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HUMAN SYSTEMS DIVISION
AIR FORCE SYSTEMS COMMAND
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TECHNICAL REVIEW AND APPROVAL

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This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Melvin E Andersen

MELVIN E. ANDERSEN, Ph.D.
Acting Director, Toxic Hazards Division
Harry G. Armstrong Aerospace Medical Research Laboratory

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This series of manuscripts from the 16th Conference on Toxicology addresses critical research and development issues for improving the scientific capability to predict potential health risks associated with various occupational and environmental exposures. To meet the needs of the Department of Defense, these contributions focus on the current knowledge of the health effects of a variety of chemical substances which are either used in military systems or generated during military operations. These manuscripts present state-of-the-art methodologies that are being used to predict subtle biochemical, physiochemical, and pathological effects with both the intact organism and specific in vitro models.

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dioxin
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explant culture
fire
fire toxicity
flow cytometry
gas generation
gasoline
groundwater
hydrazine, 1,1-dimethyl
hydrogen bromide
hydrogen chloride
hydrogen cyanide
immunotoxicology
inhalation
interaction
irritant gases
isopropylcyclohexane
lung
macrophages
marihuana
4,4'-methylene-bis(2-chloroaniline)
mixture
particle deposition
partition coefficient
perchloroethylene
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permethrin
phenobarbital
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respiratory physiology
risk analysis
skin irritation
smoke
teratogenesis
tetrachloroethylene
thermal degradation
thermoplastic
Thomas Dome
tobacco
tracheal epithelium
tributyltin
trichloroethylene
vapor generator

PREFACE

The 16th Conference on Toxicology was held in Dayton, Ohio, on 28-30 October 1986. The conference was sponsored by Northrop Services, Inc. - Environmental Services (NSI-ES), under the terms of Contract No. F33615-85-C-0532 with the Harry G. Armstrong Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio; and the Naval Medical Research Institute, Toxicology Detachment, Wright-Patterson Air Force Base, Ohio.

Dr. Donald E. Gardner, NSI-ES, served as Conference Chairman, and Linda Y. Cooper, NSI-ES, was Conference Coordinator. Lois Doncaster, NSI-ES-THRU, provided administrative support in Dayton. Acknowledgment is made to Maria Wessling Bachteal, Vivian Farah, Chuck Gaul, Linda Hesterburg, and Barbara Minton for their efforts in preparing this report.

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OPENING REMARKS

William E. Houston, Ph.D.

Northrop Services, Inc. – Environmental Sciences

Good Morning and Welcome to the 16th Conference on Toxicology. Northrop Services is proud to host this conference on behalf of the Toxic Hazards Division of the Armstrong Aerospace Medical Research Laboratory and the Toxicology Detachment of the Naval Medical Research Institute. This conference has a long history, since 1965, of bringing together distinguished scientists to exchange information on the effects of atmospheric contamination and on the toxicology of fuels, lubricants, and other compounds of Department of Defense interest. This year the focus is predictive toxicology. Each year, technical papers have been presented by nationally prominent scientists. This year we have 22 podium presentations and, to expand the scientific elements of the program, we have approximately 40 poster presentations which you will view this evening.

I would like to take this opportunity to recognize the individuals who have been responsible for managing and setting up the conference. Dr. Donald Gardner is our conference chairman from Northrop Services in Research Triangle Park, and D.. Ray Kutzman is our poster chairman. Our conference coordinators are Ms. Linda Cooper from Northrop Services in Research Triangle Park and also Ms. Lois Doncaster of our Toxic Hazards Research Unit staff here in Dayton. This year we can offer two types of continuing education credits to those interested. Through the American Board of Industrial Hygiene we can offer 2½ credits, and through the Continuing Medical Education Program at Wright State University we are offering 15 CME credits. As a final note, we are pleased to announce that the proceedings of this year's conference will be published not only in a DOD publication but also in a special issue of *Toxicology*.

It is now my distinct pleasure to introduce the welcoming speaker for the 16th Conference on Toxicology, Major General (Dr.) Fredric Doppelt, the Commanding General of the U.S. Air Force Aerospace Medical Division, Brooks Air Force Base, Texas. Dr. Doppelt received his medical degree from the State University of New York College of Medicine in Syracuse, and a Masters degree from the Johns Hopkins School of Hygiene and Public Health in Baltimore. He has had a distinguished career within the Air Force in aerospace and flight medicine. Dr. Doppelt was selected for many key positions, including the chief of the crew systems division in the man orbiting laboratory program in Los Angeles, and was a medical monitor for NASA during the Apollo program. The General has served as both vice commander and commander of the Aerospace Medical Research Laboratory here at Wright-Patterson Air Force Base and has commanded the Air Force hospital at Davis Monthan Air Force Base in Arizona, as well as having served as the command surgeon in both the Air Force

Systems Command in Washington and the Tactical Air Command at Langley Air Force Base, Virginia. General Doppelt is a Fellow of the Aerospace Medical Association and the American College of Preventive Medicine. He is a member of the American Medical Association, American Thoracic Society, and Flight Surgeons Society of Physicians of the United States Air Force. General Doppelt brings an impressive and distinguished career in leadership, in science, and in medicine to this program. It is my privilege to introduce General Fredric F. Doppelt.

WELCOME ADDRESS

Major General Fredric F. Doppelt, USAF, MC

Commander, Aerospace Medical Division, Brooks Air Force Base

Good Morning, Ladies and Gentlemen. I am pleased to have this opportunity to welcome you on behalf of the Air Force Aerospace Medical Division to this 16th Conference on Toxicology. Your host for the meeting is Northrop Services, Incorporated, under terms of a contract with the Harry G. Armstrong Aerospace Medical Research Laboratory, with the support of the Toxicology Detachment of the Naval Medical Research Institute.

For the benefit of those of you attending this conference for the first time, I'll take a few moments to provide a brief historical overview. This meeting has its roots in the 1960s, with the first five Annual Conferences on Atmospheric Contamination in Confined Spaces having been devoted to the toxicological problems associated with the early man-in-space programs.

Reflecting the national change in focus between the space-oriented '60s and the environment-oriented '70s, the name and content of the conference were changed, and in 1970 the first Conference on Environmental Toxicology was held. The 15th conference in this series took place in 1984. No conference was held last year because the contract through which the conference is supported was in the process of being competed. We are particularly happy, therefore, to be able to hold the 16th Conference this year, through the efforts of our new contractor, Northrop Services, Incorporated.

You may have noticed that this year we have dropped the word "environmental" from the title of the conference. A quick look at the conference program should convince you that this change does not mean that environmental issues are no longer addressed. Rather, the more general title was chosen to reflect the broad scope of topics that the conference has grown to include. Environmental and occupational exposures will both be addressed, and considerable emphasis will also be given to new and developing experimental and theoretical methods in toxicology.

Although the emphasis of the conference has changed over the years, all of the conferences have shared one unifying purpose: to bring together knowledgeable people to discuss current toxicologic research issues in a forum designed to promote a free and honest exchange of ideas. We very much hope that this conference will continue the tradition of its predecessors. Therefore, I strongly encourage each of you to contribute your candid opinions and constructive input to the discussions. The success of this conference depends upon your active participation.

The emphasis of this year's conference is on predictive toxicology, which, I feel, is right on target. In the past, much of toxicology was purely descriptive. Although toxicologists have come a

long way from the days of the King's poison testers, even in recent years most toxicology studies have been simply attempts to detect the adverse effects of chemicals by exposing large numbers of animals.

While it is unlikely that it will ever be possible to eliminate the need for using experimental animal studies to identify potentially hazardous materials, there are significant opportunities today to supplement traditional animal studies with newer and more reliable predictive techniques. I feel very strongly that humanely conducted animal studies for the purpose of protecting human health are justified. However, we are also continually seeking more effective alternatives to animal testing.

Our development of predictive methods will also allow us to hold down the explosive growth in the number of materials needing to be tested. As a single example of this growth, the U.S. Environmental Protection Agency's acute hazards list for 1986 includes almost 400 substances. New chemicals and materials are being developed at an ever-increasing rate. Performing extensive animal studies on all of them is simply not possible. Therefore, only those chemicals and materials that are most likely to exhibit toxicity resulting from acute or chronic exposure must be identified and targeted for further testing. Both *in vitro* assays and quantitative structure-activity relationships have shown promise for meeting this urgent need, but many uncertainties remain.

For example, the Air Force Systems Command, in a bold venture known as Project Forecast II, is aggressively pursuing the technologies and systems of the future. Many of these technologies and systems will require the development of totally new propellants and materials. Rapid evaluations of the potential health effects of these new candidates must be made as early as possible so they can be incorporated into the decision-making process.

Our new predictive methodologies potentially allow us to not only reduce the cost of these evaluations but also to provide for more timely data for them. The rapid development of these predictive methods has been stimulated by the growing recognition of the need for more scientifically valid methods of estimating the quantitative risk from a given environmental or occupational exposure.

It would be wonderful if we could totally eliminate the potential for exposure to any chemical showing evidence of toxicity; such is not the case, however. The reality of risk management is that cost/benefit decisions must be made. The scope of this task is sobering: estimates of total costs for the Air Force Installation Restoration Program reach more than 2.4 billion dollars by the year 2000.

The order in which waste sites should be cleaned up must be prioritized, because they can't all be cleaned up at once. This prioritization is critically dependent upon an accurate estimate of the risk entailed by exposure to what is often a complex mixture of chemicals with varying toxic effects.

Another facet of risk management decision-making involves the determination of whether to restrict pregnant or nursing women from workplace exposure to fuels and solvents. These decisions hinge on a determination of the potential risk to the unborn fetus or nursing infant caused by transfer of chemical from the mother.

Our predictive modeling techniques hold promise to allow the proper resolution of these and many other questions. We are proud of the advances that have been made in this area by Dr. Mel Andersen and his co-workers here in the Toxic Hazards Division of the Harry G. Armstrong Aerospace Medical Research Laboratory, but much remains to be done, as should be clear after today's sessions.

I'm excited by the tremendous potential of predictive toxicology, and I feel that this conference captures some of that potential. I hope you feel as I do and will give your full support toward making this conference fruitful. Again, Welcome.

SESSION I

PREDICTING HEALTH EFFECTS FROM GROUNDWATER POLLUTANTS

INTRODUCTORY REMARKS

Major Michael L. Shelley, USAF, BSC – Chairman

Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base

The first session today is on predicting the health effects from groundwater pollutants. To consider that question we first need to consider what the problem is. We need to ask – what are the unwanted chemicals in the groundwater? Unfortunately, the person who was to address this question could not be here today. Ms. Judy Burris from the Air Force Occupational and Environmental Health Laboratory was to present recent findings that would help to define what our problems are, particularly with Air Force installations. The Air Force has a very active program in installation restoration, and part of that effort involves a large contract to do monitoring to quantify some of the problems that we have, particularly where groundwater contamination is concerned. Ms. Burris's manuscript discussed the findings of this contract to date. Although this was not a particularly controversial manuscript, it did involve contractor findings that were not yet finalized, and there was some routine objection to that material being released at this time. So, we unfortunately will not have the benefit of Ms. Burris's presentation. Instead I would like to give some introductory remarks, including some of the information that Ms. Burris would have shared with us this morning.

The first figure depicts the classical risk management flow chart with which most of you are very familiar. Often in research one tends to lose sight of the original objectives. Quantifying the potential hazard due to exposure to a particular chemical is only a part of the risk management process. The very complicated process of exposure assessment – how materials are transported to produce an exposure to the subject – is still a very controversial issue. These two inputs lead to a risk assessment which then must be considered in the light of certain practical issues, such as cost-effectiveness, our current technology, and the funds available to take care of the problem. This question is a very serious one for the Air Force, considering the number of sites involved and the degree of environmental restoration that could be required. A very small variation in the way one arrives at a hazard assessment and exposure assessment could very well mean the difference of millions of dollars in ultimate remedial action at a particular site. This doesn't mean that we need to alter our methods of hazard assessment and exposure assessment to ensure that we come up with a remedial action of least cost, but we would at least need to make sure that our research and our efforts in predictive health effects are as accurate as possible and that they are based on a rational scientific process.

I mentioned that exposure assessment is a very difficult problem. This is particularly true in the area of groundwater contaminants. This isn't a situation in which you have a very well defined industrial process that is emitting a chemical in a defined way with a very predictable manner of exposure. Instead, there are many possible kinds of industrial wastes that have been acted on over the years under various disposal conditions which, back then, seemed to be perfectly acceptable. Now we are questioning some of those procedures. Often we can very clearly see what the problem is, what the nature of it is, what the toxicologic consequences may be, and how exposure may occur. We can also get a pretty good idea of what the magnitude of the problem is in some cases. But often we don't have even this knowledge; instead, we have chemicals of totally unknown origin. In groundwater situations throughout the United States we find low levels of organic chemicals, the origins of which are unknown. A great deal of wondering and guessing takes place as to exactly what the original source may have been or what multiple sources may have occurred. In these cases one might simply have to resort to drilling wells in various places to try to capture what the picture is underneath and what the groundwater looks like. It's a very intricate problem.

A case in point is a situation at Wurtsmith Air Force Base – perhaps one of the worst situations discovered early on. At Wurtsmith, there is a trichlorethylene plume below the surface, stretching almost a half mile. There are certain theories about how that occurred, but the complete story is still unknown. The red area in this figure indicates an area in which trichloroethylene was actually in the parts-per-million range in the groundwater underneath the Base. In this cross-sectional view of that plume, you can see it extending down as far as 70 feet, all the way down to the clay surface. This is an example of a situation that can be found in many places throughout the United States. This has also been observed with other Air Force installations, low levels of organic chemicals for which the origin is unknown. We need to determine how to handle this kind of a situation and determine the appropriate risk assessment process that will dictate the appropriate remedial action. This involves a great deal of modeling effort, prediction of environmental weathering, environmental pathways – all sorts of efforts which, in these days of the computer age can help predict exactly how this material will move. When one starts thinking about how that material is moved and how it then ultimately presents an exposure situation to the human, it gets even more complicated. How does one deal with the inhalation, ingestion, and dermal contact problems? When we think about groundwater contamination we are usually thinking about drinking water; but what about exposure when one is taking a shower, or running a dishwasher or a washing machine in the home? We need to consider such household situations and be able to predict what the exposures would be. How does one model that kind of problem? How does one come up with a risk assessment and determine what, ultimately, the groundwater levels should be and what you need for an approach as far as remedial action? This is the problem, interpreting groundwater data in terms of the

significance for human exposure. Our concern this week and in this session in particular, amid all of the exposure assessment and modeling and all of the problems in the environment, is how to incorporate that data with the available toxicology data and how to most effectively get at a reasonably rational, scientifically-based assessment of the hazard.

Let me present to you the Air Force program. The Department of Defense has an environmental restoration program that parallels the U.S. Environmental Protection Agency's Superfund program. The Air Force implementation of that is the Installation Restoration Program (IRP). The IRP is broken down into four phases of implementation that are similar but not precisely the same as those defined in Superfund. The first phase is simply a records search, to get an overview of what the problems may be at various installations. Phase 2 is a much more concerted effort, generally a large contract effort based on the Phase 1 research to actually drill wells, and to quantify the problem as precisely as possible to determine the source, the movement, and the nature of the contaminants in the groundwater. Phase 3, which is not a component of the Superfund but which the Air Force has included in its technology-based development, is the phase in which we at AAMRL and other agencies in the Air Force are involved, developing remedial technologies and also methods and hazard assessment to define exactly what levels need cleaning, and how clean is clean. Finally, Phase 4 involves actual remedial actions and is based on remedial action plans drawn up as a result of the previous phases.

Given our prioritization of chemicals that we feel are a problem and that may be a problem with the Air Force, we have made an extensive literature search effort through the Arthur D. Little Company, which has now supplied us with the Installation Restoration Program Toxicology Guide. Volume 1 of the guide is a comprehensive literature summary of 36 chemicals. The significance of this list is that not only do we suspect that these chemicals may be a problem with Air Force installations, but that they have in fact been identified in groundwater through groundwater surveys conducted for various purposes. These are the chemicals that are actually in the water at one or several sites throughout the Air Force. Not many of the chemicals on this list will come as much of a surprise to you.

Volume 1 of our toxicology guide presents a comprehensive literature search on these particular chemicals and gives us the toxicological data. It also expounds on how those data may be used in risk assessment and on the current state-of-the-art in hazard assessment as far as toxicology is concerned. It also provides a very extensive primer, as an introduction to the volume, on risk management in general, and the principles that are exercised not only in exposure modeling but also in new frontiers in toxicology hazard assessment methodology. I encourage you to obtain a copy of this excellent reference, which is now available through the Defense Technical Information Center and the National Technical Information Service as a technical report. All of our public health officials

in the Air Force have a copy of this in the field; it's intended to be a field manual. In addition to risk management fundamentals, this report also provides an updated regulatory status of the chemicals.

Volume 2, which is planned and already under way, covers chemicals that have been identified by Air Force headquarters through the Phase 1 program survey of all installations. These chemicals have not all necessarily been found in groundwater, but are certainly in heavy use and may indeed represent a problem. Note that there are many pesticides. Volume 2 should be available to us in April or May. Certain chemicals can be a particularly great challenge for us. They include things like hydraulic fluid, fuel oils, and jet fuel. How do you deal with such chemical mixtures in a hazard assessment? What chemical changes have taken place after 20 years of weathering in the environment? These questions need to be dealt with. We have a third volume coming out, using a new format, which will try to deal with these particular problems.

Our first session this morning will address our particular concerns about groundwater, the installation restoration problems, chemical interactions, and the use of risk assessment modeling to define the problem. These are some of the scientific issues that need to be dealt with and are some of our purposes for having this conference today. With that brief introduction I would like to introduce our first speaker for the day.

INTERSPECIES EXTRAPOLATIONS IN RISK ANALYSIS

Curtis C. Travis, Ph.D.

Health and Safety Research Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831

SUMMARY

Quantitative risk assessment for carcinogenic chemicals is usually based on data obtained in animal studies conducted at very high levels of exposure. A key issue in using such data is the extrapolation of results from animals to man. Recently, physiological pharmacokinetic models have been used to improve the accuracy of extrapolations. The models can also be used to aid in extrapolating between routes of administration. Model results for inhalation and ingestion of tetrachloroethylene will be presented and compared to experimental data for rats and humans.

INTRODUCTION

Risk assessment is a procedure that combines all available data and the best scientific judgment to estimate the risk associated with human exposure to chemicals. Because of gaps in our current scientific understanding of the cancer-causing process, risk assessment requires the use of a series of judgmental decisions on unresolved issues. The major assumptions arise from the necessity to extrapolate experimental results (a) across species from rats or mice to humans, (b) from the high-dose regions to which animals are exposed in the laboratory to the low-dose regions to which humans are exposed in the environment, and (c) across routes of administration. There is growing awareness of the need for an evaluation of the scientific bases upon which the assumptions used in the risk assessment process are made. Pharmacokinetics provides a tool for such an evaluation.

Pharmacokinetics is the study of the absorption, distribution, metabolism, and elimination of chemicals in man and animals. An attractive approach for interpreting empirical data relating to pharmacokinetics is the development of predictive, physiologically based pharmacokinetic (P-P) models. These P-P models utilize actual physiological parameters of the experimental animals, such as breathing rates, blood flow rates, and tissue volumes, to describe the metabolic process. The models can be used to quantitatively relate exposure concentrations to organ concentrations over a range of exposure intervals. As such, the models allow prediction of the relationship between inhaled concentrations of a chemical and the concentration found in target tissues. A chief advantage of the P-P model is that it can be utilized to describe the dynamics of chemical transport and metabolism in mice, rats, and humans simply by changing the physiological parameters.

MATERIALS AND METHODS

A P-P model divides the body into physiologically realistic compartments, all connected by the arterial and venous blood flow pathways. The model used in the present study (Figure 1 and Table 1) is patterned after a model developed by Ramsey and Andersen, who successfully predicted the behavior of styrene inhalation exposure in humans from behavior observed in rats (1). The chemical used as an illustration in this particular study is tetrachloroethylene (Perchloroethylene, PCE), an extensively used dry-cleaning solvent. Details of the model development are given in Ward et al. (2).

The tissue groups include (a) vessel-rich organs such as brain, kidney, and viscera, (b) vessel-poor organs such as muscle and skin, (c) slowly perfused fat tissue, and (d) organs (principally the liver) that have a high capacity to metabolize PCE. The model is described mathematically by a set of differential equations used to calculate the rate of change of the amount of chemical in each compartment. Metabolism of PCE, which occurs chiefly in the liver, is described by a combination of a linear metabolic component and a Michaelis-Menten component describing saturable metabolism.

The model is extrapolated to an untested species by scaling physiological parameters. The concept of scaling by body weight in pharmacokinetic modeling was introduced by Dedrick (3). Scaling is defined as the proportional variation of anatomical and physiological properties with body weight (4).

In modeling the uptake, distribution, and elimination of PCE, we used the 0.74 exponential power of the body weight to determine the scaling of cardiac output and ventilation rate. The scaling formulas obtained from Ramsey and Andersen (1) are as follows:

$$Q_{alv} = Q_{alvc}\, BW^{0.74}$$

$$Q_b = Q_{bc}\, BW^{0.74}.$$

The allometric constants Q_{alvc} and Q_{bc} are shown in Table 2. The blood flow to a given tissue group is obtained from the total blood flow Q_b by multiplying by the fraction of blood flowing to that tissue. The fractions are also shown in Table 2. Likewise, the volume of a tissue group is determined by multiplying the body weight by the volume fractions. The bone and cartilage volume (9%) is ignored in the model.

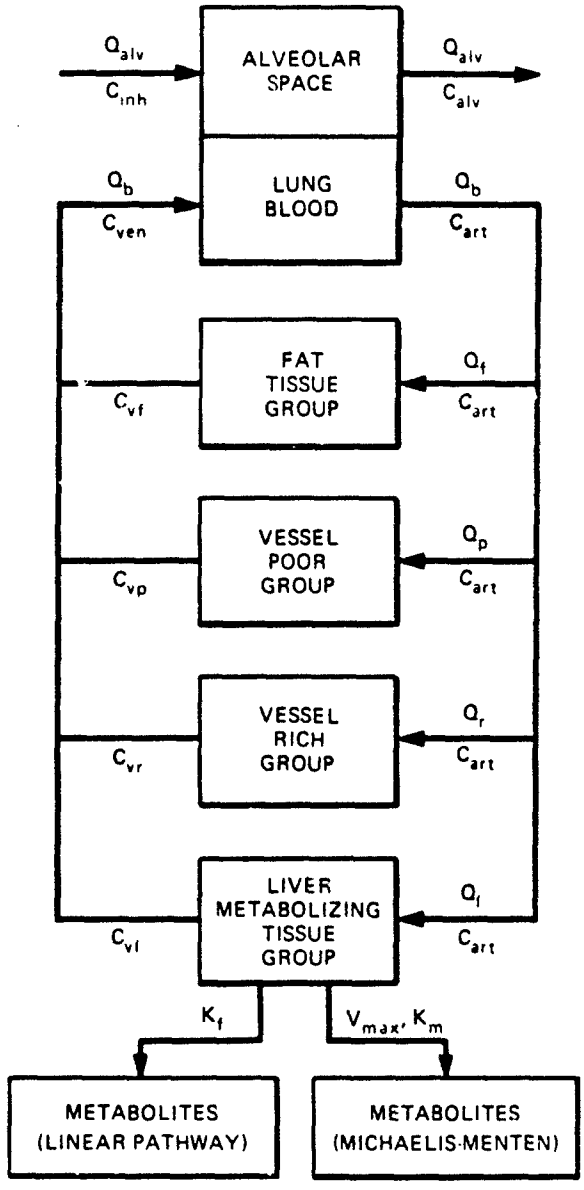


Figure 1. Diagram of the pharmacokinetic model used to simulate the behavior of inhaled PCE. The symbols are defined in Table 1 and the parmeters used to describe the model are in Table 3.

TABLE 1

ABBREVIATIONS USED IN DESCRIBING A PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL

Abbreviations	Terms
Q_{alv}	Alveolar ventilation rate (l air/h)
C_{inh}	Concentration in inhaled air (mg/l air)
C_{alv}	Concentration in alveolar air (mg/l air)
λ_b	Blood/air partition coefficient (l air/l blood)
Q_b	Cardiac output (l blood/h)
C_{art}	Concentration in arterial blood (mg/l blood)
C_{ven}	Concentration in mixed venous blood (mg/l blood)
V_{max}	Michaelis-Menten metabolism rate (mg/h)
K_m	Michaelis constant (mg/l blood)
K_f	Linear metabolism rate (h^{-1})
A_m	Amount metabolized in the liver (mg)
	Subscripts (i) for tissue groups or compartments: l Liver (metabolizing tissue group) f Fat tissue group r Vessel-rich tissue group p Vessel-poor tissue group
Q_i	Blood flow rate to tissue group (l blood/h)
V_i	Volume of tissue group i (l)
C_i	Concentration in tissue group i (mg/l)
A_i	Amount in tissue group i (mg)
C_{vi}	Concentration in venous blood leaving tissue group i (mg/l blood)
λ_i	Tissue/blood partition coefficient for tissue i (l blood/l)
k	Gavage or oral rate constant (h^{-1})
D_o	Total quantity of PCE absorbed (mg)

TABLE 2

COEFFICIENTS FOR SCALING FORMULAS

	Rats	Humans
	Allometric constants	
Ventilation (Q_{alvc} – l/h)	14	14
Cardiac output (Q_{bc} – l/h)	14	14
	Tissue volume fractions	
Liver	0.04	0.04
Fat 0.07	0.19	
VRG	0.05	0.05
VPG	0.75	0.63
	Blood flow fractions	
Liver	0.25	0.25
Fat	0.09	0.09
VRG	0.51	0.51
VPG	0.15	0.15

The tissue/blood and tissue/air partition coefficients used in our model were made available by Gargas and Andersen (5). The partition coefficients were measured using a vial equilibration technique (6) in which the chemical was added to a closed vial containing blood or tissue, and the partitioning was determined by estimating the amount that disappeared from the headspace after equilibration at 37°C (7). The tissue/blood partition coefficients used in the model (Table 3) are obtained by dividing the tissue/air partition coefficients by the blood/air partition coefficients. Gargas et al. (7) have also proposed that the metabolic parameters V_{max} and K_f can be scaled using body weight. The Michaelis-Menten metabolic rate, V_{max}, is presumed to be proportional to surface area (8):

$$V_{max} = V_{maxc}\, BW^{0.7}.$$

The linear metabolic rate constant is scaled as follows.

$$K_f = K_{fc}\, BW^{-0.3}.$$

Values for the coefficients V_{maxc} and K_{fc}, and for the Michaelis-Menten constant, K_m, are shown in Table 4. The rat metabolic parameters presented in Table 4 were determined from limited data. Because accurate metabolic data are critical if the PCE model is to be used in risk analysis, further experimental determination of these parameters is necessary. I suggest an experiment in which rats are exposed to a range of doses (via either inhalation or gavage), and both exhaled air concentrations of PCE and urinary metabolites are quantified.

TABLE 3

PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS USED IN DESCRIBING THE BEHAVIOR OF PCE IN THE PHARMACOKINETIC MODEL SHOWN IN FIGURE 1

End Point	Parameter	Rat	Human
Body weight (kg)	BW	0.25	70.0
Alveolar ventilation (l air/h)	Q_{alv}	5.02	325.0
Blood flow rates (l blood/h)			
Total blood flow rate	Q_b	5.02	325.0
Blood flow rate in liver	Q_l	1.26	81.3
Blood flow rate in fat	Q_f	0.45	29.3
Blood flow rate in vessel-rich tissues	Q_r	2.56	165.7
Blood flow rate in vessel-poor tissues	Q_p	0.75	48.7
Tissue group volumes (l)			
Volume in liver	V_l	0.0100	2.8
Volume in fat	V_f	0.0175	13.3
Volume in vessel-rich tissues	V_r	0.0125	3.5
Volume in vessel-poor tissues	V_p	0.1875	44.1
Blood/air partition coefficient	λ_b	18.9	10.3
Tissue/air partition coefficients			
Liver/air partition coefficient	$\lambda_{l/a}$	70.3	70.3
Fat/air partition coefficient	$\lambda_{f/a}$	2060.00	2060.00
Vessel-rich/air partition coefficient	$\lambda_{r/a}$	70.3	70.3
Vessel-poor/air partition coefficient	$\lambda_{p/a}$	20.0	20.0

TABLE 4

METABOLIC PARAMETERS USED IN DESCRIBING THE BEHAVIOR OF PCE

End Point	Parameter	Rat	Human
Body weight (kg)	BW	0.250	70.0
Michaelis-Menten metabolic rate coefficient	V_{maxc}	0.12	0.12
Michaelis-Menten metabolic rate (mg/h)	V_{max}	0.046	2.35
Michaelis-Menten constant (mg/l blood)	K_m	0.30	0.30
Linear rate coefficient	K_{fc}	2.0	2.0
Linear metabolic rate (h^{-1})	K_f	3.03	0.559

RESULTS

In this section, we will determine whether the metabolic scaling factors described above allow for both interspecies extrapolation and dose-route extrapolation. To accomplish this, we will first determine metabolic parameters so that model predictions will reproduce rat data published by Pegg et al. (9). Then, using scaling parameters, we will attempt to reproduce rat ingestion data published by Pegg et al. (9) and human data published by Fernandez et al. (10).

Rat Inhalation

Adult male Sprague-Dawley rats weighing 250 g were exposed to ^{14}C-labeled PCE by inhalation for a duration of 6 h in experiments conducted by Pegg et al. (9). In the 72 h following exposure to 10 ppm PCE, metabolism accounted for 20% of total radioactivity recovered, whereas unchanged PCE in expired air accounted for 70%. Pulmonary elimination of PCE had a half-life of about 7 h.

The biological parameters and partition coefficients used to model the empirical data of Pegg et al. (9) are presented in Table 3. We used Andersen's values for the $\lambda t/a$ (tissue/air) partition coefficients, except for fat. It was necessary to increase the fat/air partition coefficient from Andersen's measured value of 1638 to 2060 to account for increased alveolar concentrations of PCE after exposure. Because empirical values for the metabolic parameters are not available, we determined the values that produced the best fit with the inhalation data from Pegg et al. (9). Metabolic scaling coefficients that produced the best fit with the empirical data were V_{maxc} = 0.12 mg/h, K_m = 0.3 mg/l blood, and K_{fc} = 2.0/h.

In Figure 2, model predictions (shown as solid points) are compared with the empirical data (shown as vertical bars) of Pegg et al. (9). The figure shows the percentage of PCE recovered in expired air in rats (for a number of different time intervals) following an exposure to 10 ppm PCE for 6 h. The vertical bars represent the range of the empirical data. The predictions and empirical data are integrated from the beginning of each time interval to the time at which the points and bars are drawn. Note that the length of the time intervals varies, causing the data points to be non-monotonic. The model predicted that 68% of the body burden of PCE would be recovered in expired air during the 72 h after exposure; this value is in good agreement with the experimentally determined value of 70%.

Rat Ingestion

Adult male Sprague-Dawley rats weighing 250 g were orally administered ^{14}C-labeled PCE in corn oil in experiments conducted by Pegg et al. (9). In the 72 h following exposure to 500 mg/kg PCE, metabolism accounted for 5% of total radioactivity recovered, while unchanged PCE in expired

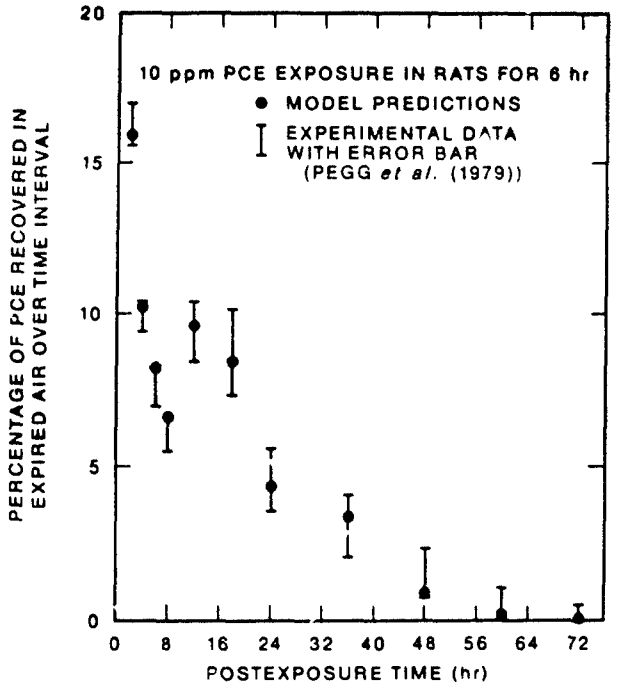


Figure 2. Percentage of PCE expired by rats following exposure to 10 ppm in air for 6 h. The percentage plotted at 8 h represented the percentage expired from 6 to 8 h, etc. Note that the time intervals are longer after 8 h. • = Model predictions; I = Range of experimental data.

air accounted for 90%. There was no significant difference in elimination half-life (approximately 7 h) with dose or route of administration.

In Figure 3, model predictions of expired air concentrations (solid line) are compared with the empirical data of Pegg et al. (9). The model, based on parameters obtained from the inhalation study, slightly overestimated the rate of elimination of PCE. This may be due to the effects of corn oil as a carrier vehicle on the pharmacokinetics of PCE. Withey (11), Withey et al. (12), and Angelo et al. (13) have shown that an oil carrier results in a slower elimination pattern for dichloromethane (methylene chloride).

Human Inhalation

Fernandez et al. (10) exposed 24 subjects to concentrations of 100 ppm PCE for 1 to 8 h. During the first hours after exposure, the concentration of PCE decreased rapidly in alveolar air; however, more than 2 weeks is necessary to eliminate the PCE retained following an exposure of 100 ppm for 8 h.

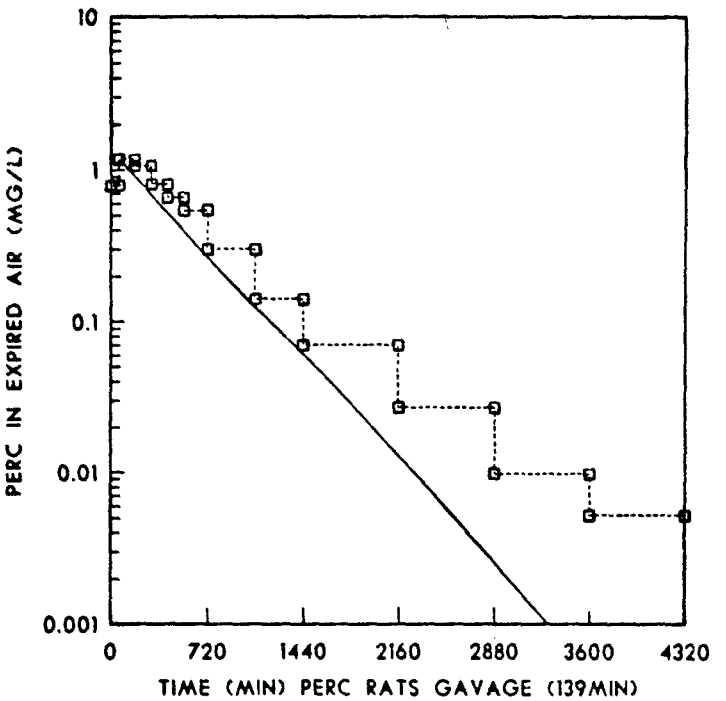


Figure 3. Concentration of PCE expired by rats following oral administration of 500 mg/kg of ^{14}C-labeled PCE in corn oil for 6 h. (–) = Model predictions; □ = Experimental data.

The biological parameters and partition coefficients used to model the data of Fernandez et al. (10) are also presented in Table 3. A body weight of 70 kg was used in all model calculations. The metabolic parameters for humans were obtained using the scaling formulas and the V_{maxc} and K_{fc} determined for rats (Table 4).

Figure 4 is a graph of the Fernandez data and shows alveolar concentrations resulting from exposure to 100 ppm PCE for various durations versus our model results (solid lines). The model results were calculated presuming 19% fat; the agreement between the model and the data is very good.

DISCUSSION

We have shown that it is possible to extrapolate pharmacokinetic behavior to the chemical PCE by scaling of physiological parameters. Simple scaling of metabolic parameters, originally adjusted to account for the effects in rats, also accounted for the responses in human subjects to doses of PCE administered by inhalation. We have also shown that it is possible to extrapolate between routes of administration.

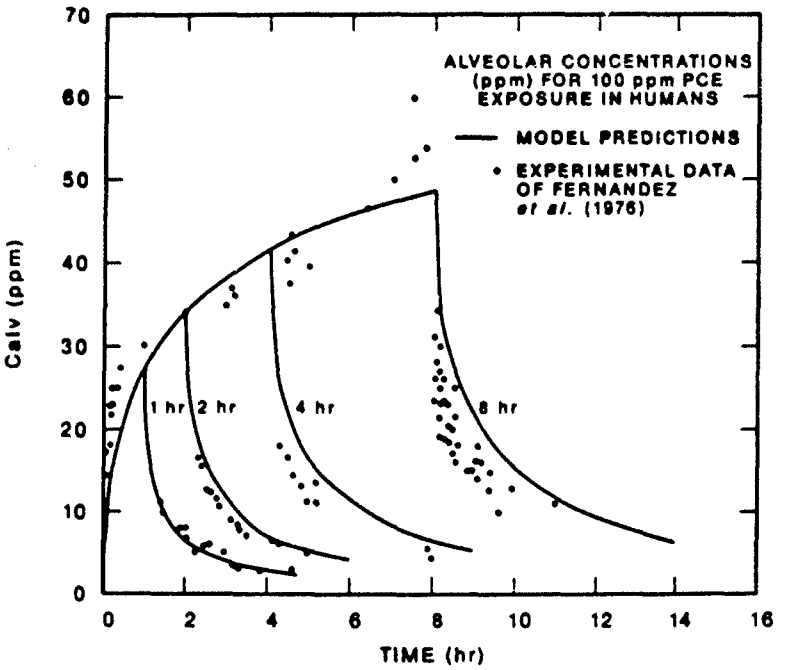


Figure 4. PCE concentrations in alveolar air of humans after an exposure concentration of 100 ppm for periods of 1-, 2-, 4-, and 8-h durations. (–) = Model predictions; • = Experimental data.

Because pharmacokinetic models allow quantitative extrapolation of exposure data across species and between routes of administration, they provide a tool to quantitatively evaluate assumptions currently used in the risk assessment process.

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QUESTION AND ANSWER SESSION

DR. YANG (NIEHS): In your comparison of the bioassay data, you are comparing rats and mice, inhalation and gavage, and cancers at different sites. Isn't that like comparing apples and oranges?

DR. TRAVIS: That's a good question. The data that I presented were just for liver cancer in both species, but you're asking if we are comparing apples and oranges. My answer to that is we may be – we don't know if we are or not. One of the questions that we are investigating here – and that's why I wasn't saying that a straight line would fit the data – is whether there is some consistency between the results for rats and the results for mice. It looks like there is. After we adjust for route of administration and compute the effective dose to both species, the bioassay data seem to be consistent across species.

DR. YANG: Oh, it's much better if you are only comparing the liver. I didn't realize that.

DR. TRAVIS: We were just looking at liver in both species.

DR. GARDNER: (NORTHROP SERVICES, INC.): In your slide that showed the comparison between inhalation dose and gavage, for example, you used the word inhalation dose. Do you mean inhalation dose, or do you mean inhalation concentration in the chamber? It could make quite a difference in your slides; because, what you were exposing the animals to, if you measure the chamber concentration, is not necessarily what would be expected to be deposited in the lungs.

DR. TRAVIS: That's true. The pharmacokinetic models can be used to extrapolate between any one of those units. I think I actually had parts per million as the unit for the inhalation exposures. I could have used any unit for the drinking water exposures also. I could have used parts per million in the water. We actually used milligrams per kilogram because for the drinking water pathway it's much easier to compute if you assume 100% absorption. In the inhalation pathway it is not so easy to compute the dose in terms of milligrams per kilogram so we gave an air concentration. But the model can be used to do any of those extrapolations.

DR. GARDNER: But wouldn't that make a big difference because such a small fraction may really be deposited in the lungs and may throw off your line considerably?

DR. TRAVIS: Well, yes, it will change the lines if we change the units, but the biological data in terms of no-effect levels is probably expressed in terms of parts-per-million exposures. You had a certain parts per million in exposure and you got a no-effect level. So that's the kind of data we would want.

DR. MEHENDALE (UNIVERSITY OF MISSISSIPPI): The slide, the one before the last one, had four points in the middle, and I saw that you had generated a curve spanning from the bottom all the way to the midline. I wonder how you got that curve with those four points clustering in the middle. This was the slide where you had gavage.

DR. TRAVIS: The line was simply the metabolized dose resulting from various exposures through the gavage pathway or the inhalation pathway. That line was generated with the Pharmacokinetic model. Basically that line was for a six-hour termination of the exposure, and then followed for 24 hours to determine the total metabolite or the nonlinear metabolite. This is the same protocol that was used in the animal bioassay. We wanted to see how much metabolite was produced under a condition of a six-hour exposure followed by no exposure for the rest of the 24-hour period. You can do that for each air concentration, it gives you a data point for metabolite production, and you can draw that line. It's a lot of computer runs. That line represents 20 or so computer runs. The point of those slides and our whole study of tetrachloroethylene and the other chemicals is that right now I'm not willing to say that, for instance, we can extrapolate from mice to rats or that we can extrapolate from the mice and rats to humans. We are investigating pharmacokinetics as a tool in risk assessment. We are looking at various data sets and seeing how consistent the data are from mice and rats. Can we predict blood concentrations and exhaled air concentrations in humans from the mouse and rat data? We are just now investigating pharmacokinetics as a tool in risk assessment. Down the road we may be willing to say after this investigation that yes, these models look like they are an extremely valuable tool for risk assessment. I can already say that they appear to be an extremely valuable tool because they allow us to do many things that we couldn't do before. One is the dose route extrapolation problem.

DR. LISS (STATE UNIVERSITY OF NEW YORK): In your last slide you plotted your data against your linear enzymes. You said you ignored--

(Dr. Travis interrupted)

DR. TRAVIS: That was the nonlinear.

DR. LISS: The nonlinear. In either case, in your model the box for both types of enzymes was of equal size. Do you have any indication that there are equal amounts of enzymes contributing to the metabolism?

DR. TRAVIS: Well, we can estimate the amount of metabolism that takes place through each pathway by trying to reproduce the data we have on pharmacokinetics. The type of pharmacokinetic data we have are the blood concentrations or exhaled air concentrations that I showed you for the rat. If we change the metabolism parameters in the model it will change our fit to the data. It's an optimization problem of trying to determine the best metabolic parameters that fit the data.

DR. LISS: But other than data fit, are you following any other established parameters when you eliminate one set of enzymes versus the other that indicate you are actually modeling a true animal?

DR. TRAVIS: We're not presently. We are just trying to reproduce the observed data in the animals which are basically exhaled air concentrations, blood concentrations, fat concentrations, or tissue

concentrations as a function of time. The models are able to do that fairly well. We are indirectly inferring metabolism production. Now for some of the experiments we do have measured metabolism production; like in rats or mice for tetrachloroethylene, there is a study and we are able to reproduce the measured metabolite productions. Also, in humans we have metabolite production data and we are able to reproduce that.

DR. NEWELL (ELECTRIC POWER RESEARCH INSTITUTE): Curt, one of the first slides you put on showed differences in excretion rates. I believe it was TCE between mice and rats. I think that slide showed that with the varying times and concentrations, the ratio of retention to excretion was quite similar in the rat. There was a marked difference in the mouse, and I think that's a good example of where one should come back then. Further, there are a number of assumptions that are made in risk estimation of saying that you can extrapolate well between species. I strongly suggest that we shouldn't oversell a lot of this. Each compound really has to be looked at under all these parameters and get enough data and then say we can make a go of it. The work of Dr. Andersen, Harvey Clewell, and the rest here at Wright-Patt on methylene chloride is a beautiful example of those differences of concentration and how they are handled. So I think what we are doing is very important, but we also have to realize that there are many limitations and until we understand those parameters broadly, we can't make the broad claims.

DR. TRAVIS: I agree 100%. Certainly we don't want to give anybody the idea that these models solve all of the problems and that if we investigate the pharmacokinetics of tetrachloroethylene that we will understand the pharmacokinetics of every other chemical. It has to be done on a chemical-by-chemical basis. You have to have a lot of information about how the chemical is metabolized. You have to determine the metabolic pathways and which metabolite is possibly the carcinogen. The more information you have the better. The pharmacokinetic models are simply a tool for collecting all of this information and analyzing it. They are not a substitute for knowledge. As a matter of fact, they tend to drive experiments in the sense that pharmacokinetic models raise a lot of questions about the chemical. Then we have to do more experiments to gather the data to answer the questions that the models raise. But they also provide a theoretical framework in which to interpret toxicological data and pharmacokinetic data.

PREDICTIVE ONCOGENIC MODELING INCLUDING PHARMACOKINETICS

Thomas B. Starr, Ph.D.

Manuscript Not Submitted

TOXICOLOGICAL STUDIES OF CHEMICAL MIXTURES OF ENVIRONMENTAL CONCERN AT THE NATIONAL TOXICOLOGY PROGRAM: HEALTH EFFECTS OF GROUNDWATER CONTAMINANTS

Raymond S.H. Yang, Ph.D., and Elmer J. Rauckman, Ph.D.

National Toxicology Program, National Institute of Environmental Health Sciences,
Research Triangle Park, NC 27709

SUMMARY

In cooperation with the Agency for Toxic Substances and Disease Registry, the National Toxicology Program is participating in a Public Health Service activity related to the Comprehensive Environmental Response, Compensation and Liability Act (Superfund Act) by conducting toxicology studies on chemicals found in high-priority hazardous waste sites and for which adequate toxicological data are not available. As part of this effort, a project on the toxicology of chemical mixtures of groundwater contaminants was initiated. The first study, centered on the health effects of groundwater contaminants, is at the contractual stage. Nineteen organic and six inorganic chemicals, selected from more than 1000 known groundwater contaminants, will be given in drinking water to Fischer 344 rats and B6C3F$_1$ mice for 3 or 6 months. Controls and five dose levels, based on average concentrations (i.e., baseline level) of individual component chemicals, or 0.1-, 10-, 100-, or 1000-fold of the baseline level, will be used. Toxicological end points include mortality, clinical signs, water and food consumption, body and organ weights, clinical pathology analyses (e.g., hematology, clinical chemistry, and urinalysis), gross and histopathology, neurobehavioral tests, sperm morphology and vaginal cytology evaluations (SMVCE), and cytogenetics. This paper summarizes the rationale behind our experimental design and the factors one must consider when designing studies of complex chemical mixtures.

*The detailed technical aspects regarding materials and methods for the proposed studies in this paper may be found in the NTP General Statement of Work (NTPGSW) At the time of preparing this manuscript, the most recent version of NTPGSW was that of July 1984.

INTRODUCTION

Human exposure to chemicals, be it an occupational or an environmental exposure, is rarely limited to a single chemical. Even in a strictly controlled situation such as a production plant for one single chemical, workers are exposed daily to a variety of starting materials and process intermediates as well as other chemicals in food, drinks, and personal hygiene products. In recent years, the concern for potential toxicity from the exposure to multiple chemicals has been amplified by various environmental problems, including those associated with hazardous waste sites (1-9).

One of the most critical environmental issues today is groundwater contamination. Between 40 and 50% of the population in this country depends upon groundwater as its primary source of

drinking water, and approximately 75% of the cities in the United States derive their supplies, in total or in part, from groundwater (10,11). The following processes may contribute to the deposition of a variety of organic and inorganic chemical contents in the groundwater: (a) natural processes such as leaching of minerals; (b) waste-disposal practices; and (c) other human activities such as accidental spills and leaks, agricultural practices, and mining.

Although the phenomenon of drug and/or chemical interactions is not new, and a number of reviews have been available (2,4,12-25), most past studies involved only two chemicals, and the emphasis was, in general, on the acute biochemical, physiological, or toxicological effects. Several notable exceptions where chemically defined mixtures of 10 or more components were studied are the investigation of amino acid toxicity by Gullino et al. (26), the subchronic toxicity studies of persistent contaminants in the Great Lakes by Chu et al. (27) and Cote et al. (28), and the chronic toxicity/carcinogenicity study of 11 volatile halogenated hydrocarbon drinking water contaminants by Webster et al. (29). The relatively limited scope of investigation in this area can be attributed to the complexity of experimentation required for a chemical mixture that has more than two components. On the other hand, there have been some recent studies dealing specifically with the toxicity of complex mixtures such as dump site samples (7,30), combustion products from tobacco (31-33), diesel fuel and gasoline (34,35), contaminated water samples (36,37), contaminated fish (38,39), and by-products from synfuel operations and coal combustion (40-49).

The purpose of this study is to investigate the long-term (up to 6 months) health effects in laboratory animals given drinking water containing chemical contaminants frequently found in U.S. groundwater sources. A National Toxicology Program (NTP) proposal to conduct studies of chemical mixtures was reviewed at the National Institute of Environmental Health Sciences (NIEHS) on August 8, 1985, by an expert panel of scientists (see Acknowledgments). In that meeting, a consensus was reached on the following directions.

Health Effects of Drinking Water Contaminants. Short-term repeated-dose and 13-week subchronic animal toxicology studies should be conducted using the most frequently seen drinking water contaminants at the known human exposure level and profile via drinking water exposure. Certain safety factors (i.e., 10×, 100×, 1000×) should be incorporated in the experimental design because of probable interspecies and interindividual variations. Chronic toxicology studies should be considered after reviewing the data from these proposed short-term and subchronic studies.

Investigation of Frequency of Occurrence of Toxicological Interactions. Short-term repeated-dose animal toxicology studies should be conducted using groups of two or more chemicals in combination (based on known target organ toxicity and/or toxic

mechanisms). Such studies should be designed to investigate the frequency of occurrence of synergism, potentiation, or antagonism among the high-priority chemical contaminants in hazardous waste sites and those frequently detected in groundwater.

Mechanistic Approach to the Study of Toxicological Interactions. Modulation of representative drinking water contaminants on the specific biological end points (e.g., bioavailability, enzyme induction, covalent binding, DNA repair) of one or more model compounds (such as known carcinogens) should be studied.

This report represents an effort on the first directive noted above. In this report, the terms "drinking water" and "groundwater" are considered synonymous for the following reason. Public water supplies, as defined in the Safe Drinking Water Act, are those that serve 25 or more people. It is estimated that in the United States there are currently in excess of 45,000 public groundwater supplies that serve more than 100 million people. Furthermore, there are more than 11 million private wells that provide drinking water to an additional 25 to 30 million residents, mainly in rural areas (50). Because groundwater is not stationary, contaminated groundwater resulting from hazardous waste disposal could conceivably become the drinking water source for a large segment of the U.S. population.

EXPERIMENTAL APPROACH

Rationale for Selecting Chemical Mixture

The Lockheed Engineering and Management Services Company, Inc., (51) and the Mitre Corporation (52) conducted, for the EPA, two surveys on the contamination of groundwater in the vicinity of hazardous waste disposal sites. From the results of these surveys we selected a mixture of 28 chemicals (Table 1) that broadly represent the drinking water contaminants most frequently found.

In the Lockheed survey, the data base includes analyses of groundwater samples in the vicinity of 180 hazardous waste disposal sites located throughout the United States, covering all 10 EPA regions. The information given in the survey includes total numbers of analyses (i.e., total analytical attempts), total numbers of positive analyses (i.e., the number of analyses that exceeded the detection limits for that chemical), percentage of positive analyses (i.e., frequency of detection), and maximal and average concentrations of each contaminant (in micrograms per liter). Selection of chemicals for the mixture was made after excluding some of the commonly known minerals (e.g., calcium, magnesium, sodium) because of their lower toxicity and ubiquitous presence. Cut-off points were set at $\geq$ 10% positive analysis and a minimum of 100 positive analyses to limit the number of chemicals. This selection process yielded 37 chemicals (15 organics and 22 inorganics) out

TABLE 1

SELECTED CHEMICALS REPRESENTING MOST FREQUENTLY DETECTED GROUNDWATER CONTAMINANTS

Chemicals	Percent Positive	Total Positive	Total Analysis	Max. Conc. (ppm)	Avg. Conc. (ppm)
Acetone	12.4	117	944	250	6.90
Arochlor 1260	0.9	14	1,576	2.9	0.21
Arsenic	25.9	712	2,747	3,670	30.6
Benzene	11.2	464	4,151	1,200	5.0
Cadmium	25.7	653	2,545	225	0.85
Carbon tetrachloride	7.0	378	5,372	20	0.54
Chlorobenzene	5.5	225	4,077	13	0.10
Chloroform	28.4	1,565	5,512	220	1.46
Chromium	36.3	1,021	2,812	198	0.69
Cyanide	19.8	179	906	181	1.42
2,4-D[a]	7.7	32	415	1,700	53.15
DEHP[b]	11.5	176	1,524	5.8	0.13
1,1-Dichloroethane	17.8	931	5,218	56.1	0.31
1,2-Dichloroethane	14.2	750	5,292	440	6.33
1,1-Dichloroethylene	25.2	1,346	5,343	38.0	0.24
1,2-*trans*-Dichloroethylene	29.1	1,565	5,383	75.2	0.73
Ethyl benzene	5.9	230	3,933	25	0.65
Lead	32.2	861	2,676	31,000	37.0
Mercury	14.4	312	2,168	50	0.34
Methylene chloride	19.2	777	4,043	7,800	11.2
Nickel	34.0	600	1,764	95.2	0.50
Phenol	33.4	324	969	380	3.27
	(13.6	237	1,764	7,713	34.0)
Tetrachloroethylene	36.0	2,296	6,383	21,570	9.68
	(92.9	461	496	0.5	0.02)
Toluene	11.6	472	4,061	1,100	5.18
1,1,1-Trichloroethane	18.9	1,007	5,337	618	1.25
Trichloroethylene	51.3	3,011	5,866	790	3.82
Vinyl chloride	8.7	219	2,524	516	0.80
Xylenes	42.6	43	101	150	4.07

Information Source: Survey conducted for the U.S. EPA by Lockheed Engineering and Management Services Company, Inc., July 1985. Numbers in parentheses represent duplicate survey results.

[a] 2,4-D = 2,4-Dichlorophenoxyacetic acid.
[b] DEHP = Di(2-ethylhexyl)phthalate.

of a total of more than 1000 organic and inorganic chemicals on the survey list. These 37 chemicals were compared against the Mitre survey list (52), which is a compilation of the contaminants most commonly detected in and around (i.e., in groundwater, surface water, and air) the 881 hazardous waste sites on the National Priority List. Based on the frequency of occurrence and the known toxic potential, 21 chemicals (14 organics and 7 inorganics) were selected from these 37 candidates to be included in the chemical mixture for the proposed studies. In addition, seven other chemicals – Arochlor 1260; carbon tetrachloride; monochlorobenzene; 2,4-dichlorophenoxyacetic acid (2,4-D) sodium salt; ethyl benzene; vinyl chloride; and xylenes – were also chosen even though they did not meet one or both of the cut-off points (see Table 1). The selection of these chemicals was based on additional consideration of criteria including extensive use, large commercial volume, potential impact to the environment, representation of certain classes of chemicals, and/or frequency of detection at and near hazardous waste sites.

Following preliminary work on chemistry development, however, it became apparent that potassium cyanide, 2,4-D sodium salt, and vinyl chloride should be deleted from the mixture because of their respective formulation problems, including evolution of highly toxic vapor, chemical interactions, and extreme volatility. Therefore, a final mixture of 25 chemicals (Table 2) will be used for the proposed toxicity studies.

Dosage Setting and Rationale

Five dosage groups plus one control group (deionized water) will be employed for the proposed studies (Table 2). The baseline group (i.e., 1 ×) is set at the average concentrations for individual component chemicals (see Table 1). The other four dosages are set at 0.1-, 10-, 100-, or 1000-fold (i.e., 0.1 ×, 10 ×, 100 ×, or 1000 ×) concentrations of those of the baseline group except those chemicals with known high acute toxicity (see item 5 below). If use of the higher dose formulations presents problems (e.g., insolubility, chemical interaction) in the initial phase of chemistry development, the experimental design of the toxicology studies will be adjusted accordingly (e.g., fewer dosage groups will be used).

Although the original purpose of this study was to investigate the health effects of drinking water contamination on the basis of a worst-case scenario, some consideration was given to experimental practicality when setting the dosage levels. The guidelines for dose selection were generated based on the following considerations.

1) We chose to use the *average concentration* rather than the *maximal concentration* detected for each groundwater contaminant as the baseline (i.e., 1 ×, see Table 2). For instance, the maximal concentration recorded for lead was 31,000 ppm (Table 1). Because

TABLE 2

A CHEMICAL MIXTURE AND THE DOSAGES FOR THE SUBCHRONIC TOXICITY STUDIES IN FISCHER 344 RATS AND B6C3F$_1$ MICE

Chemicals	CAS No.	Dose Levels (ppm)				
		0.1 ×	1 ×	10 ×	100 ×	1000 ×
Acetone	67-64-1	0.69	6.90	69	690	6,900
Arochlor 1260	11096-82-5	0.02	0.21	2.1	21	210
Arsenic trioxide[a]	1327-53-3	8.08	80.8	200	200	200
Benzene	71-43-2	0.50	5.0	50	500	5,000
Cadmium chloride[a]	10108-64-2	0.14	1.39	13.9	139	880
Carbon tetrachloride	56-23-5	0.05	0.54	5.4	54	540
Chlorobenzene	108-90-7	0.01	0.10	1	10	100
Chloroform	67-66-3	0.15	1.46	14.6	146	1,460
Chromium trioxide[a]	1333-82-0	0.13	1.33	13.3	133	900
DEHP[b]	117-81-7	0.01	0.13	1.3	13	130
1,1-Dichloroethane	75-34-3	0.03	0.31	3.1	31	310
1,2-Dichloroethane	107-06-2	0.63	6.33	63.3	633	6,330
1,1-Dichloroethylene	75-35-4	0.02	0.24	2.4	24	240
1,2-*trans*-Dichloroethylene	156-60-5	0.07	0.73	7.3	73	730
Ethyl benzene	100-41-4	0.07	0.65	6.5	65	650
Lead acetate[a]	301-04-2	5.81	58.1	581	1,200	1,200
Mercuric chloride[a]	7487-94-7	0.05	0.46	4.6	10	10
Methylene chloride	75-09-2	1.12	11.2	112	1,120	1,670
Nickel sulfate[a]	7786-81-4	0.13	1.32	13.2	132	210
Phenol	108-95-2	0.33	3.27	32.7	327	3,000
Tetrachloroethylene	127-18-4	0.97	9.68	96.8	968	9,680
Toluene	108-88-3	0.52	5.18	51.8	518	5,180
1,1,1-Trichloroethane	71-55-6	0.13	1.25	12.5	125	1,250
Trichloroethylene	79-01-6	0.38	3.82	38.2	382	3,820
Xylenes	1332-20-7	0.41	4.07	40.7	407	4,070
Total concentration of all chemicals		**20.45**	**204.47**	**1,436.7**	**7,921**	**54,670**

[a] The baseline dosages for these chemicals were adjusted to give the respective elemental or ion concentrations (average concentrations) as shown in Table 1; safety factors were then incorporated accordingly.
[b] DEHP = Di(2-ethylhexyl) phthalate.

it is already 3.1%, the application of safety factors (i.e., 10 x, 100 x) would have made the high dosage levels unrealistic for experimentation.

2) In applying the safety factors (i.e., 10 x, 100 x, 1000 x) for each individual component, the maximal concentration will not exceed 10,000 ppm (i.e., 1%).

3) Because there are 25 chemicals in the mixture, the combined total concentration of all chemicals at the highest dosage will not exceed 10%. This consideration alone, therefore, ruled out the group with a safety factor of 10,000 x, because, even with an upper limit of 1% for any given chemical, the combined total concentration of all chemicals in the 10,000 x group would have been greater than 13%.

4) The baseline group (i.e., 1 x in Table 2) has a combined total concentration of all chemicals of approximately 204 ppm. To anticipate possible synergistic effects of chemicals, a lower dosage group (i.e., 0.1 x) was incorporated.

5) Even though the pharmacokinetics of a chemical are different when dosed via the drinking water route or a single bolus (e.g., gavage, i.p.), the dosage of any chemical at any level in the mixture will not exceed the LD_{50} (p.o. or i.p.) or other similar, available acute toxicity indices of that chemical. The LD_{50} values and other acute toxicity indices were obtained from the Registry of Toxic Effects of Chemical Substances, 1980.

6) In the event of solubility limitation of some of the components at the highest achievable dosage level, a saturation solution for these compounds, in the presence of other solubilized components, will be prepared. After this highest dose with saturation levels of certain components has been formulated, the lower dose levels will be prepared by diluting this dosing solution of greatest concentration by factors of 10 until the anticipated lowest dosage level has been reached.

Animals

Fischer 344 rats and $B6C3F_1$ mice (randomized into treated and control groups) will be individually housed.

Route of Administration

Dosed drinking water will be employed. The chemicals will be solubilized in deionized water without using any solubilizing agent. The control animals will be given deionized water.

Chemistry Development

Prior to the animal experiments, methods will be developed for dose formulation of the 25-chemical mixture and for dose analysis. The purity of each of the 25 chemicals and the stability

and chemical speciation of the mixture under normal laboratory conditions will be determined. We understand that the proposed concentrations of a number of chemicals in the 1000 x group (Table 2) exceed the reported water solubility of these individual chemicals. In the absence of any knowledge of the solubility of these chemicals under the conditions of a 25-chemical mixture as proposed, we will rely on empirical findings to guide the design of the subsequent sections. Accordingly, the information generated from our work on chemistry development will be utilized to adjust, if necessary, the experimental design of the animal studies described below.

Initial concepts of the dosing solution preparation procedure are as follows: Several stock solutions, each containing several of the 25 chemicals, will be prepared. Stock solutions will then be diluted to the proper concentrations (i.e., dosing solutions) for experimentation. The dosing solutions will be analyzed for one or more "marker chemicals" (i.e., chemicals representative of each stock solution) to confirm accuracy of dose formulations.

14-Day Palatability and Mortality Study in Fischer 344 Rats and B6C3F$_1$ Mice

The palatability and preliminary mortality information of the chemical mixture of drinking water contaminants proposed for the subchronic studies will be investigated.

Treatment and Observation. The basic scheme for animal assignments to the various groups is shown below in Table 3.

TABLE 3

ANIMAL ASSIGNMENTS FOR 14-DAY PALATABILITY AND MORTALITY STUDY

	Number of Animals		Sexes		Species		Dose Levels		Totals
Test Groups	10	x	2	x	2	x	3	=	120
Controls	10	x	2	x	2	x	1	=	40
Total									**160**

The dose levels for this study will be the three highest levels (e.g., 1000 x, 100 x, and 10 x of the average concentrations of each component contaminant, if feasible) proposed for the subchronic studies (see Table 2). Administration will be continuous (7 days/week) for 14 days using dark glass bottles with as little headspace as possible, to avoid photodecomposition and volatility. Animal body weights will be recorded on days 0, 1, 4, 7, and 14 of the study. Dosing solutions or deionized water (for control groups) will be changed daily to minimize possible instability of the chemicals in the mixture. The frequency with which dosing solutions will be prepared will depend upon the outcome of the chemistry development work. Twice per week, each animal's 24-h water

consumption will be measured. Animal food consumption will be measured weekly. During this 14-day study, the dosing solutions will be analyzed once as follows: Two drinking water samples of (1) the mixing room sample, at the beginning of a dose-preparation period while the solution or water is fresh, and (2) the animal room sample, at the end of the last exposure day for that dose-preparation period, are to be taken from each dosage group (note: each dose level is the same for both sexes of both species) and immediately analyzed for concentration of marker chemicals. During this 14-day study, one referee sample will be analyzed for all the chemicals in the mixture. Based on the preliminary chemistry development work, it is anticipated that five marker chemicals in the dosing solutions will be analyzed (three by GC, one by HPLC, and one by atomic absorption spectroscopy).

This is a palatability and mortality study only, the reason for which is given in detail in the Discussion section. Therefore, other than daily clinical observations (including checking for mortality) and the measurements outlined above, no other procedures will be done. Because one of the first signs of reduced water intake is that the animals stop grooming themselves, particular attention will be paid during the daily observation for roughness and "brownish reddish" discoloration of fur. To conserve resources, no gross pathology or histopathology will be done on these animals. (See Discussion section for details.)

Subchronic Drinking Water Toxicity Studies in Fischer 344 Rats and B6C3F$_1$ Mice

The health effects of a chemically defined mixture of primary drinking water contaminants following subchronic exposure to Fischer 344 rats and B6C3F$_1$ mice will be evaluated.

Treatment and Chemical Analyses. In this study, rats and mice of both sexes, 20 per dosage group, will be used. The five dosage levels (same for both rats and mice) of the chemical mixture of drinking water contaminants in deionized water are as shown in Table 2; however, the doses may be adjusted depending on the solubility of the chemicals, the results from the chemistry development work, and the results of the palatability and mortality study. The control groups will be given deionized drinking water. Animals will be dosed for 13 weeks. If dose-related toxic signs (e.g., mortality, body weight depression, poor clinical state, neurobehavioral signs) are evident, one-half of each group (i.e., 10 animals/sex/group/species) will be sacrificed at the end of 13 weeks, and the other one-half will be maintained for six months without further dosing (for recovery studies). If there are no toxic signs at the end of 13 weeks, one-half of each group will be sacrificed as described above, and the other one-half will be dosed continually for six months (for six-month subchronic studies), at which time they will be sacrificed. Dosing will be continuous (7 days/week) via drinking water using dark glass bottles with as little headspace as possible, for the same reasons given earlier. Dosing solutions or deionized water (for control groups) will be changed daily to minimize loss

and/or possible instability of the chemicals in the mixture. The frequency with which dosing solutions will be prepared will depend upon the outcome of the chemistry development work. Once per week, each animal's 24-hour water consumption will be measured. Animal food consumption will be measured weekly. The dosing solutions will be analyzed at the beginning of the study and monthly thereafter. For each analysis, two drinking water samples from (1) the mixing room sample, at the beginning of a dose-preparation period while the solution or water is fresh, and (2) the animal room sample, at the end of the last exposure day for that dose-preparation period, will be taken from each dosage group (note: each dose level is the same for both sexes of both species) and immediately analyzed for concentration of marker chemicals. Two referee samples, one each at the beginning and end of this subchronic study, will be analyzed for all the chemicals in the mixture. Based on the preliminary chemistry development work, it is anticipated that five marker chemicals in the dosing solutions will be analyzed (three by GC, one by HPLC, and one by atomic absorption).

The basic scheme for animal assignments to the various groups is shown in Table 4.

TABLE 4

ANIMAL ASSIGNMENTS FOR SUBCHRONIC DRINKING WATER STUDIES

Study	Group	Number of Animals		Sexes		Species		Dose Levels		Totals
Main Toxicity	Test	20	×	2	×	2	×	5	=	400
	Control	20	×	2	×	2	×	1	=	80
Clinical Path.[a]	Test	10	×	2	×	1[a]	×	5	=	100
	Control	10	×	2	×	1[a]	×	1	=	20
Neurobehavioral[b]	Test	10	×	2	×	2	×	5	=	200
	Control	10	×	2	×	2	×	1	=	40
Subtotal										840

[a] Rats only; these animals are to be used for clinical pathology evaluation (i.e., hematology, clinical chemistry, and urinalysis). Serial blood sampling will be carried out on these rats, and they will be held until the 6-month sacrifice. No gross pathology or histopathology will be done on these animals.

[b] These animals are for neurobehavioral and special neuropathology studies only and will be held until the 6-month sacrifice. No routine gross pathology or routine histopathology will be done on these animals

Observations and Pathology. See 14-day study above for the daily clinical observations. Complete necropsies will be performed on all treated and control animals that die or that are sacrificed, and all tissues will be saved in formalin for possible histopathologic examination. Hematoxylin and eosin (H&E) slides will be prepared for complete histopathology of all animals in all groups (List 1).

LIST 1
TISSUES FOR WHICH COMPLETE HISTOPATHOLOGIC EVALUATIONS ARE CONDUCTED IN A 13-WEEK STUDY

Gross lesions and tissue masses (and regional lymph nodes, if possible)
Mandibular and mesenteric lymph nodes
Salivary gland
Femur, including marrow and epiphysis
Thyroid
Parathyroids
Small intestine (duodenum, jejunum, ileum)
Large intestine (cecum, colon, rectum)
Liver
Gall bladder (mouse only)
Prostate
Testes/epididymis/seminal vesicle
Ovaries
Lungs and mainstem bronchi
Nasal cavity and nasal turbinates (3 sections)
Preputial or clitoral glands (paired)
Heart
Esophagus
Stomach (including forestomach and glandular stomach)
Uterus
Brain (three sections, including frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons)
Thymus
Trachea
Pancreas
Spleen
Kidneys
Adrenals
Urinary bladder
Pituitary
Spinal cord and sciatic nerve (if neurologic signs were present)
Eyes (if grossly abnormal)
Mammary gland (to include surface skin)
Pharynx (if grossly abnormal)
Thigh muscle

A complete histopathologic examination inclusive of gross lesions will be done on all control animals, all animals in the highest dose group with at least 60% survivors at the time of sacrifice, and all animals in higher dose groups including the early deaths and the survivors. Chemically related lesions (target organs) will be identified, and these organs plus gross lesions will be examined in lower doses until a no-observable effect level is determined.

Clinical Pathology Parameters. A separate group of 10 rats will be reserved for serial measurements of clinical pathology parameters. Therefore, all measurements of hematologic, clinical chemistry, and urinalysis parameters will be performed on those animals. In mice, all measurements are done at terminal sacrifice in the "Main Toxicity" group; no separate groups of mice are designated for clinical pathology studies.

A. ***Hematology (Rats and Mice).*** Red blood cell (RBC) count, packed cell volume or "hematocrit" (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) count, WBC differential count (absolute), absolute reticulocyte (RETIC) count, platelet (PLAT) count, and qualitative evaluations of RBC, WBC, and platelet morphologic features will be made at the 13-week and 6-month terminal sacrifices.

B. ***Clinical Chemistry (Rats and Mice).*** Sorbitol dehydrogenase, creatine phosphokinase, alanine aminotransferase, γ-glutamyl transpeptidase, creatinine, and albumin will be measured for rats at 3, 15, 45 days; 13 weeks; and 6 months of the study in the animals designated for clinical pathology studies. For mice, clinical chemistry analyses will be performed at 13 weeks and 6 months of the study.

C. ***Urinalysis and Urinary Enzyme Determination (Rats only).*** Volume (16 h), appearance, specific gravity, glucose (quantitative), protein (quantitative), microscopic examination of sediment from centrifuged urine, and two urinary enzymes, alkaline phosphatase and aspartate aminotransferase, will be measured at 3, 15, 45 days; 13 weeks; and 6 months of the study in rats designated for clinical pathology studies.

Special Analyses and Special Studies

A. ***Neurobehavioral Studies.*** Because many of the chemicals in the proposed mixture cause acute or chronic neurotoxicity, neurobehavioral studies and, possibly, neuropathological investigations will be conducted.

   Neurobehavioral toxicity tests (forelimb and hind limb grip strength, startle-electronic measurement, tail flick, motor activity in the Automex, and foot splay) will be performed on the animals designated for neurobehavioral testing in the subchronic studies at

preexposure (i.e., 1 to 2 days before dosing) and every 6 weeks after the initiation of dosing.

If neurobehavioral effects are evident, the special neuropathologic procedures described below will be carried out. Whole-body perfusion will be carried out on 10 randomly selected animals (five males, five females) from each group (i.e., animals designated for neurobehavioral studies) following anesthesia. The animals will be perfused with buffered glutaraldehyde fixative preceded by an initial flush with heparinized saline (53, 54). After the perfusion, nerves will be removed from both the right and left sides; these will include the hind limb-sciatic nerve with tibial and sural branches, the sciatic nerve-related dorsal root ganglia (one on each side), the spinal cord, and the brain. These tissues will be further immersed in the fixative for 24 h. Teased nerve sections (54) will also be prepared on these animals.

Nerve tissues from three control animals and from three of the five animals/group/species in the highest dose group with at least 60% survival rates will undergo neuromorphological examination by light microscopy. The tissues will be prepared for examination with H&E, Luxol fast blue, and cresyl violet stains (6μ paraffin sections). If lesions are seen, a no-observable effect level will be established. In preparation for electron microscopic examination, epon sections (1μ toluidine blue stain) of the nerve tissues of the other two animals of the highest dose group and two control animals of each sex will then be prepared and examined by light microscopy. This examination will aid the sampling in the subsequent ultrastructural examination by electron microscopy. If histopathologic abnormalities are seen, morphometric analyses will be conducted on the teased nerve sections of selected animals.

B. ***SMVCE.*** This procedure will be performed (at 13 weeks and 6 months) on rats and mice from the control and three dose groups. The three dose groups are to be selected on the basis of the data on survival, body weight gain, and clinical signs after weeks 11 and 24, respectively, for the 13-week and 6-month sacrifices.

C. ***Blood Smears.*** These will be prepared (at 13 weeks and 6 months) from all mice for the micronucleus assay.

DISCUSSION

There are a number of unusual factors that must be considered in the design of a toxicology study on a complex, chemically-defined mixture. Specific points that are unique to our effort of testing chemical mixtures related to hazardous waste sites include (1) the narrowness of focus and

rationale for end points measured, (2) the "grab sample" acquired directly from hazardous waste sites versus chemically defined "cocktail," (3) the comprehensive 14-day study versus a 13-week study as a "first line" investigation, and (4) the rationale for the 25 chemicals used in the mixture. The discussion below reflects our philosophy toward this controversial area of the toxicology of chemical mixtures.

In this study, the objective is to investigate the possible adverse health effects in rodents following subchronic ingestion of a prototype mixture of groundwater contaminants. The adverse health effects will be directly related to any treatment-related toxic responses observed in the proposed subchronic toxicity study. For instance, significant body weight depression without obvious differences in food consumption would suggest a growth retardation effect; marked elevation of alanine aminotransferase might suggest liver damage; and distinct neurobehavioral signs followed by Wallerian degeneration in the nervous system would suggest neuropathy. When a chemical mixture has more than two components, it is difficult to speculate on the experimental outcome. With a mixture of 25 chemicals, it is impossible to predict the target organs. Therefore, all the toxic end points routinely employed under the NTP Task I in the 13-week subchronic toxicity study (see the NTP General Statement of Work) were automatically included. The neurobehavioral end point was included because (1) lead and mercury are well-known neurotoxicants, (2) there are other chemicals on our list that were suggested to have produced or been involved in the development of neurotoxicity upon repeated dosing, and (3) many of the chemicals on the list certainly possess the capacity to induce neurotoxicity after acute administration.

There are two extreme outcomes of the study that warrant further discussion. The first case is that no observable effects are seen after subchronic exposure of the animals to the 25-chemical mixture at the proposed dose levels. The other extreme is that severe toxicity results at even some of the lower doses. In either case, such information will be useful in the risk assessment of contaminated groundwater and might stimulate further research into the mechanisms of toxicity induced by chemical mixtures. Investigating the toxicology of complex mixtures is particularly relevant because the general public is demanding information on chemical mixtures and toxicological interactions (e.g., Federal Register 50:30517-30519, 1985).

In designing our experiment, the consideration of whether to test grab samples directly from hazardous waste sites versus a chemically defined "cocktail" was a central issue. With so many hazardous waste sites and with each of those having a unique set of conditions (i.e., chemical content, geographical and geological features), it is impossible to find a grab sample that could be considered representative of the various sites. Furthermore, it is often impossible to chemically characterize and quantify all the contents for such a sample. A chemically defined mixture based on

an extensive survey of groundwater contamination, on the other hand, can be used to build a worst-case scenario and can be analyzed both qualitatively and quantitatively.

In this report, the experimental design is such that a limited 14-day palatability and mortality study will be followed by a comprehensive subchronic toxicity study with an exposure time of as long as six months.

Scientifically, the single most important reason to conduct a 14-day study leading toward subchronic and chronic studies is to set the dose levels for the longer term studies. Because of the unique nature of our mixture study, the dose levels have already been set for subchronic and chronic studies (i.e., a baseline level for known human exposure levels for a mixture of chemicals with safety factors such as 10 x , 100 x , incorporated). Other than variations of the number of dose levels and the upper boundary for the highest dosage at the stage of each of the three studies, the dose levels for 14-day, subchronic, and chronic studies will be identical. Without the issue of dosage setting, the utility of a 14-day study, as in this instance, would be mainly to explore palatability and mortality in preparation for the subsequent subchronic study. Furthermore, it is unlikely that any target organ toxicity, which is detrimental to the animal, would be detected in a 14-day study and not detected in a subsequent 13-week study under identical dose levels and exposure frequencies. Because the most pertinent health issue of drinking water contamination is the effects of long-term, repeated exposure, our approach is to conduct a limited 14-day study and shift the first-line thorough examination to the 13-week study stage.

In addition, there are economic and logistic reasons for not conducting a more thorough 14-day study. The most expensive part of an animal toxicity study is generally the histopathological evaluation. Considering the issues discussed above and the fact that clinical observation, water and food consumption, and body weight measurements have already been included in the 14-day palatability and mortality study, it is not prudent to conduct full-scale clinical and anatomical pathology evaluations in the 14-day study. The NTP contractual process for toxicology studies is a time-consuming one. The time it takes to design an acceptable protocol, prepare and issue a Request For Proposal, carry out the competitive bidding process, and award the contract to an NTP Master Agreement Laboratory is approximately eight months. Between the time of award and the first day of dosing, there may be another two months for chemistry development work, animal quarantine, and other issues. Therefore, it is helpful to minimize delay in obtaining the most critical information.

At the outset we knew that the number of chemicals used in the mixture would be a controversial issue; therefore, we chose to remain an open minded about that matter but to adhere to our goal of exploring the health effects of a worst-case scenario of groundwater contaminants.

Consequently, the facts of the survey results governed that the number of chemicals in the mixture would be large, and that the high doses in the experimental design would have relatively high chemical concentrations. Information from two very comprehensive surveys conducted by the EPA was utilized to come up with 21 candidate chemicals. Because of the rigidity of our self-imposed guidelines and preliminary chemistry development work, we decided to revise the list to 25 chemicals (this exercise was detailed in the sections on Rationale for Selecting Chemical Mixture and Dosage Setting and Rationale).

In terms of experimental logistics, perhaps a mixture of fewer chemicals would be easier to manage. But we feel that we can manage a study with such a complex chemically defined mixture. Scientifically, however, even with binary mixtures, depending on the concentrations of each component given to the animals and the temporal relationship between the two treatments, results may be unpredictable. It is, therefore, debatable whether a 10-chemical mixture is scientifically more "manageable" than a 25-chemical mixture. A logical and scientifically sound approach to studying a 25-chemical mixture is to systematically investigate all single chemicals, and then all binary, tertiary mixtures, et cetera, until reaching the mixture with all 25 chemicals. Furthermore, these studies are to be conducted according to a protocol that takes into consideration the probable dose- and time-related toxicological interactions. However, to calculate all the possible combinations of a 25-chemical mixture, one would follow the formula $(2)^{25}-1$, which would result in 33,554,431 combinations. Using a very conservative estimate of $100,000 for a 13-week subchronic study with a single species, this alone translates into more than 3 trillion dollars for such a project.

CONCLUSION AND PERSPECTIVE

Throughout our many deliberations during the development of this project, we came to the realization that there is no perfect protocol for this study. However, we felt that research work must be initiated on this very important issue of groundwater contamination from hazardous waste sites. Our philosophy, therefore, is to take the first step despite the inevitability of a less than perfect protocol and the limitation of knowledge and resources. We believe that, as in the case of other scientific endeavors, little by little we will be able to contribute to the understanding of the mosaic of the toxicology of chemical mixtures.

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An expert panel of scientists for reviewing the chemical mixture study proposal at the NIEHS/NTP consists of Dr. G.C. Becking (World Health Organization/International Programme on Chemical Safety), Dr. D. Couri (Ohio State University), Dr. R. Griesemer (Oak Ridge National Laboratory), Dr. C. Halder (Amoco Corp./American Petroleum Institute), Dr. D. Hughes (Proctor & Gamble Company/American Industrial Health Council, unable to attend the meeting), Dr. R. Kimborough (Centers for Disease Control/Agency for Toxic Substances and Disease Registry), Dr. R.J. Kociba (Dow Chemical Company), Dr. R.W. Niemeier (National Institute for Occupational Safety and Health), Dr. G. Plaa (University of Montreal), Dr. A. Turturro (National Center for Toxicological Research), Dr. H. Vainio (International Agency for Research on Cancer/World Health Organization), Dr. D.C. Villeneuve (Health and Welfare Canada), Dr. Craig Zamuda (U.S. Environmental Protection Agency/Superfund Program, unable to attend the meeting).

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PANEL DISCUSSION I

Lyman Condie, Ph.D. – Rapporteur

U.S. Environmental Protection Agency

DR. CONDIE: This session was entitled "Predicting Health Effects from Groundwater Pollutants." I think we all agree that we have a long way to go to predict health effects issues in groundwater pollutants. I would like to give an example. When I joined the Environmental Protection Agency (EPA) about eight years ago, the attorneys in the regional office were thrilled. There was a toxicologist who had joined the staff, and he would be able to answer all their questions and provide them guidance and evidence and testimony to legal suits against contaminating responsible individuals. Well, when the attorneys would march in my office they would give me lists and lists of chemicals that are usually found in waste dump barrels, and they would want me to give a definitive explanation of the toxicology so they could bring lawsuits against these chemicals. I asked two questions regarding the same two issues, human exposure and risk assessment. I asked what is the exposure of these chemicals to humans, because I needed an exposure to estimate the toxicology, and I wanted a reasonable list of the chemicals. Obviously they only had a partial list. Why use this as a smoke screen? I knew, as a toxicologist, that I could not really interpret the toxicology of the myriad of chemicals found in these waste sites. I only had one meeting with these attorneys; they never came back to my office because, obviously, it's a common problem that we lack data. We lacked data seven years ago and we still lack data. If you look at the models, the various speakers, the one thing that is generally lacking in the toxicology of environmental chemicals is good-quality data. One reason why it is important to study groundwater pollutants is because groundwater is the drinking water source for half the people in this country. The groundwater pollution is increasing, as witnessed by various surveys. In preparation for a talk I plan to give next week, I surveyed and compared different lists. I compared the IRP chemical list with some of the EPA lists, and, in general, there is about an 80% correlation of the common chemicals among the list. So one approach would be to look at the more common chemicals. One thing that was alluded to by the last speaker is that there is an attempt among Federal Government agencies to try to address the toxicology of complex mixtures. Up until now the Government has looked at contamination problems from a chemical-by-chemical basis, and we are moving into the complex mixture area. So my first question for the panel is to comment on their views of the utility of conducting chemical-by-chemical specific toxicology assessments versus whole-mixture toxicology evaluations. That will be the first question of the panel, and we would like to lead into questions from the audience. So, which of you gentlemen would like to address that question of chemical-specific evaluations versus complex-mixture evaluations?

DR. YANG (NIEHS): One of the issues we are repeatedly confronted with is the definition of a complex mixture. How do you define a complex mixture? I am not trying to dodge the question, but in our daily life I really cannot think of any single way in which we are being exposed to a single chemical; because you can take a pill or aspirin, but there are binding agents and you usually use water and some use iced tea and so on. You are dealing with quite complex mixtures. Even in chemical plants where they produce only one single chemical, your personal hygiene, your food, and so on are going to contribute to the overall homeostasis of the body response. Therefore, this is a thing with which we at NTP always struggle. Because on the one hand we want to pinpoint what is the bad actor. Particularly, for example, in pesticide preparation. Whether the main active ingredient is the troublemaker and so on. On the other hand, could there be some synergistic effect or antagonistic effect? I really don't know the answer. We have traditionally paid more attention to the single chemical, but I think now there is a tendency to look more and more at various formulations and mixtures. This is part of our effort, at the very least, to address this problem.

DR. TRAVIS (OAK RIDGE NATIONAL LABORATORY): My reaction to this proposal to test the complex mixtures is mixed. I understand the reason for doing so, because we are exposed to these complex mixtures and hopefully a bioassay like this would give us some sort of upper boundary estimate of risk of complex mixtures. We all recognize the limitations in that they have 28 compounds in it and somebody at a different site might be only exposed to 25 of those. That might change the risk estimate. Those things aside, my first reaction after having talked on pharmacokinetics and heard a talk on sort of biologically based low-dose extrapolation models – we need more information to interpret a long-term animal bioassay with 28 compounds. It would seem, for instance, that we'd like to know cell turnover rates. For that you're giving a complex mixture to all of these animals and it could be that there is going to be some toxicity, and increased cell turnover rates might account for a large part of the carcinogenicity that is manifested. You would like to at least know if there is any increase in cell turnover rates as a result of those exposures. It would be interesting to know about pharmacokinetics of various compounds – even in the mixture if you could radiolabel some of the 28 compounds and look at the pharmacokinetics of them within the mixture. But I know those things probably cannot be done. My basic reaction is that I would like to see them pull back and build up. Start with two or three compounds, understand the pharmacokinetics of those and the implication of those for long-term animal bioassays. In other words, get more understanding in the process and work up so that we could predict what would happen if we gave an exposure of 25 of these instead of the 28 or we could predict what would happen if we only had 7 of them. I guess that may be a long-term research goal.

DR. STARR (CIIT): I don't have much that is of any consequence to say about this. I'm reminded of a statement by Will Rogers that it's not what we don't know that gets us into trouble it's what we

know that ain't so. In this respect, experiments like Ray Yang described are going to tell us something about what we don't know. If we make assumptions that are unjustified, we are likely to make mistakes. We heard a paper yesterday in Washington by Dan Kruske, a very fine statistician from Health and Welfare Canada who has looked at possible interactive effects of simple mixtures and binary mixtures on low-dose extrapolation. What he was able to show was that in the context of this kind of oncogenic model that I have been talking about – the very simple cases where the transition rates are linear in the individual concentrations and the product of the two concentrations – that at very very low doses the interactive effects go away. So it's quite possible that if you do these experiments at 1000 times and detect interactions, those may actually be confounding with the true effects of exposure at the low doses that you are interested in.

DR. YANG: Can I make a comment? I think one thing I want to stress is this: we should consider this our first step toward this very complex problem; and my personal philosophy is that even if this experiment is totally fruitless, if there is such a thing, then I would like this to serve as an example for people who follow us. So they don't have to make that mistake again. I think in our institute we are also planning on using this mixture, if we can formulate it, for reproductive toxic assessment and for immunological toxicology assessment as well. Earlier I alluded that we certainly want to look at the chronic effects of something like this. Maybe even from the point of view of promotional effects toward some known carcinogens and so on. I think your question would be very interesting for those heavy smokers. Let's say if they were exposed to drinking water contaminants at very low levels for a lifetime. Would that have any effect on the possibility of carcinogenesis?

DR. CONDIE: One reason why I raise this issue is because of passage of the new legislation the EPA is being forced into – an assessment mode of going out and assessing individual waste sites; not only from a chemical approach but from a bioassay approach. You will probably see in the future more and more research proposals from the EPA to look at complex mixture research, and I believe that the military arm of the environmental research also is moving into looking at some complex mixture research. I would like to open the session up to questions from the audience.

DR. McKONE (LAWRENCE LIVERMORE LABORATORIES): I address this question to the full panel, but I think it was raised in my mind by the talk that Curtis Travis gave. The issue is that the regulatory process currently is pretty well entrenched with the use of low-dose extrapolations and multistage sequel linearized multistage models. We are now suggesting better approaches. To what extent do you anticipate a lot of resistance to this because the process is firmly entrenched right now in some of the more traditional approaches? How can we cope with some of the resistance we are going to run into when we try to refute some of the ideas in the regulatory process?

DR. TRAVIS: I believe there is some caution in adopting these new methodologies, but EPA is investigating pharmacokinetics for instance. They are funding me. I think that EPA will endorse pharmacokinetics within a year or so and start using it as a routine tool when it can be used – when we have chemical-specific information. The biologically based low-dose extrapolation models will take much longer to be incorporated into the regulatory process, I believe, because they are such a departure from what we have been doing. I think EPA is interested in them, they are looking at them, they are evaluating them. What we need in both of these areas, pharmacokinetics and biologically-based low-dose extrapolation models, is more experience. We need to apply them to more chemicals, get a bigger data base of experience, and from that experience identify the biological experiments that need to be done and the biological parameters that need to be measured to validate and give us more assurance about the use of the models. Then I believe they will be incorporated into the regulatory process.

DR. PEIRANO (U.S. ENVIRONMENTAL PROTECTION AGENCY): More of a comment on that. I'm with the Environmental Criteria and Assessment Office in Cincinnati which deals with deriving criteria for drinking water and air, etc. I can assure you that we are starting to incorporate pharmacokinetics and other types of biomarkers into assessments. The biggest stumbling block in doing this is, number one, input data on such simple things as absorption of material into the body; in other words, from external exposure to what is absorbed into the body in the systemic circulation. So just in that very rudimentary part of pharmacokinetics where we have that information we are starting to incorporate it into the assessments. It is a very early process that we are starting with, but we are progressing and as more data become available and as the models become enhanced with more partition coefficients and so forth, you are going to find much better assessments being done. Of course with a lot of criticism by different people.

DR. MEHENDALE (UNIVERSITY OF MISSISSIPPI): Just a comment concerning Ray's presentation, which was excellent. I wonder if you have thought of looking at the products of such a mixture rather than feeding the entire mixture to the animals. I can visualize even with 25 chemicals you might have at least 25, but may be not even 25 at times, depending upon the chemicals, or perhaps exceed that number in actuality. There are going to be reactions. So is it really 25 chemicals or is it 150 or is it 13?

DR. YANG: Well, as I show in one of my slides, the chemistry development work will address the issue of chemical speciation. I think maybe most of you are not familiar with the NTP system; we do our chemistry development work by a special contractor, Midwest Research Institute. What we want to have is a formulation that is stable, and that could be transported. We have to take into consideration, say in mid-December or January, a transport of the stock solution to the testing laboratory. Somehow it was sitting on the loading dock and all of a sudden something precipitated

out. Now that is the sort of question we want to address. Then in the testing laboratory we want to take reference samples after mixing the stock solutions. When I say stock solutions, we are thinking along the lines of preparing several stocks of certain chemicals, each of which is stable. Then, in the testing laboratory, they will be mixed together to prepare the dosing solutions. So there will be reference samples sent back analyzing all 28 chemicals and whatever else might in there. Also, the dosing solution in the animal room that we will take regular samples for analysis. But those analyses will be done in the testing laboratory, probably by marker chemicals. So the answer to your question is yes, we have considered that.

MAJ. CLEWELL (AAMRL – WPAFB): I was wondering if the panel could comment on the question about going even further in the use of modeling in the risk assessment process back up to the consideration of the identification of the chemical as a carcinogen. What I am referring to is, for example, a fellow at Arnold Engineering Development Center asked me if he should use methylene chloride or trichloroethylene for a process down there. He had gone through all the numbers and the numbers said use methylene chloride; I said I would suggest TCE because I don't think there is as much evidence that it is carcinogenic. He didn't have any way to fold that in. He didn't have any way to use that information in order to come up with a quantitative reason why he should select TCE versus methylene chloride. There is a similar problem, for example, with vinylidene chloride; the action levels for that are lower than for vinyl chloride, and I think most people would suggest that vinyl chloride is more likely to be hazardous than vinylidene, for people at any rate, at the low levels we are talking about. Do you think there is any hope the pharmacokinetics so far have just addressed the question of doing the quantitative assessment assuming that it is in some fashion carcinogenic, but can you take it back further to incorporate probabilities?

DR. STARR: There are certain aspects of it that you could utilize that way. The physiological components of the model can allow you to make statements in certain cases about specific target organs not being affected by exposure according to different routes. It's possible to make statements for highly reactive chemicals that are active only at the site of deposition that you wouldn't expect toxicities at other places. I think that's an important contribution that pharmacokinetics could in the long-run make that it doesn't yet do. As I understand it, what these models are capable of doing is they can predict target organ concentrations if you tell them what target organs you should be doing it for. The problem still to be resolved is to find out which are the target organs.

DR. TRAVIS: I think the biologically based low-dose extrapolation models, this Moolgavkar model, offers a lot of promise to investigate the type of question you are asking. What about chemicals that don't appear to be carcinogenic? And what is actually the mechanism for the carcinogenicity? It seems to me that if you believe the Moolgavkar model, there are two ways that a chemical can be

carcinogenic. One is that it can be genotoxic and cause mutation or some type of an event to initiate a cell and then finally transform it. The other is that it increases the cellular dynamics, and this can enhance the expression of natural tumors or initiated ones. It increases the rate at which they appear. So, probably, most carcinogens are a combination of both of those mechanisms. You can sort of see a sliding scale in which some carcinogens are just genotoxic but don't cause any increase in cell turnover rates. In the middle there will be carcinogens that are probably genotoxic and increase the cell turnover rates. Therefore, the increase in cell turnover rates enhances the carcinogenicity of the chemical, and on the far side there will be compounds that just cause increased cell turnover rates and enhance natural background rates of cancer. I think by investigating the data for several classes of compounds we are going to have to gather a lot more biological data – there is no doubt about that. The model provides a framework for determining what kind of biological data should be gathered. Then we can apply the model to the animal bioassay data and try to understand, in general, the types of mechanisms that are operating for these carcinogens. Then I think we can answer questions like you are posing.

DR. ZUCKER (NORTHROP SERVICES, INC.): This is addressed to Dr. Yang and the other two gentlemen that have implications. I am questioning your end point analysis. I run a flow cytometer at Northrop, and with the flow cytometer I think we are able to get a little more sensitivity for low-dose testing. Some tests that are very apparent are testes evaluation, sperm evaluation. These are things that can test at very low levels. We can get some very good data on the effect of toxic substances. The other aspect that is quite apparent with the flow cytometer is the immunological aspects of monitoring of T & B cells. You can monitor changes of these things at very low doses. It's a question of whether this should be applied when you do your end point analysis. The other question is with *in vitro* analysis. I noticed that in the talk there weren't any *in vitro* methods mentioned. In a poster from our lab that I am presenting this evening we've shown that we can pick up lead and cadmium and zinc toxicity with a standard *in vitro* system by using a flow cytometer in sort of inventive ways. I would just like you to comment on that if you can.

DR. YANG: Let me address the second point first. The *in vitro* testing, yes. In fact, other than our plan for contractual work, which most of these if not all will be, in-house we are doing certain developmental work. In fact we are doing primary hepatocyte culture work and we are thinking about doing kidney primary cell culture work. There I have some differences of opinion with my colleagues. I like to hear their arguments and so on. We have the problem of accepting the extrapolation of the animal toxicity testing results to human, let alone a Petri dish of liver cells. I think it is a good tool, depending upon how you use it. So, to answer your question, yes we are doing that and we are even doing some interaction studies at the binary mixture level at the institute on a smaller scale. Now the earlier question in terms of sensitivity, in fact I have that on one

of the slides. One of the questions also very frequently encountered is what is your toxic end point? They say use a health effect. What? Then the follow-up of that question is maybe you don't know anything. You are taking a shotgun approach. Well, I really detest that word but, yes, it is a shotgun approach because I don't know any better. Who can predict what will happen? Therefore we use subchronic testing, the normal NTP end point such as body weight and so on. If we detect certain body weight depression without food consumption loss or water consumption loss, then I'd conclude there might be a growth retardation effect of some sort. If there are neurobehavioral signs followed by some Wallerian type of degeneration of nerve system, then I say there is a possibility of neuropathy. So anything is possible, and I think at our stage our philosophy is that we open our minds and detect as wide a range of possibilities as possible and let the results guide us.

DR. TRAVIS: I might comment on short-term tests. It's one of my soap boxes that I like to get on. We've been talking about pharmacokinetic models and these biologically based low-dose extrapolation models. It may give you the wrong idea about risk assessment. That's sort of the Mercedes Benz of risk assessment. We certainly can't do it for very many chemicals now, and in the near future we probably aren't going to do it for very many chemicals. By very many, I mean 20 or 30 at the most, but we are dealing in hazardous waste sites with maybe 60,000 chemicals plus or minus a few thousand. For most of those chemicals we don't have any toxicological information, and I think we need to develop short-term tests to predict long-term toxicity from the exposure to those chemicals to be able to develop preliminary guidance values for acceptable concentrations in the environment. There are some moves in that direction. The Air Force has adopted a method that predicts toxicity of chemicals from short-term tests. I think we need more research in that area, not just in the Mercedes Benz end of the research area.

DR. FURST (UNIVERSITY OF SAN FRANCISCO): I wish you would also consider hard water for your background, because I am finding more and more papers and literature where calcium and magnesium can antagonize the toxicity and even carcinogenicity of some of these metals. I think this is an important fact as far as facing what's going on.

DR. YANG: I think we should once again stress that this is the first step and that we certainly believe there are many more studies to be done. We are thinking about it and we are proposing. If you folks have any suggestions, please write to us.

SESSION II

APPLICATION OF PREDICTIVE TOXICOLOGY FOR OCCUPATIONAL EXPOSURES

INTRODUCTORY REMARKS

CAPT David E. Uddin, MSC, USN – Chairman

Naval Medical Research Institute, Toxicology Detachment, Wright-Patterson Air Force Base

On behalf of the Navy I would like to thank the Air Force and Northrop Services for inviting us to participate in this conference. Such collaboration has been not only enjoyable but extremely productive for all.

This afternoon's session is on the application of predictive toxicology for occupational exposures from a variety of viewpoints. One of these viewpoints, composites, is a new area for which there is not a lot of toxicological information and for which we hope to gain a better understanding of the kinds of material that may be involved. Hydrazine is a material that both the Navy and the Air Force use, although the Air Force has used it for many years and the Navy has plans to implement it in certain applications over the next several years. It was more difficult finding a way to make tri-*n*-butyltin relative to the Air Force, since it is used as an ingredient in antifouling paints; but I suspect that if the Army has airplanes someplace, the Air Force must have boats. Finally, our last speaker this afternoon will talk about another way of predicting exposure from a biological standpoint.

OVERVIEW OF EMERGING COMPOSITE MATERIALS AND PROCESSES

Anne R. Beck

Northrop Corporation, Aircraft Division, Hawthorne, California 90250

WHAT ARE COMPOSITES?

Composite materials are combinations of materials that differ in form on a macroscale. The separate constituents retain their identities in the composite; that is, they do not dissolve or otherwise merge completely into each other, although they act together as a group. Normally, the components can be physically identified and exhibit an interface between one another. Advanced composites, which are composite materials made by embedding high-strength, high-modulus fibers in an essentially homogeneous matrix, are used in aerospace component construction.

The cross section of a typical graphite/epoxy composite laminate is shown in Figure 1. Multilayers of graphite fiber bundles or tows are embedded in an epoxy matrix. Typically, fiber tows like these contain 2,000-10,000 fibers in each tow or bundle. The fiber tows that combine with resin in a single layer are known as "prepreg." During manufacture, the prepreg is precured to an intermediate stage of chemical reaction called B-stage. Prepreg materials are normally precured to this stage to speed handling and processing before finally curing them into a composite laminate or structure. Figure 2 magnifies the laminate cross section to show the different ply orientations found in a composite structure. By placing the prepreg layers or plies in different orientations or angles during the lay-up, the properties of the composite can be altered. Hence, the composite strength is increased in the load-bearing direction.

WHY COMPOSITES?

Advanced composites have been developed during the past 15 years as a primary material for use in near-term and next-generation aircraft structures, because composites provide greater structural efficiency at lower weights than equivalent metallic structures. The role of composites in the design and manufacture of next-generation high-performance fighters will increase significantly. All recently produced military aircraft (i.e., F-14, F-15, F-16, F/A-18A, AV-8B, B-1, and F-20) possess composite components. As shown in Figure 3, up to 50% of the structural weight of the next-generation aircraft will be made from composite materials. Other materials are also considered in this review.

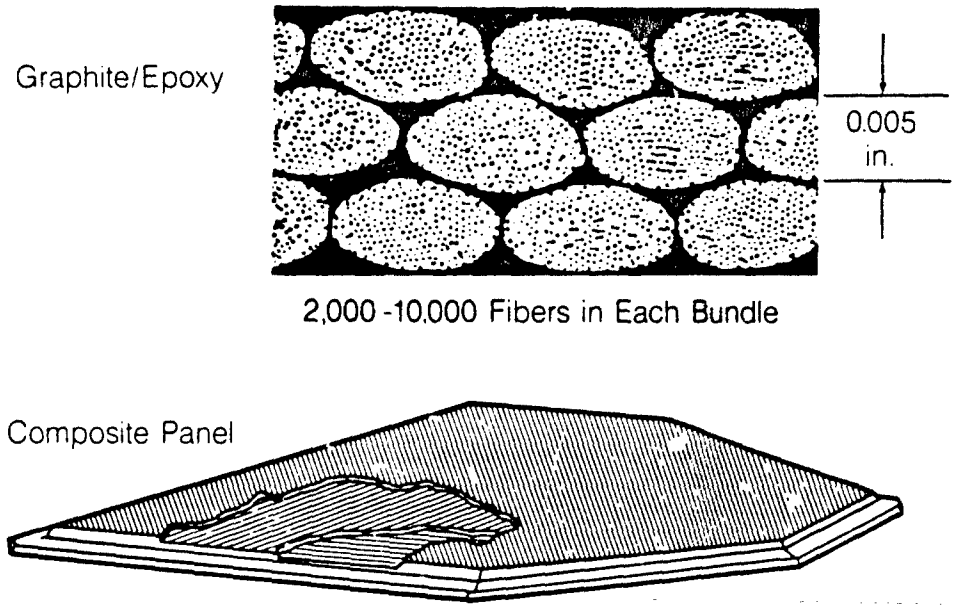


Figure 1. Cross section of a typical composite laminate.

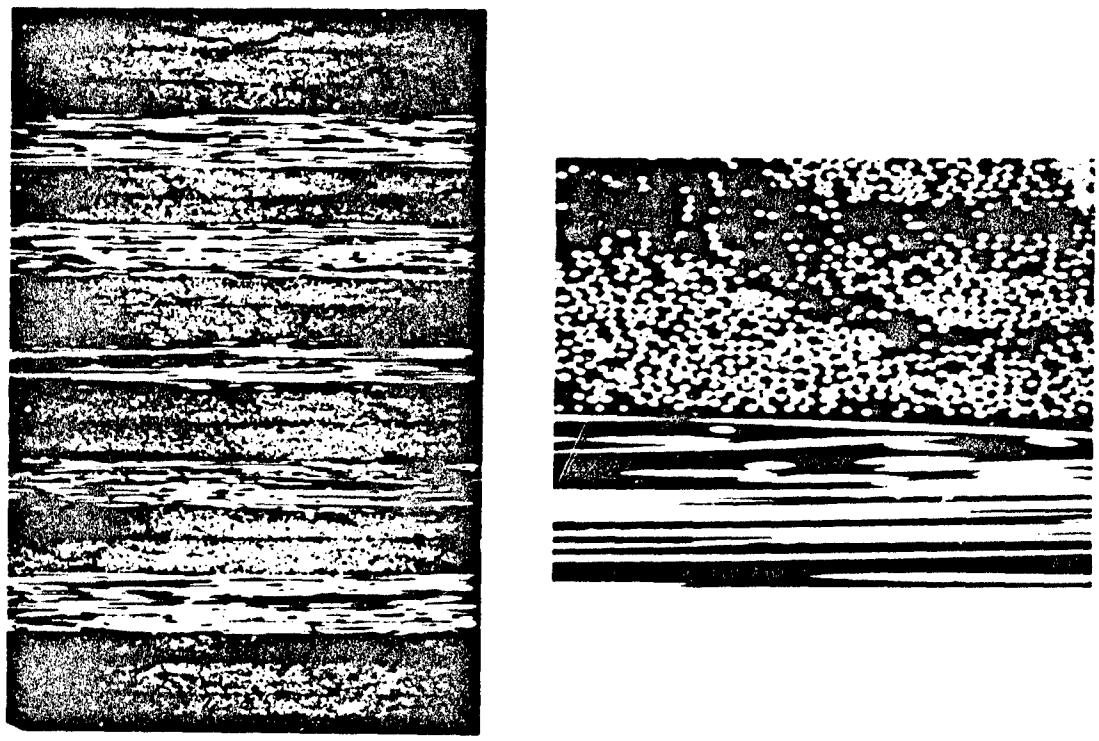


Figure 2. Magnified views of actual composite laminate.

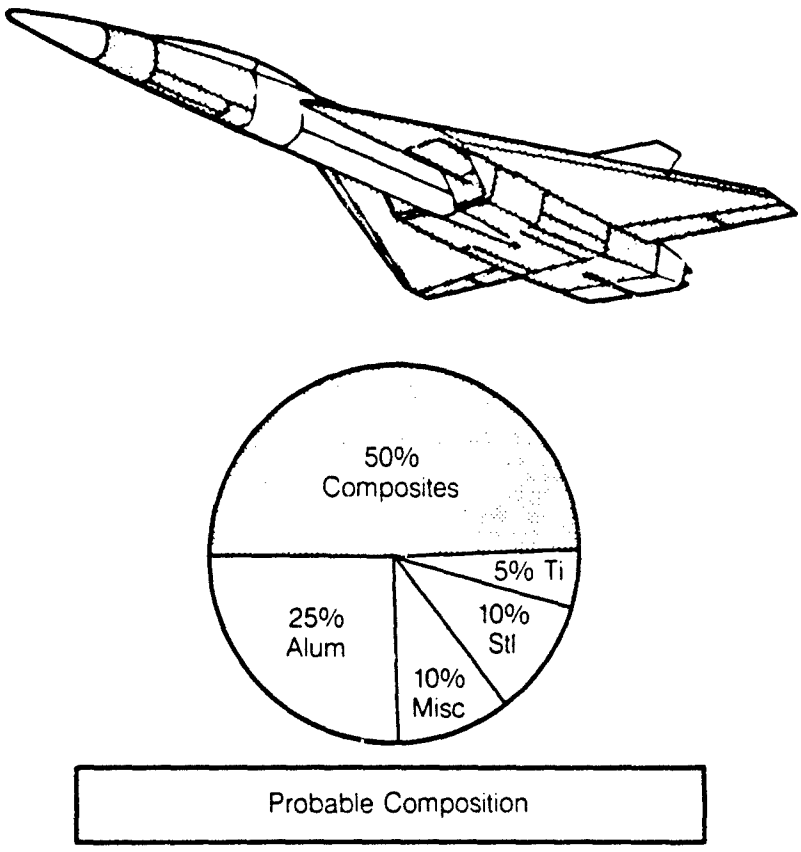


Figure 3. Projected advanced fighter material usage.

Composites have distinct advantages over the more traditional metals used in aircraft. First, they have higher strength and stiffness-to-weight ratios, and the part-ply orientation can be altered to accommodate increased strength and/or stiffness in a required direction. Second, because the matrix material is organic and the fibers are either organic or inert, the material is corrosion resistant. Third, composites are less susceptible to the fatigue that is common in metals.

MATRIX MATERIALS

The fibers or filaments of the composite are embedded in a homogeneous matrix material. An organic polymer is the matrix material widely used in production aircraft. Other matrices with metals and ceramics are being developed for high-temperature and stiffness in aerospace applications. Because the matrix is the weakest part of the composite, it determines the operating limits of the composite structure.

There are two major classes of organic matrices: thermosets and thermoplastics. Thermoset matrix materials are composed of organic polymers that react chemically to make parts. After lay-up

(or placing successive layers of prepreg), the material is transferred into an autoclave, a closed vessel that applies heat and pressure. The chemical reaction is thus controlled by a predetermined cure cycle, and a high-quality part is produced. On the other hand, thermoplastics are fully reacted chemically. They only need to be consolidated by heat and pressure.

Thermosets were the first matrix materials to be used in aerospace structures. Current resin systems include epoxies, BMIs (bismaleimides), and polyimides. Table 1 outlines the differences between them. Epoxy matrix composites, the most widely used matrix material in production aircraft, are used on the McAir/Northrop F/A-18 fighter.

TABLE 1

COMPARISON OF THERMOSET MATRIX MATERIALS

Characteristic	Epoxy	BMI	Polyimide
Polymerization	Addition	Addition	Condensation
Morphology	Linear or Cross-linked	Cross-linked	Cross-linked
Prepreg process	Hot melt	Hot melt	Solvent
Moisture content (end-of-life)	1.4%	1.2%	1.6%
Service temperature	To 220°F	To 425°F	600°F
Damage tolerance	Moderate	Moderate	Moderate

The newly emerging thermoplastic matrices have many advantages over the thermosets. They are more damage tolerant and more cost effective. Fast, automated forming processes are used in contrast to the long processing cycles typical of thermoset materials processed in an autoclave. Thermoplastics only require heat and pressure to consolidate the prepreg into a laminate or structure. The processing cycle for thermoplastics is short (30 min to 4 h depending on part size) compared to thermosets (8 h and up). Thermosets must be stored in a freezer to prevent further reaction before the part is made. Because thermoplastics are a completely reacted matrix, the prepreg may be stored at room temperature.

Thermoplastics, however, do have some disadvantages. They lack resistance to some organic solvents (such as paint-stripping products) and aircraft fluids. Current U.S. Environmental Protection Agency/Occupational Safety and Health Administration guidelines indicate that some of these solvents may be withdrawn from use in the near future, making solvent resistance less of an issue High processing temperatures (600-800°F) are often required to process thermoplastics (350-475°F is typical for thermosets). The thermoplastic prepreg is stiff, making it difficult to bend into corners

and curves for a complex-shaped part. The prepeg for thermosets tends to be soft and pliable, making it easier to form complex curves.

Current thermoset materials have their limitations, yet as demands increase for service temperature, durability, and damage tolerance, thermoplastic composites will become more widely used in future military aircraft.

FIBERS

The fiber of the composite is the part with the highest axial strength and modulus. Four fibers are currently being used in aerospace structures: graphite (or carbon), S-2 fiber glass, Kevlar (an organic aramid), and boron. The differences between these fibers are described in Table 2. By volume produced, graphite is the most widely used today. Although boron was the first fiber used in advanced composites (Grumman's Navy F-14), graphite replaced boron in later aircraft. Graphite is less expensive, and the boron composites are more difficult to machine. However, because boron has a high modulus, it is currently being used in metal matrix composites for space applications. Kevlar is widely used in commercial aircraft construction. Because of its low service temperature (300°F) and highly hygroscopic nature, it will not be used in advanced fighter aircraft that demand a higher temperature tolerance. Both graphite and S-2 glass will be used extensively in future aircraft.

TABLE 2

COMPARISON OF TYPICAL COMPOSITE FIBERS

Characteristic	Graphite	S-2	Kevlar	Boron
Strength	Good	Fair	Fair	Fair
Stiffness	Good	Poor	Fair	Excellent
Density, g/cc	1.8	2.5	1.4	2.5
Service temp.	High	High	Low	High
Other factors	Most potential	Low conductivity	High impact	Expensive

PROCESSING

A flow chart in Figure 4 outlines the process of composite part production from manufacturing the prepreg through installing the part on the aircraft. The prepreg manufacturer combines fibers and resin into the prepreg and ships it to a company such as Northrop for part fabrication. After receiving and inspecting the prepreg, it is placed into the part configuration, cured and/or consolidated, inspected for quality, then drilled and trimmed for final fit. Some smaller

parts are combined into larger assemblies and installed on the aircraft. Each step is described in the following paragraphs.

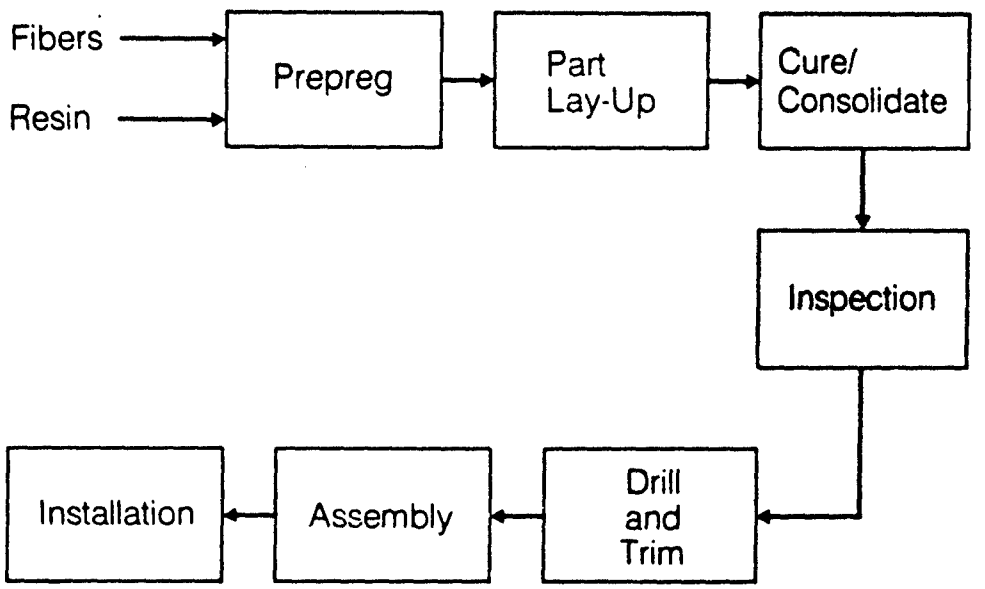


Figure 4. Flow chart of the processes involved in composite part production.

Solvent Method

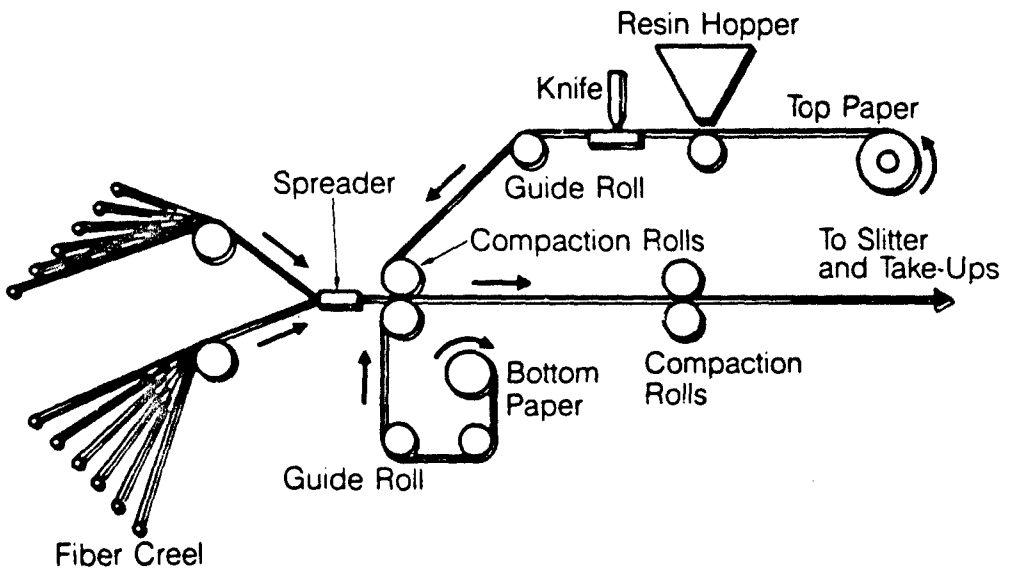


Figure 5. Schematic of the solvent method of prepregging.

Prepreg is manufactured by one of two methods: solvent or hot melt. In the solvent method (diagrammed in Figure 5), the resin is applied to a carrier paper and the thickness adjusted with a

knife edge. Once the resin is spread to a thin and uniform thickness, the fibers are applied. The two materials are then pressed together by compaction rolls and slit to the desired width before rolling onto a core or mandrel for shipment. If the product is formed from single, unidirectional fibers, the prepreg is called unitape (Figure 6a). If woven fiber (fabric) is used, the prepreg is called broadgoods or woven prepreg. A variety of weaves are available in prepreg fabric form. One of the most common, five-harness satin, is illustrated in Figure 6b. Unitape is the most common because it is thinner and more tailorable than woven materials, although both forms of prepreg are widely used.

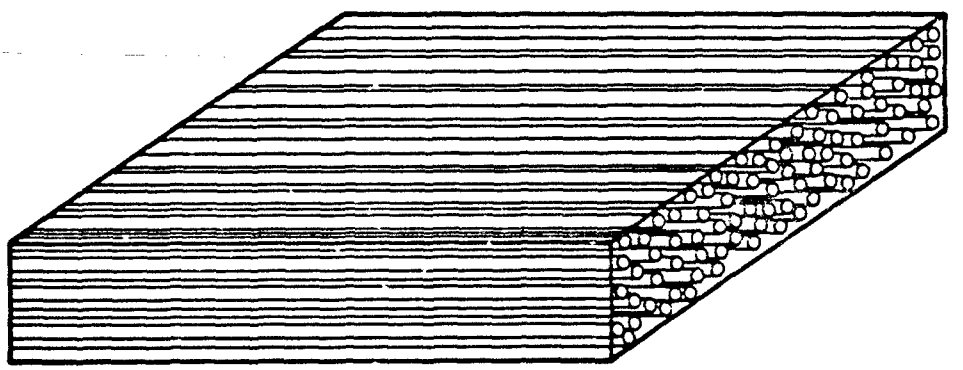


Figure 6a. Continuous fiber reinforcement prepreg.

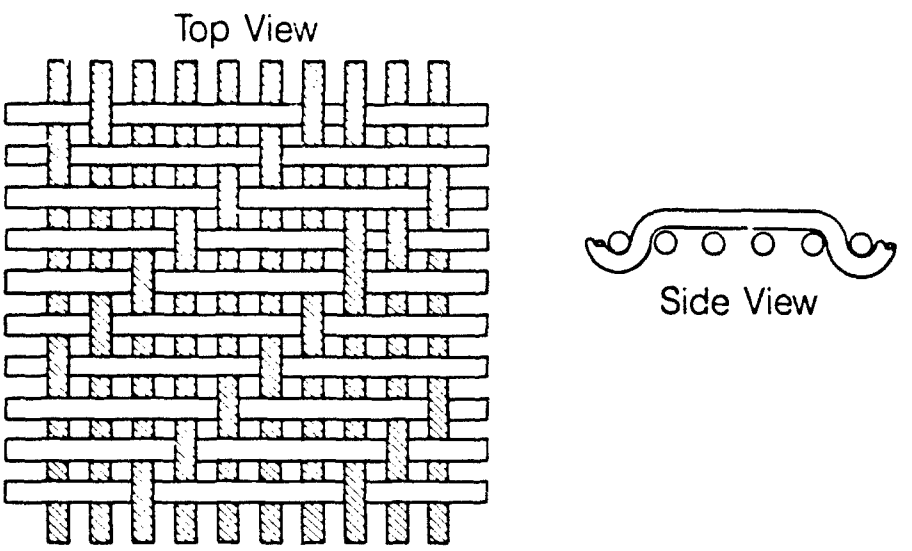


Figure 6b. Woven fiber prepreg.

Distinct differences exist between thermoset and thermoplastic prepreg (Table 3). While thermoset prepreg is wound on hollow circular cores for shipment and storage, thermoplastic prepreg is available either on circular cores or as flat sheet stock. Because thermoset resins are

reactive, the prepreg must be stored at 0°F to prevent the reaction from advancing before the part is made. Thermoset resins must also be handled in a humidity- and temperature-controlled clean room to prevent contamination. For the same reason, personnel must wear protective clothing and, in some cases, respirators. Gloves may also be required. Because thermoplastic prepregs are not reactive and are stored at room temperature, parts are fabricated in a low-level clean room. Protective clothing is not required. As previously mentioned, thermosets are pliable and have good tack (stickiness of the prepreg where one ply of prepreg adheres to another). These qualities are needed to make parts with complex curvatures. Thermoplastics are generally stiff and unpliable. They also have little or no tack.

TABLE 3

COMPARISON OF THERMOSET AND THERMOPLASTIC PREPREG MATERIALS

	Thermoset	Thermoplastic
Incoming Form	Roll	Roll or flat sheet stock
Storage	0°F	Room temperature
Lay-up room	Clean	Low-level clean
Personnel protection	Lab coats, hats (respirators, gloves)	None
Tack and drape	Good to fair	Poor

PART FABRICATION

A part is made by successively placing prepreg layers (called the lay-up procedure) on a shaped tool in a specified orientation to give the desired strength needed for the finished part. Typically, the lay-up is done by hand; however, the aerospace industry is currently seeking to replace much of this labor-intensive process with automated ply-placement using robotics. After lay-up, the part is bagged to prepare for curing the resin in an autoclave. A typical composite fabrication bagging procedure, shown in Figure 7, is used to 1) control the resin content of the finished part, 2) allow the air and any reactive volatiles which may be trapped between the prepreg plies to escape, and 3) assist in the full consolidation of the part. The bagging procedure for thermoplastics is much simpler because thermoplastics are nonreactive and form no by-products during the high-temperature processing.

Both thermoset and thermoplastic composites may be processed in an autoclave (Figure 8). Thermoplastic composites can also be processed in a hydraulic press (Figure 9). This press applies

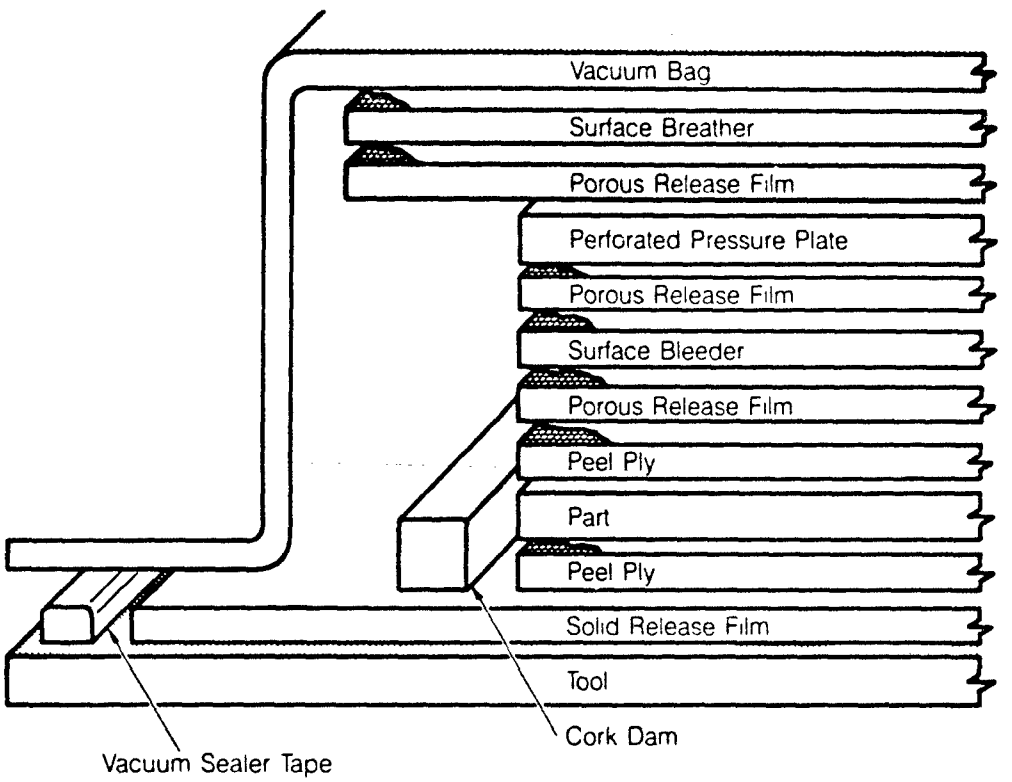


Figure 7. Typical thermoset composite fabrication bagging procedure.

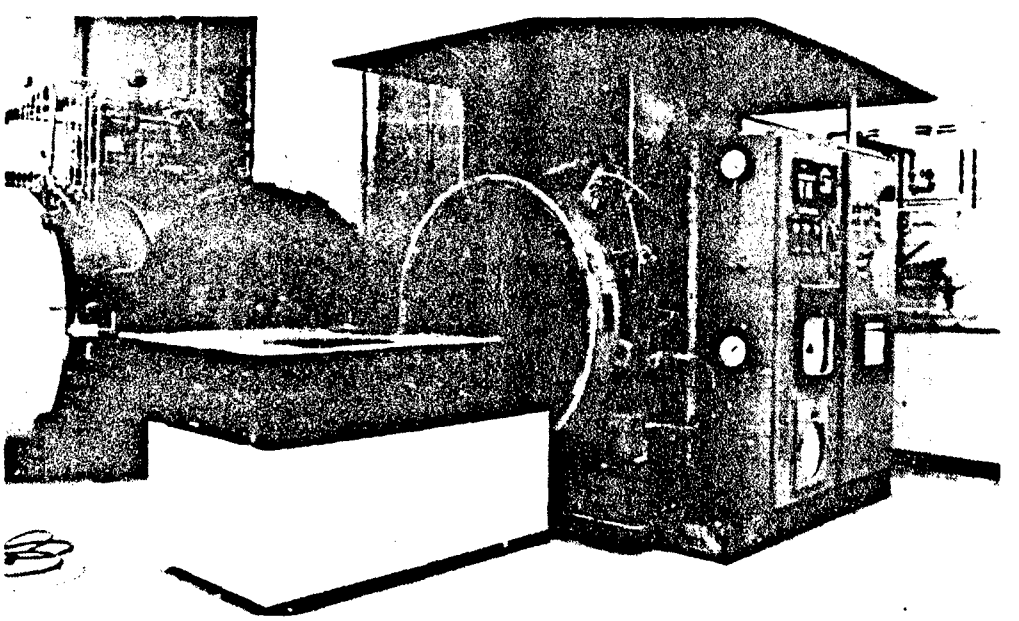


Figure 8. Research autoclave used in processing composite parts.

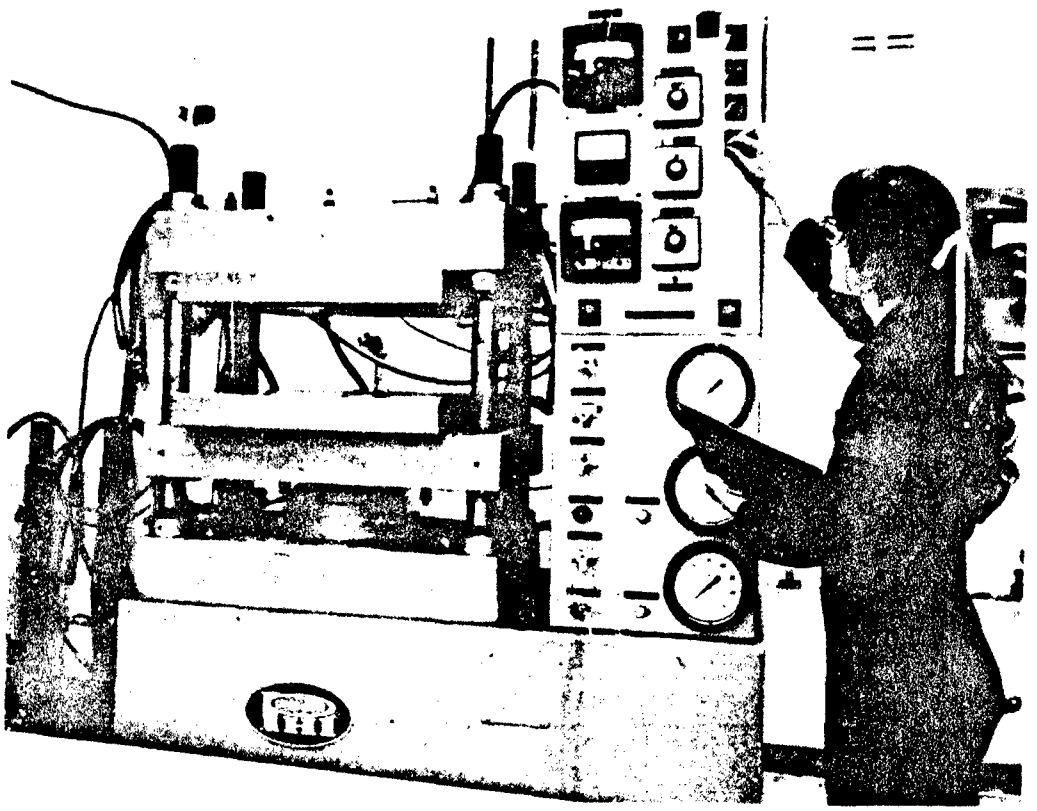


Figure 9. Research hydraulic press used in processing thermoplastic composite parts.

heat through resistance-heated platens to soften the material and pressure to consolidate (melt-fuse) the prepreg plies into the desired shape. Because thermosets undergo a chemical reaction during processing, their cure cycles must be longer to ensure complete reaction. Typical processing cycle profiles are shown in Figure 10. In addition to the autoclave curve, some thermosets need an additional 4-10 h postcure at an elevated temperature to drive the chemical reaction (cross-linking) to completion and fully attain composite properties.

After the part is fabricated, it is inspected by either radiographic or ultrasonic methods. In an ultrasonic inspection (Figure 11) the part is placed between water jet streams (the water couples the composite part to the transducer). A sonic wave is transmitted through the part. This transmitted wave is measured as it emerges on the other side of the part. Any imperfection in the part causes a change in the wave velocity which can be related to the type and size of defect.

Once the part has passed quality inspection, it is trimmed for fit and assembled with other parts for final installation. Care must be taken to prevent damage when transporting the part from one work station to another. During final machining of the composite part, special tool fixtures are needed to avoid splintering the material and to remove the carbon dust produced. During

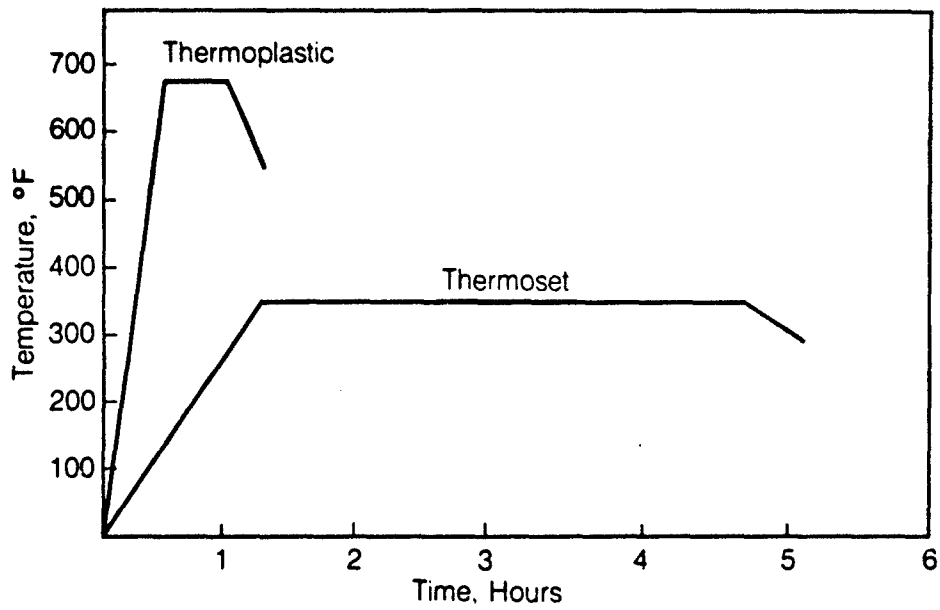


Figure 10. Profile of typical processing cycles for thermoplastic and thermoset composites.

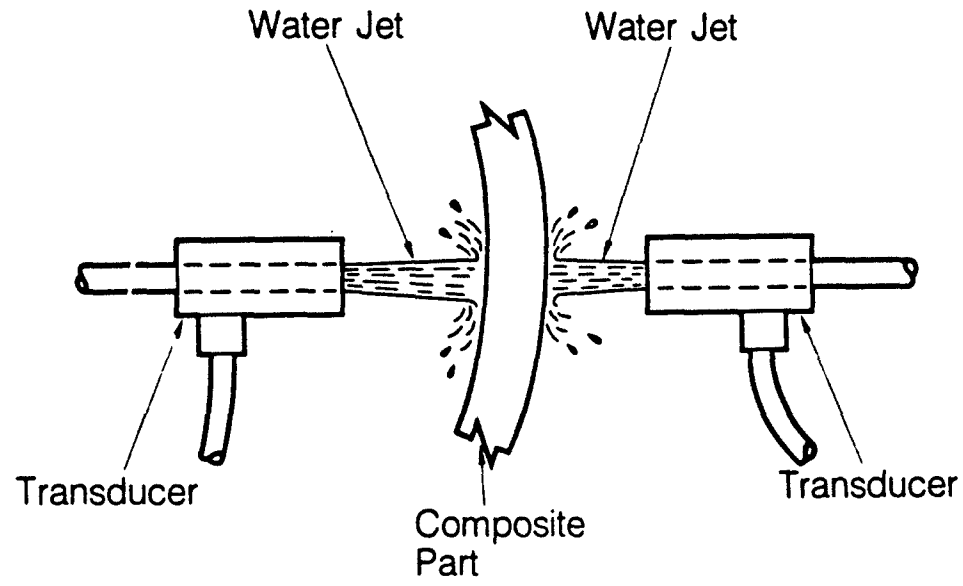


Figure 11. Ultrasonic inspection techniques used to verify composite part quality.

subassembly, adhesives rather than mechanical fasteners are frequently used to join the part details. Since these adhesives are unreacted chemicals they must be handled carefully in well-ventilated areas.

COMPOSITE APPLICATION

The McAir-Northrop F/A-18 (Figure 12) uses the highest percentage of composites in a traditional production fighter aircraft. This thermoset composite is primarily a graphite/epoxy. Of all production VSTOL (vertical and/or short takeoff and landing) aircraft the McAir AV-8B (Figure 13) uses the highest percent composite, again mostly graphite/epoxy. Future fighter aircraft will use as much as 50-60% composites including the newly emerging higher temperature thermosets and thermoplastics.

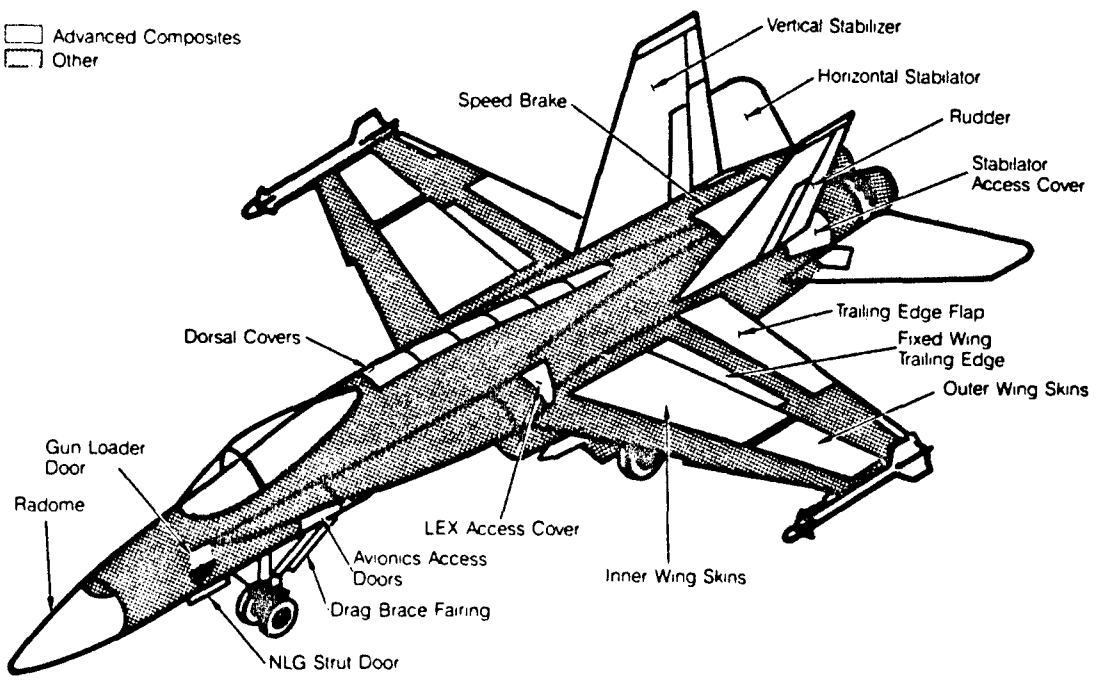


Figure 12. F/A-18A composite material usage.

To demonstrate how thermoplastic composites can be used, Northrop recently fabricated an F-5E landing gear door assembly (Figure 14) from thermoplastic composite materials. A significant portion of the door (measuring approximately 2 ft x 3½ ft) was fabricated as a single piece of composite. This door will be installed on an F-5E and tested for flight-worthiness in late 1987.

In summary, thermoset and thermoplastic composites are the aircraft materials of the future. A greater percentage of these composites will be used in future aircraft designs as new ones are developed. Also, as future aircraft construction starts to demand materials with high-temperature tolerance, composite structures will be made with other matrices such as metals and ceramics.

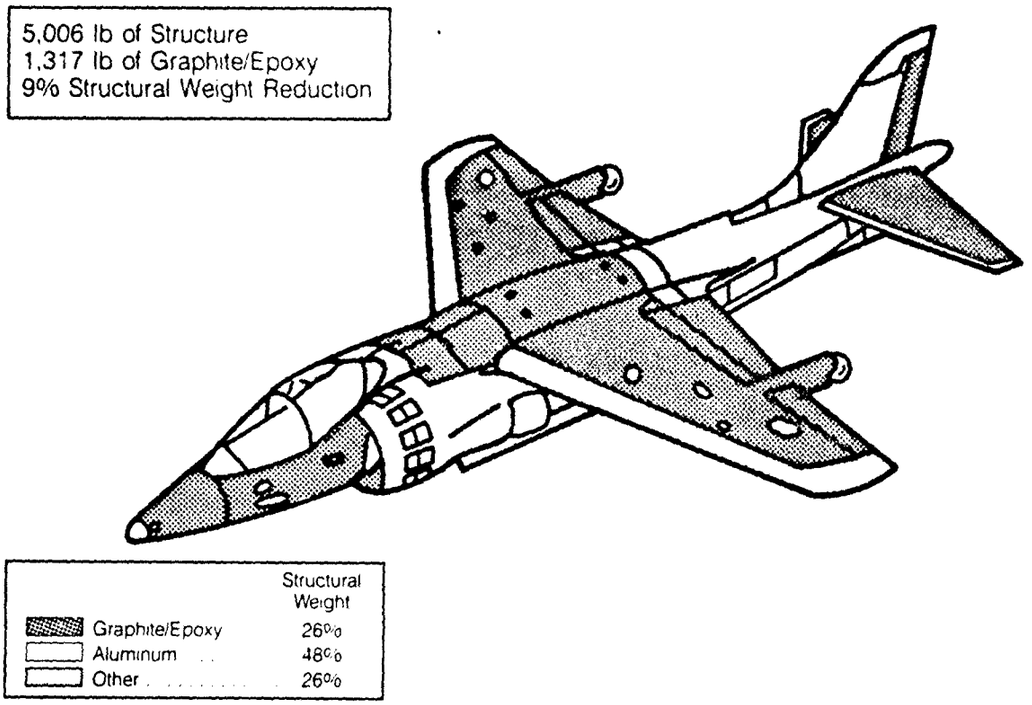


Figure 13. AV-8B composite material usage.

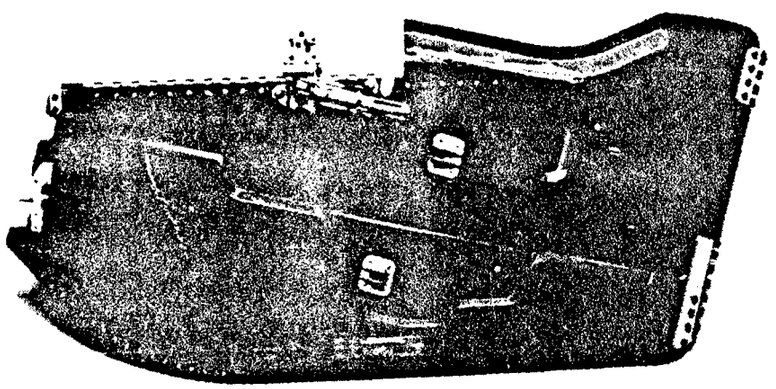


Figure 14. Application of thermoplastic composite in an F-5E landing gear door prototype.

QUESTION AND ANSWER SESSION

CAPT UDDIN (NMRI/TD): It seems like during commercial manufacturing there is a lot of safety and health protection. One of the questions that we have had as toxicologists is, "What processes are used to repair damage to those materials?"

MS. BECK: Repair is a very critical issue and is one that involves a number of things, including crash site investigations. Typically, on board ship would be your worst possible case. What happens currently is that on board ship they are taking out preformed patches and adhesives. The part that needs to be repaired is cleaned. If it can be removed from the aircraft, it is removed from the aircraft, taken down into a semi-clean environment, and repaired there. If it is damaged, it requires thorough cleaning before a good repair is made. A temporary repair is put on the aircraft, it is flown back to one of the rework facilities, and that's where it is repaired.

CAPT UDDIN: Are there unique occupational safety considerations during the repair process?

MS. BECK: Because most of the adhesives have high solvent contents, they are unreacted chemicals and must be handled very carefully. Again, because you have to handle drilling, routing out, those kinds of things, the personnel that are doing this have to be aware, they have to know that they must indeed wear their face masks. They must clean up the area behind themselves and keep the area clean.

OCCUPATIONAL SAFETY CONSIDERATIONS WITH HYDRAZINE

Major James N. McDougal, Ph.D., USAF, BSC

Manuscript Not Submitted

TRI-*n*-BUTYLTIN: A MEMBRANE TOXICANT

LCDR Brian H. Gray, Ph.D., MSC, USN[a], LCDR Martin Porvaznik, Ph.D., MSC, USN[b], Carlyle Flemming, M.A.[c], Lanfong H. Lee, M.S.[b]

[a]Naval Dental School, Naval Dental Clinic, NMCNCR, Bethesda, Maryland 20814-5007

[b]Naval Medical Research Institute/Toxicology Detachment, Wright-Patterson Air Force Base, Ohio 45433-6503

[c]Northrop Services, Inc. - Environmental Sciences, 101 Woodman Drive, Dayton, Ohio 45431-1482

SUMMARY

Increased use of the biocidal compound tri-*n*-butyltin (TBT) in antifouling paints has prompted research aimed at determining the mechanism for TBT toxicity. Past investigations indicate that the primary cellular target for TBT is the cell membrane. Erythrocyte suspensions treated with TBT concentrations ≥ 5 μM undergo hemolysis described by a sigmoidal kinetic pattern. Transformation of cell shape from discocyte to echinocyte occurs at TBT concentrations ≥ 0.1 μM, indicating that the compound enters the outer membrane bilayer. TBT at concentrations ≥ 10 μM forms electron-dense aggregates that are intercalated within plasma membranes as viewed in ultrathin sections by transmission electron microscopy. Qualitative X-ray microanalysis of these aggregates confirms the presence of tin. The size of these structures can be modified by either 10 mM cyanide or 2,3-dimercaptopropanol (British Anti-Lewisite, BAL). Adding 10 mM cyanide to hemolytic TBT concentrations resulted in a synergistic stimulation of hemolysis attributable to high cyanide anion concentrations in or near the cell membrane. The elevated cyanide anion levels are thought to contribute to membrane lysis. The lipophilic dimercapto compounds BAL, dithiothreitol, and 2,3-dimercaptosuccinate are effective inhibitors of TBT-induced lysis. Water-soluble 2,3-dimercapto-1-propane sulfonate, a BAL analog, was largely ineffective as an inhibitor. The detailed molecular mechanism for TBT-induced membrane lysis is not yet clear. Cellular ATP depletion could be induced by TBT as well as by delipidation of anionic phospholipids or even formation of tributylstannylperoxy radicals, resulting in lipid peroxidation.

INTRODUCTION

Organotin compounds such as TBT (the chemical structure for which is shown in Figure 1) have been used as constituents in numerous formulations, including pesticides and antifouling paints (1). TBT is frequently incorporated into polymers (Figure 2) before it is added to antifouling paint formulations (2) and is believed to be the primary biocidal component responsible for the effectiveness of such paints (3). TBT is released from the painted surfaces by hydrolysis, forming species such as TBT hydroxide and TBT chloride. Several organotin-based antifouling paint

formulations have been tested and are effective in reducing biological encrustations. Large quantities of antifouling paints containing TBT have already been applied to ship and boat hulls, and future use of these effective paints can be anticipated (1). Increased use of organotin-based antifouling paints has led to environmental concerns about TBT levels in harbors, marinas, and coastal waters (4). These concerns have stimulated research into the mechanism of TBT toxicity.

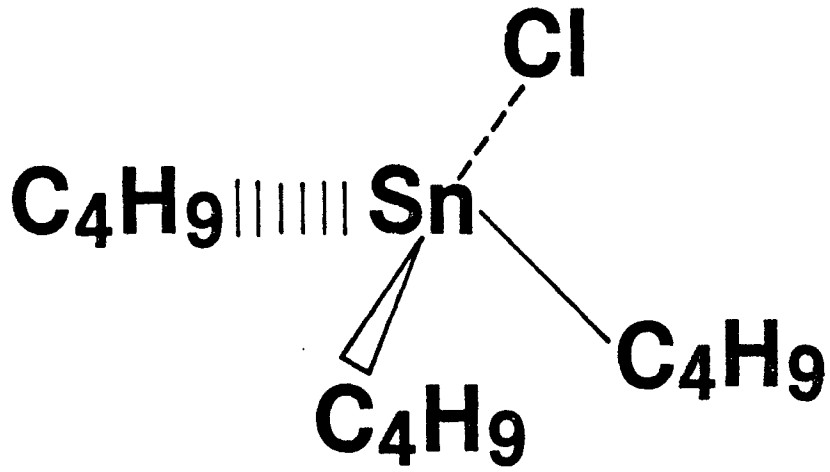


Figure 1. Structural formula for tetrahedrally shaped tributyltin chloride.

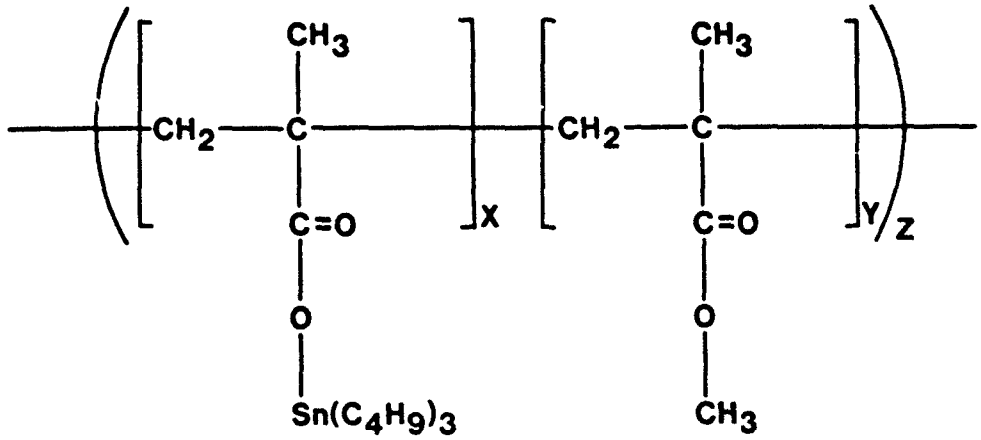


Figure 2. Organometallic polymer. The polymer shown contains TBT methacrylate/methyl methacrylate in a 1:1 mole ratio.

TBT's cellular and biological effects have been examined to elucidate its mechanisms of toxicity. TBT at concentrations ≥0.1 μM inhibited neutrophil migration toward known chemotactic attractants (5). At ≥1.0 μM, TBT inhibited mitochondrial oxidative phosphorylation by transporting hydroxyl anions across membranes, resulting in abolition of pH gradients required for ATP

production (6,7). TBT at 10 μM and at higher concentrations effectively shut down both sodium-potassium and calcium pumps in cell membranes (8). Concentrations of TBT ≥ 5 μM led to membrane lysis, which was extensively studied in erythrocytes (9,10). TBT-mediated membrane effects were clearly demonstrated by inflammation and skin irritation at the application sites (11,12). Both acute and chronic toxicity studies strongly suggested that TBT effects were primarily at cell membrane sites of exposure (13). Chronic feeding of TBT to rats resulted in rosettes of erythrocytes around mononuclear cells in lymph nodes, and reductions of T cells in spleens (14,15). These findings support the contention that TBT is primarily a membrane effector.

The erythrocyte model has been used extensively to investigate effects of xenobiotics on cell membranes (16). Studies described in this report employed the erythrocyte membrane model to determine rates of TBT-mediated hemolysis and to examine erythrocyte shape transformation. And finally, the ability of TBT to induce the formation of tin-containing aggregates in membranes was investigated.

MATERIALS AND METHODS

Human blood was collected and washed, and erythrocyte suspensions were prepared as previously described (17). Hemolysis assays using erythrocyte suspensions were done by published methods (10,18). Samples were examined by electron microscopy using methods outlined in published papers (10,17,18). Data analysis employed BMDP software and statistical methods previously described (10,18).

RESULTS

Figure 3 is a graphic representation of percent hemolysis as a function of time at various TBT concentrations in suspensions containing approximately 2.3×10^8 human erythrocytes per milliliter. Red blood cell suspensions containing 1.0 μM TBT exhibited hemolysis rates similar to control suspensions containing 1% ethanol (171 mM) for periods up to 24 h at 35°C. TBT concentrations ≥ 5 μM led to hemolysis rates significantly higher ($p = 0.0059$) than control suspensions by 4 h. Hemolysis rates in red blood cell suspensions increased with increasing TBT concentrations greater than 5 μM. Erythrocyte suspensions containing TBT concentrations ≥ 25 μM had hemolysis rates too rapid to measure using the techniques employed. Both 10- and 25-μM TBT concentrations elicited erythrocyte lysis with a definite lag following addition of the insult, and the kinetic profile appeared to be sigmoidal.

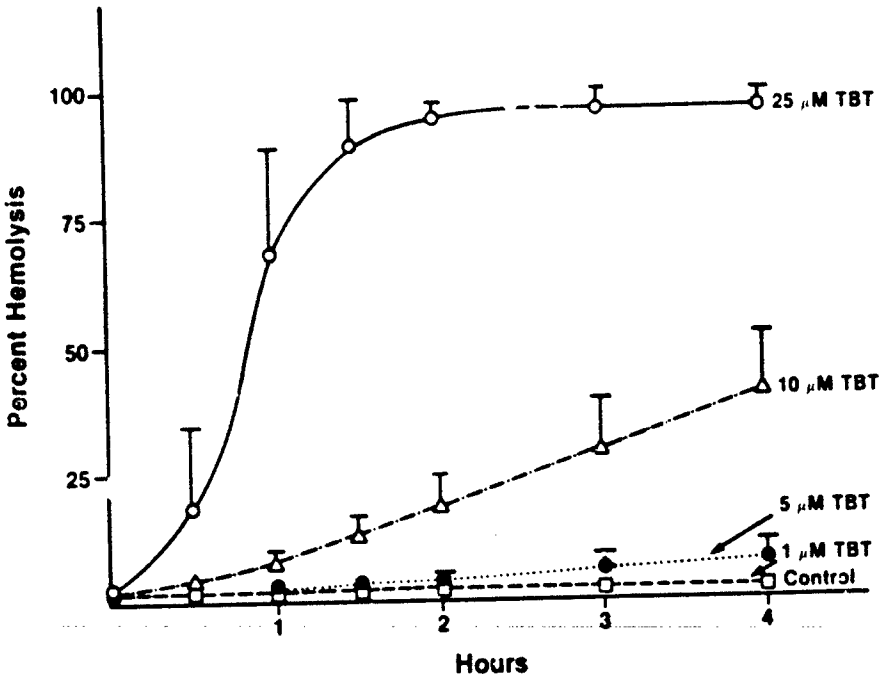


Figure 3. Percent hemolysis as a function of time for various TBT concentrations. Human erythrocyte suspensions had about 2.3 x 10^8 cells per ml plus 171 mM ethanol. (□ - - - □) control (171 mM ethanol), (● ····· ●) 5 μM TBT, (Δ - · - · - Δ) 10 μM TBT, and (O ——— O) 25 μM TBT.

TBT at concentrations ≥ 0.1 μM induced rapid shape transformation in red blood cells. Figures 4a and 4b demonstrate a rapid conversion of erythrocytes from the resting discocyte to the echinocyte form 5 min after the addition of 25 μM TBT. Real-time shape transformation was monitored using phase contrast light microscopy, which indicated that echinocyte transformation occurred within 30 s following the addition of 25 μM TBT. The effect of TBT concentration on erythrocyte shape transformation is shown in Table 1. At 0.1 μM TBT, shape transformation was reversible with time. TBT at 1 μM produced shape transformation, but hemolysis did not occur above control levels. Higher TBT concentrations (≥ 5 μM) produced irreversible cell shape transformation that progressed to hemolysis. Erythrocyte suspensions with hemolytic TBT concentrations contained cell shapes ranging from discocytes to crenated discocytes, echinocytes, spherocytes, and cell ghosts.

Transmission electron microscopy of cell preparations treated with ≥ 10 μM TBT followed by glutaraldehyde fixation and osmium tetroxide postfixation regularly displayed spherical to ellipsoid-shaped aggregates absorbed within the erythrocyte plasma membrane or free in the extracellular space (Figure 5). The average size of these membrane-associated aggregates was 71.5 ± 18.2 nm in diameter. Erythrocyte suspensions treated with ≤ 5 μM TBT did not display these spherical

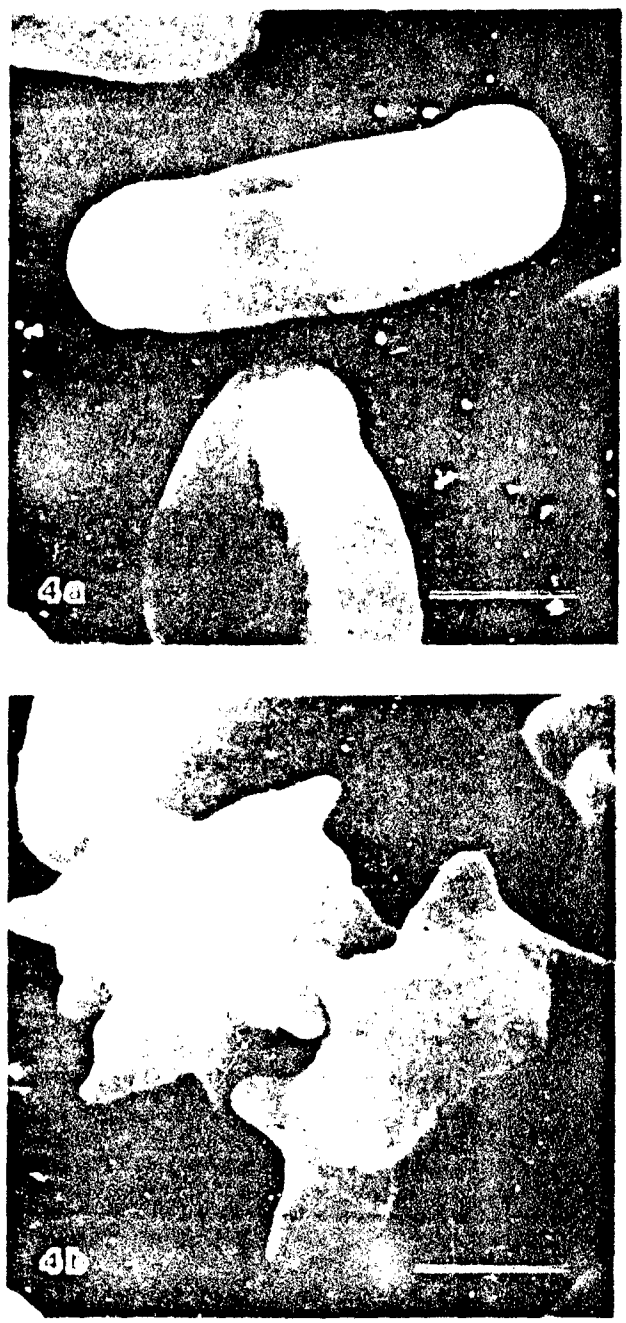


Figure 4a. Control population of human RBCs before the addition of 25 µM TBT. Notice the characteristic biconcave discocyte morphology. Bar = 2.0 µm; x 10,600.

Figure 4b. TBT-treated (25 µM) population of human RBCs 5 min after the addition of TBT. Notice the various echinocyte forms. Bar = 2.0 µm; x 11,000

TABLE 1

SHAPE TRANSFORMATION INDUCED BY TBT

Cell suspension addition	Percent discocytes at 20 min[a]
Control (171 mM ethanol)	95 ± 1.7
0.1 μM TBT	78 ± 1.7
1 μM TBT	22 ± 5.6
10 μM TBT	6.7 ± 6.8
100 μM TBT	7.2 ± 4.6

[a]Cell shape was assayed by scanning electron microscopy; n = 243 ± 42 for each treatment group.

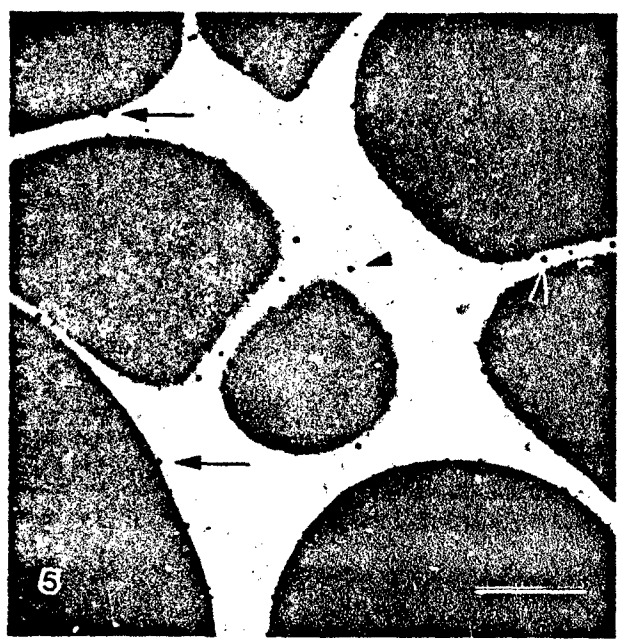


Figure 5. TBT aggregates are observed in the extracellular space (arrowheads), but the most probable location to observe these structures is in the plasma membrane intercalated within the membrane bilayer (arrows). The debris in the extracellular space is extruded hemoglobin after hemolysis. Bar = 2.0 μm; ×7,900.

aggregates. Intramembranous aggregates were visible in sections prepared without additional heavy metal staining by virtue of their affinity for osmium complexes during the postfixation. Erythrocyte samples prepared with glutaraldehyde fixation, without osmium tetroxide postfixation, and followed by dehydration through an increasing series of ethanol concentrations, lacked the electron-dense intramembranous aggregates. Rather, these samples had translucent, spherical areas in the plasma membrane and extracellular space approximately the same size as aggregates

observed in osmium-fixed preparations. Presumably, the ethanol dehydration extracted any tin aggregates from the membranes because of the solubility of organotin molecules in ethanol. Erythrocyte suspensions treated with 25 μM TBT, centrifuged 30 min later, washed in sterile saline, and subsequently prepared for electron microscopy had fewer membrane-associated aggregates and a reduced rate of hemolysis compared to TBT treatment alone. Nucleated, peripheral blood cells treated with ≥ 10 μM TBT displayed membrane-associated aggregates not only in the plasma membranes but also in the membranes of the internal organelles, including the nuclear membrane.

Transmission electron microscopic examination of freeze-fracture preparations of erythrocyte suspensions treated with ≥ 10 μM TBT revealed unique intramembranous structures (Figure 6). These structures were observed in the absence of osmium fixation or ethanol dehydration and were approximately 60 nm in diameter. Membrane fracture faces prepared from control (171 mM ethanol) suspensions were devoid of any large intramembranous structures. Protoplasmic membrane faces of both control and TBT-treated erythrocyte membranes also displayed the typical 6 to 10 nm diameter intramembranous particles normally observed by freeze-fracture analysis.

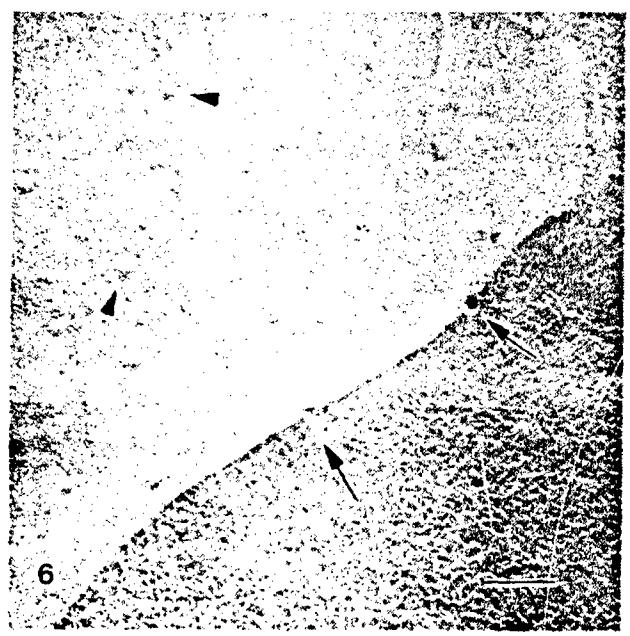


Figure 6. The TBT aggregates are most likely to be observed intercalated within the lipid bilayer. This is demonstrated in the freeze-fracture preparation of TBT-treated erythrocytes. Notice the large, particulate structures on the outer membrane half (arrowheads) approximately the same size as those observed in thin sections. When these structures are cross-fractured along the periphery, they can be observed protruding into the extracellular space (arrow). Notice that these cross-fractured aggregates are, in fact, intercalated within the plasma membrane bilayer. Bar = 200 nm; x 53,700.

X-ray energy-dispersive spectrometry (EDS) was used for metal analysis in membrane-associated aggregates. Spectra of samples treated with TBT and examined following osmication (Figure 7a) differed greatly from samples that were not osmicated (Figure 7b). Only osmicated aggregates produced a spectrum characteristic of osmium. When the electron beam was focused either on intracellular spaces or within cells, and not on a membrane-associated aggregate, only a background spectrum was observed. Tin was not detected in osmicated samples, yet a structure was observed. In response to this puzzling finding, a similar analysis, performed using water-soluble fixation and an embedding process with glutaraldehyde-carbohydrazide, showed tin present in the membrane-associated aggregates without osmium. This confirmed our suspicion that tin was present in an aggregated form that partitioned from aqueous compartments into the more hydrophobic biological membranes.

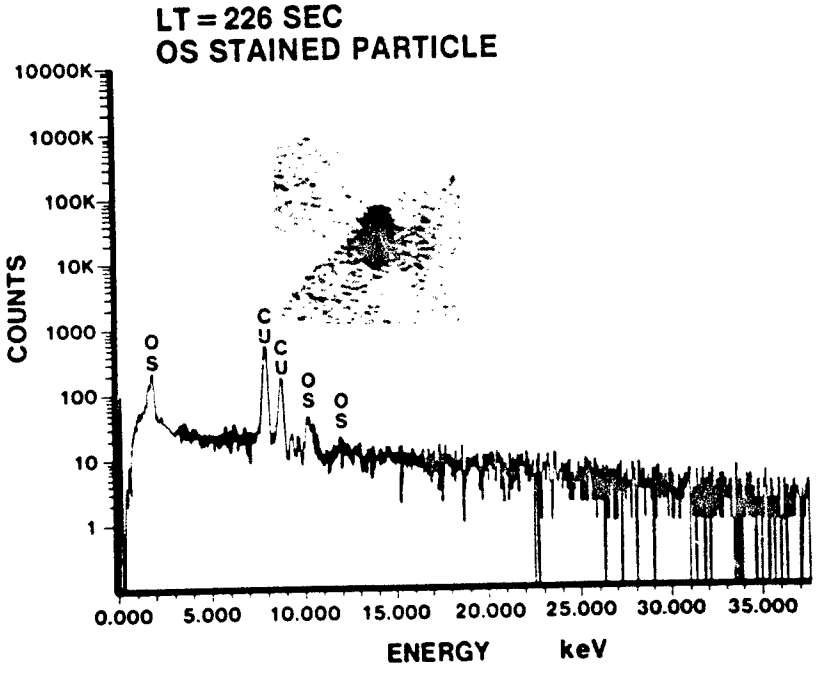


Figure 7a. An X-ray EDS spectrum produced from a membrane-associated aggregate, as shown in the inset. The aggregate in this osmicated sample produced an energy spectrum displaying osmium L-lines at 8.91, 10.596, 11.68, and 12.522 keV, and an M-line at 2.042 keV. The copper mesh grid supporting the unstained thin section produced a background spectrum that did not interfere with an L-line at 0.930 keV and K-lines at 8.048 and 8.905 keV.

A number of sodium salts, organic compounds, and proteins were examined to determine their effect on TBT-mediated hemolysis rates. Ten millimoles sodium nitrite, sodium thiocyanate, and sodium azide each slightly reduced TBT-mediated hemolysis rates. Ten millimoles sodium

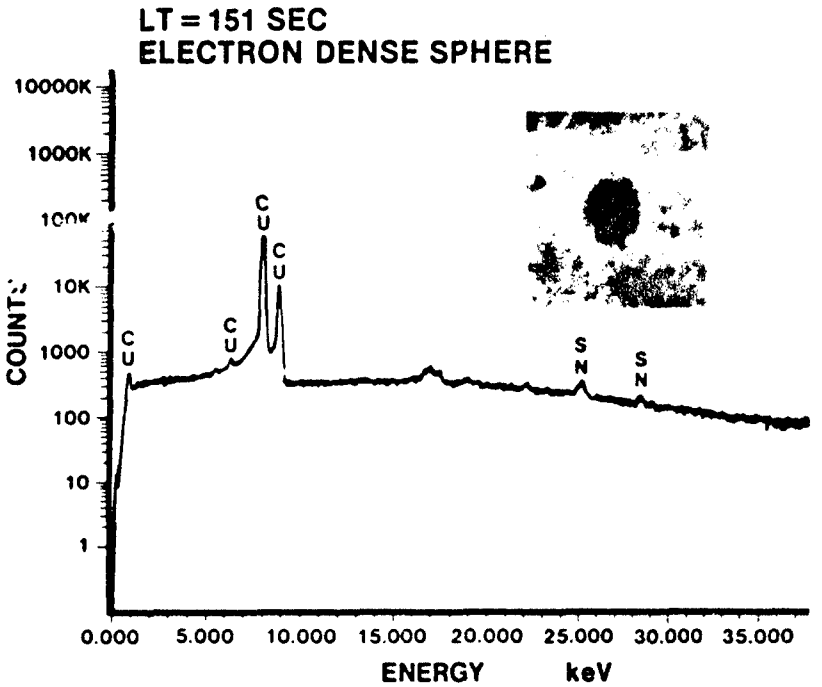


Figure 7b. An X-ray EDS spectrum produced from the membrane-associated aggregate, as shown in the inset. The organotin aggregate is preserved in this glutaraldehyde-carbohydrazide-embedded sample, producing an energy spectrum characteristic of tin with K-lines at 25.27 and 28.483 keV. Again, the copper background spectrum did not interfere with the analysis.

bromide, sodium nitrate, or sodium iodide did not affect TBT-mediated hemolysis. There was a slight decrease in TBT-induced hemolysis rates by 1 mg/ml catalase or superoxide dismutase. However, the decrease was the same for both native and denatured enzymes. Concentrations of 1 mg/ml *d*-α-tocophero. acetate and 100 mM mannitol both decreased hemolysis rates only slightly.

Erythrocyte suspensions treated with hemolytic TBT concentrations plus sufficiently high sodium cyanide levels had greatly accelerated hemolysis rates. The data plotted in Figure 8 demonstrate that 10 μM TBT in the presence of increasing sodium cyanide concentrations stimulated hemolysis rates. Hemolysis was stimulated slightly above control (171 mM ethanol) by 10 mM sodium cyanide in the absence of TBT. Also, 1.0 μM TBT plus 10 mM sodium cyanide produced the same slight increase in hemolysis. TBT at 10 μM plus 1 mM sodium cyanide produced hemolysis rates similar to 10 μM TBT alone. Erythrocyte lysis by 5 mM sodium cyanide in the presence of 10 μM TBT was greatly increased over that induced by 10 μM TBT alone. TBT at 10 μM plus 10 mM sodium cyanide increased the hemolysis rate to the level of 25 μM TBT. No other compound tested produced stimulation of TBT-mediated hemolysis in erythrocyte suspensions.

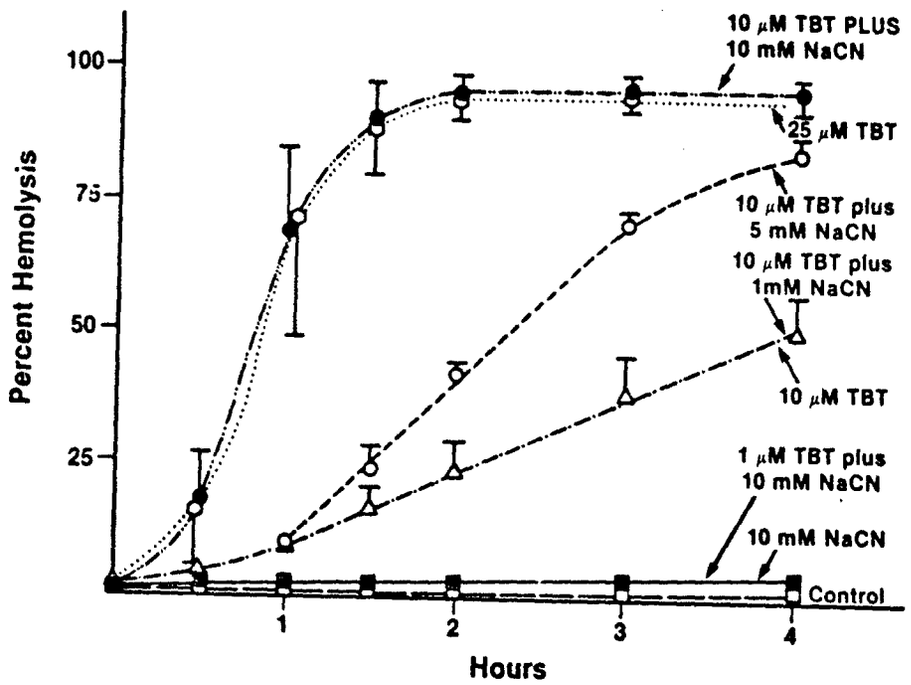
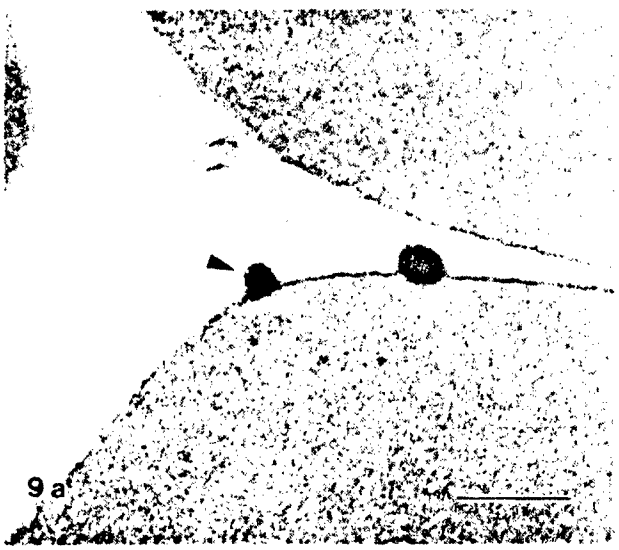


Figure 8. Percent hemolysis as a function of time for various TBT concentrations in the presence of sodium cyanide. Human erythrocyte suspensions contained about 2.3×10^8 cells per ml plus 171 mM ethanol. (□ – – – □) control (171 mM ethanol), (□ – – – □) 10 mM NaCN or 1 μM TBT plus 10 mM NaCN, (Δ – • – • – Δ) 10 μM TBT or 10 μM TBT plus 1 mM NaCN, (O – – – O) 10 μM TBT plus 5 mM NaCN, (● – ··· – ●) 10 μM TBT plus 10 mM NaCN, (*•••••*) 25 μM TBT.

Figure 9a shows membrane-associated aggregates in erythrocytes exposed to 25 μM TBT, and Figure 9b shows similar structures in erythrocytes exposed to 25 μM TBT plus 10 mM sodium cyanide. There is a size difference in aggregates observed following these two treatments: Aggregates in erythrocyte membranes exposed to 25 μM TBT were approximately 67 nm in diameter, and structures in cells exposed to 25 μM TBT plus 10 mM sodium cyanide were approximately 88 nm in diameter.

Selected mercapto compounds were assayed using the erythrocyte model to determine their effects on TBT-mediated hemolysis. Figure 10 shows formulae for two lipophilic compounds (BAL and DL-dithiothreitol [DTT]) and two water-soluble dimercapto compounds (sodium 2,3-dimercapto-1-propane sulfonate [DMPS] and *meso*-2,3-dimercaptosuccinic acid [DMSA]) examined. The single sulfhydryl-containing compound, β-mercaptoethanol (BMER), was ineffective in blocking TBT-mediated hemolysis. However, BAL, DTT, and DMSA were effective inhibitors of TBT-mediated



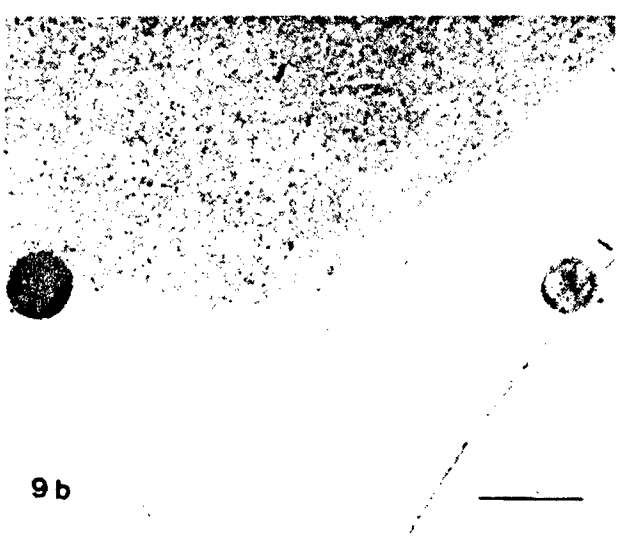
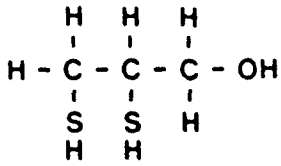


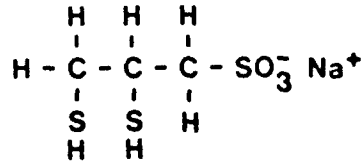
Figure 9a. Human erythrocytes exposed to 25 µM TBT will typically exhibit electron-dense, membrane-associated aggregates that have a mean diameter of 71.5 nm. Notice that the membrane-associated aggregate is truly intercalated within the membrane bilayer (arrowhead). Only the outer membrane half is clearly visible protruding into the extracellular space. Bar = 200 nm; ×80,500.

Figure 9b. Similar structures may be observed intercalated in membranes that have been exposed to 25 µM TBT and 10 nM NaCN; however, the mean diameter of 88 nm is significantly larger compared to TBT treatment alone. Bar = 200 nm; ×80,900.

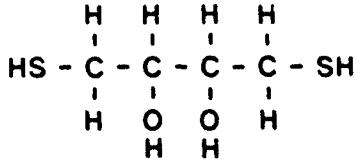
hemolysis when present at twice the TBT concentration (Figure 11). Table 2 demonstrates how BAL was effective in blocking TBT-mediated hemolysis for up to 3 h when present at twice the TBT concentration. DMPS, a water-soluble analog of BAL, was almost ineffective as an inhibitor of cell lysis. BAL at tenfold the TBT concentration, added 30 min after TBT, slowed hemolysis but did not stop it.



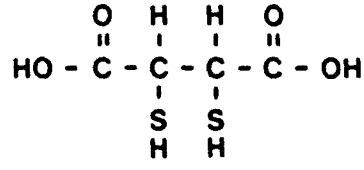
BAL



DMPS



DTT



DMSA

Figure 10. Formulae for dimercapto compounds 2,3-dimercaptopropanol (BAL), sodium 2,3-dimercapto-1-propane sulfonate (DMPS), DL – dithiothreitol (DTT), and *meso*-2,3-dimercaptosuccinic acid (DMSA).

BAL effects were examined in detail because this compound was the most effective inhibitor of TBT-mediated hemolysis. There was a progressive increase in the size of membrane-associated aggregates as the BAL concentration increased. Concomitantly, a reduction in the number of membrane-associated aggregates per cell profile occurred as the BAL concentration increased. Figure 12 most clearly shows the BAL-induced size increase in aggregate diameter.

DISCUSSION

TBT is a lipophilic compound that acts primarily as a membrane toxicant. Hemolysis induced by TBT has been demonstrated both *in vivo* and *in vitro* (9,19). The sigmoidal kinetic pattern indicates a complex process representing accumulation of sufficient membrane damage to permit leakage of hemoglobin from cells (10). In addition, a sharp cut-off concentration between 1 and 5 μM TBT exists for human erythrocyte lysis (~2.3×10^8 cells/ml). Therefore, human erythrocytes must

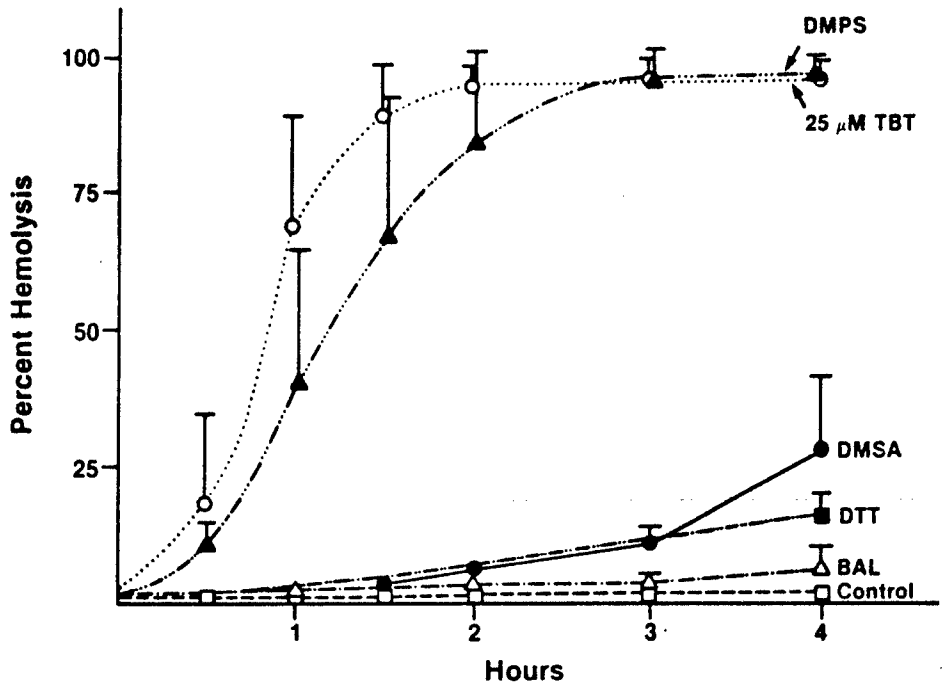


Figure 11. Percent hemolysis as a function of time for 25 μM TBT in the presence of selected dimercapto compounds. Human erythrocyte suspensions contained approximately 2.3×10^8 cells per ml plus 171 mM ethanol. (□ – – – □) control, (Δ – – – Δ) 25 μ TBT plus 50 μM BAL, (□—·—□) 25 μM TBT plus 50 μM DTT, (●——●) 25 μM TBT plus 50 μM DMSA, (Δ – ··· – Δ) 25 μM TBT plus 50 μM DMPS, (O····O) 25 μM TBT.

TABLE 2

SCHEFFE PAIRWISE COMPARISON PROBABILITIES THAT CONTROL (171 mM ETHANOL) TREATMENT YIELDS EQUAL VALUES OF HEMOLYSIS FOR LISTED TREATMENTS

Comparisons	Hour						
	0	0.5	1.0	1.5	2.0	3.0	4.0
25 μM TBT	1.00	0.00	0.00	0.00	0.00	0.00	0.00
50 μM BAL[a]	1.00	0.99	0.99	0.99	0.99	0.98	0.52
50 μM DTT[a]	1.00	1.00	0.99	0.98	0.72	0.01	0.00
50 μM DMSA[a]	1.00	1.00	0.99	0.99	0.96	0.04	0.00
50 μM DMPS[a]	1.00	0.65	0.00	0.00	0.00	0.00	0.00

[a]Erythrocyte suspensions with mercapto compounds also contained 25 μM TBT.

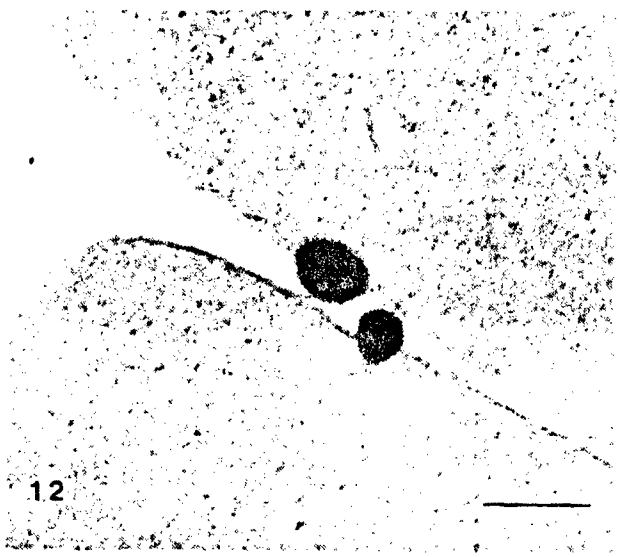


Figure 12. Human erythrocytes exposed to 25 µM TBT and 100 µM BAL. A 4.5-fold reduction in the number of these structures/cell profile occurs after 100 µM BAL treatment; however, note the significantly larger diameter aggregates (119 nm) associated with the membranes. Bar = 200 nm; x 78,500.

be treated with between 2.6×10^6 and 1.3×10^7 molecules of TBT per cell before the levels associated with the hemolysis above control (171 mM ethanol) levels occur. These calculated values are accurate if the assumption is made that all the TBT is in the erythrocyte membrane. Partition coefficients for TBT indicate that this assumption is a reasonable approximation of reality (20,21). Limited membrane incorporation of TBT without cell lysis indicates an accommodation mechanism for the compound by erythrocyte membranes through sequestration or possible metabolic conversion to a less toxic form.

Echinocyte shape transformation at TBT concentrations ≥ 0.1 µM substantiates the hypothesis that the compound is a membrane effector. Crenation of discocytes to echinocytes is usually induced by anionic or non-ionized compounds (16). These compounds are distributed asymmetrically in the outer layer of the membrane bilayer couple, producing cell shape transformation to echinocytic forms (22). Reversible shape transformation produced by 0.1 µM TBT is another indication of membrane accommodation of this compound (22).

Electron microscopic evidence demonstrates that tin is present in erythrocyte membranes treated with TBT (17). Tin-containing aggregates were the first demonstration of a stable structure produced by a xenobiotic intercalated in membranes. The observed aggregates are similar to vesicles formed by didodecyldimethylammonium hydroxide and other hydrophobic compounds in

aqueous suspensions (23). The presence of stable, hydrophobic aggregates within membranes is unexpected. Forces stabilizing these aggregates in a hydrophobic milieu may reflect the hierarchy of hydrophobic forces proposed for vesicles (24). Positively charged liposomes have recently been observed to bind to erythrocyte membranes and fuse with one another on membrane surfaces (25). Apparently, liposome interactions with membrane surfaces and TBT intercalation within membranes reflects variations in the interactions between liposomes or TBT aggregates with biological membranes.

We suggest that osmium forms complexes with organotin molecules in the TBT aggregates during the postfixation step. Perhaps tin is reduced by osmate anions during fixation, resulting in accumulation of osmium at aggregate sites (17). Ethanol dehydration removes alcohol-soluble TBT, leaving only osmium deposits at aggregation sites. Certainly, erythrocytes treated with TBT, fixed without osmium tetroxide, and embedded in glutaraldehyde-carbohydrazide have tin in the aggregates. TBT aggregates may be useful for further investigations of mechanisms of osmium staining.

Hydrogen cyanide is rapidly taken up by erythrocytes at pH 7 and causes a modest increase in hemolysis (26). However, hemolytic TBT concentrations in the presence of sodium cyanide (NaCN; $\geq$ 5 mM) stimulated hemolysis synergistically (10). The most reasonable explanation for this result depends on the known function of TBT as an electrically silent anion carrier in erythrocyte membranes (7,27). TBT-cyanide ion pairs would greatly increase the concentration of cyanide anion in or near membranes. This increased cyanide anion concentration could inhibit catalase, superoxide dismutase, or glutathione peroxidase located at or near membranes (28,29,30). In addition, increased cyanide anion concentrations could react with disulfide bonds of other erythrocyte proteins (31). Increases in TBT aggregate sizes in the presence of 10 mM NaCN are analogous to size increases in didodecyldimethylammonium hydroxide vesicles upon HBr addition (23). This analogy supports the theory that the size of TBT aggregates increases when the aggregates are bound to CN anions. The use of TBT plus cyanide may help explain cyanide-induced hemolysis and increase our understanding of membrane structure at the molecular level.

We found that certain dimercapto compounds effectively inhibited TBT-mediated hemolysis, which supports the hypothesis that TBT is primarily a membrane effector. The relative order of effectiveness for inhibition of TBT-mediated hemolysis by mercapto compounds is BAL>DTT>DMSA>DMPS>BMER (18). This order of effectiveness is also the order of hydrophobicity for dimercapto compounds. The very water soluble sulfonic acid compound, DMPS, is ineffective and the lipid soluble oil, BAL, is highly effective in reducing TBT-mediated hemolysis. These results suggest that dimercapto compounds must enter lipid reservoirs to react with TBT located in these reservoirs. BAL reduces the number of TBT-containing membrane aggregates per

cell, but those present are increased in size. In addition, BAL, added 30 min after TBT, reduced rates of hemolysis compared to erythrocyte suspensions with TBT alone. These observations also support the contention that TBT is a membrane effector.

The single sulfhydryl-containing compound, BMER, was ineffective in reducing TBT-mediated hemolysis. This result is similar to the observation that BMER did not reduce Lewisite toxicity (32). BMER is sufficiently lipid-soluble to enter membranes; and TBT, which is a weak Lewis acid, could allow BMER to act as an electron pair donor (33). However, the single sulfhydryl-containing compound, BMER, would be expected to form a five-coordinate complex with tin in TBT. Dimercapto compounds such as BAL have two potential sulfhydryl-combining sites and may form true chelates with tin in TBT. The coordination number of tin in TBT with BMER or BAL has not been determined. It would certainly be useful to know whether dimercapto compounds form such chelates with tin in TBT and what the relative water solubilities of TBT-dimercapto chelate compounds would be. This basic information could be useful in choosing BAL, DMSA, or other dimercapto compounds as possible therapeutic agents following TBT exposure.

A detailed molecular mechanism for TBT-mediated erythrocyte membrane lysis has not been determined. One possible explanation is rapid cellular ATP depletion, possibly due to inhibition of membrane calcium or sodium-potassium pumps at TBT concentrations $\geq 10\ \mu M$ (8). Cells depleted of ATP would form echinocytes rapidly, and lysis could follow (34). A second interesting explanation involves erythrocyte shape dependence not only on the lipid bilayer, but on cytoskeletal proteins as well (35). It is known that cytoskeletal protein 4.1 binding to the transmembrane protein glycophorin depends on phosphatidylinositol-4,5-diphosphate as a cofactor (36). TBT is a well-known anion carrier in membranes (8,27). Perhaps TBT strips anionic phosphatidylinositol-4,5-diphosphate from glycophorin, breaking crucial bonds with the cytoskeleton (35). The resulting disruption of cytoskeletal-lipid bilayer bonds would lead to shape transformation and hemolysis. A third possible mechanism involves production of tri-*n*-butylstannylperoxy (TBTOO) free radicals in membranes (37). The proposed mechanism shown in Figure 13 has the advantage of a relatively long half-life for TBTOO in aprotic environments (38). Lipid peroxidation due to TBTOO treatment could result in sufficient membrane alteration to permit hemoglobin leakage from cells. Finally, an unproposed mechanism may be operating to facilitate TBT-mediated membrane toxicity. Future mechanistic studies of TBT effects on membranes should add information concerning both TBT toxicity and membrane physiology.

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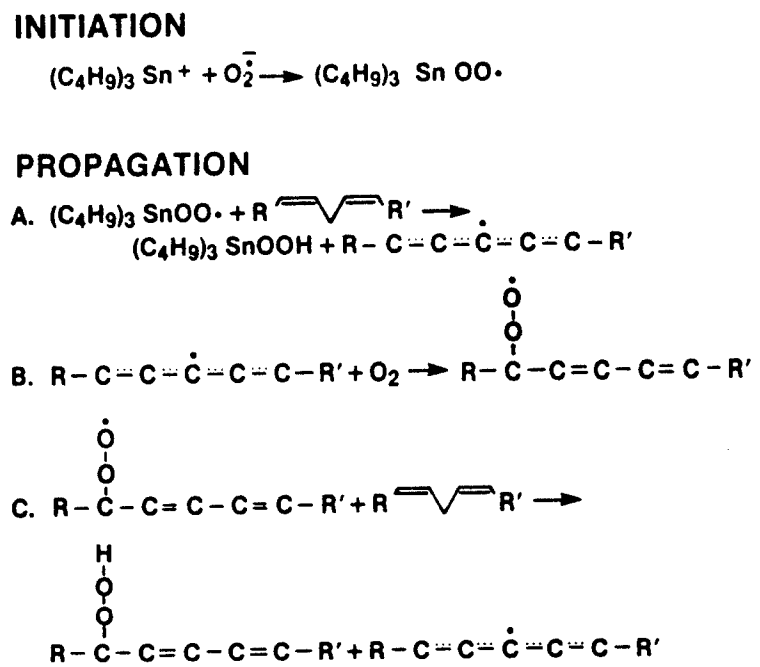


Figure 13. Possible tri-*n*-butylstannylperoxy free radical mechanism for lipid peroxidation in membranes.

electron microscope facilities at Wright-Patterson Air Force Base. We are grateful to Dr. W. Larsen for the use of his freeze-fracture facility at the University of Cincinnati.

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QUESTION AND ANSWER SESSION

DR. ZUCKER (NORTHROP SERVICES, INC.): Being an avid boater, this paint issue is very relevant to me. When sanding a boat, you are aware of the toxicity and you wear your face mask. In some application, possibly one that you may not have thought about, flow cytometry really could be very relevant to your work. I was curious whether you tested this out on the mammalian system such as tissue culture. In other words, what would tin be doing in other tissues rather than the erythrocyte, which is a perfect osmometer?

LCDR GRAY: We haven't tested it in a tissue culture system. But the white blood cell that we showed was undergoing lysis.

DR. ZUCKER: Okay. Just some application as you were talking. You can measure with a flow cytometer things like pH, mitochondria function, membrane permeability in membrane structure in addition to calcium studies which we relate to the structure of the membrane. I think the system that you mentioned here is very applicable to flow cytometry, and you may want to apply it in that particular direction.

LCDR PORVAZNIK (NMRI/TD): I just wanted to comment on the cell culture system. We did use a cell culture system, it was a mouse connective tissue cell. We looked at cell death using a tetrasolean blue dye. The concentrations did produce cell death and lysis.

LCDR GRAY: But there were strange things going on with the tetrasolean dye and the tri-*n*-butyltin.

THE BIOLOGICAL EXPOSURE INDEX: ITS USE IN ASSESSING CHEMICAL EXPOSURES IN THE WORKPLACE

Larry K. Lowry, Ph.D.

Division of Biomedical and Behavioral Science, National Institute for Occupati[illegible]afety and Health, Cincinnati, Ohio 45226

SUMMARY

Human exposure to chemicals in the workplace has traditionally been assessed by determining the concentration of an airborne chemical in the workroom air. More recently, biological monitoring has been used to assess worker *uptake* of chemicals by all routes of exposure. Both approaches for the assessment of exposure and uptake are complementary. This relationship is examined, along with the advantages and limitations of using biological monitoring.

The concept of the biological exposure index (BEI), developed by the American Conference of Governmental Industrial Hygienists (ACGIH), and information on the intended use and interpretation of BEIs are described. Examples are presented on the use of biological monitoring in NIOSH Health Hazard Evaluations (e.g., carboxyhemoglobin in blood to assess exposure to carbon monoxide, urinary metabolites of trichloroethylene to assess exposure to trichloroethanol, and 2-ethoxyacetic acid in urine to assess exposure to 2-ethoxyethanol). The progress of current research studies on the biological monitoring of volunteers exposed to paint spray solvents is presented, along with speculation on the future directions of biological monitoring research.

INTRODUCTION

The Concept of Biological Monitoring

Human exposure to chemicals found in the workplace has traditionally been evaluated by measuring the concentrations of these chemicals in workroom air or in the workers' breathing zone. Environmental limit values (e.g., threshold limit values [TLVs]; permissible exposure limits [PELs]; maximum allowable concentrations [MACs]; recommended exposure limits [RELs]) have helped keep worker exposure within so-called safe limits.

Environmental Limit Values

Environmental limit values (ELVs) are usually based on the relationship between the airborne environmental concentration and some adverse health effect of the industrial chemical in some laboratory animal or in humans. Epidemiology data gathered from actual workplace exposures are often available to support ELVs that are based on animal toxicology studies.

Measurement of the concentration of substances in breathing zone air does not ensure that the worker is totally protected from adverse health effects from chemicals in the workplace. The actual body burden of the chemical resulting from all routes of exposure is more directly related to potential toxicity. The uptake of the workplace chemical by the inhalation route, absorption of the chemical through the skin or the gastrointestinal tract, and nonoccupational exposure to the chemical all influence the body burden. Interaction of the chemical with other environmental and workplace chemicals may stimulate or inhibit its metabolism and elimination, and thus potentially influence the toxicity of the chemical in the worker.

Biological Limit Values

Biological limit values (BLVs) are based on the relationship between some measure of internal dose and either the environmental exposure or some measure of a health effect. BLVs provide information useful in assessing a worker's individual response and in measuring his or her overall exposure (1-3).

BLVs are subject to many factors that can affect the results: important biochemical factors such as the metabolism and pharmacokinetics of the chemical, and individual biological factors such as body build, workload, and life-style. BLVs also must take into account the uptake of a chemical by all routes of exposure, including dermal, oral, and nonoccupational sources, and background levels of the chemical or its biotransformation products in body fluids. All significant factors that contribute to biological variability must be considered when attempting to interpret biological monitoring test results for a particular chemical.

BLVs and ELVs

Bernard and Lauwerys have developed a concept to explain the interrelationships between environmental exposure, internal dose, and health effects (3). Bernard and Lauwerys recommend that the BLV be based on the relationship between the internal dose and an adverse health effect (Figure 1), whereas others suggest that the BLV be directly related to the assessment of exposure. It should be emphasized that the measurement of environmental exposure by traditional industrial hygiene methods does not measure the uptake of the chemical by the worker, nor does the environmental measurement take into account the absorption of the workplace chemical by all routes of exposure. BLVs should not be considered bioequivalent to ELVs because uptake and exposure are not equivalent. The two limit values approach equivalence when inhalation is the primary route of exposure, and only under carefully controlled conditions.

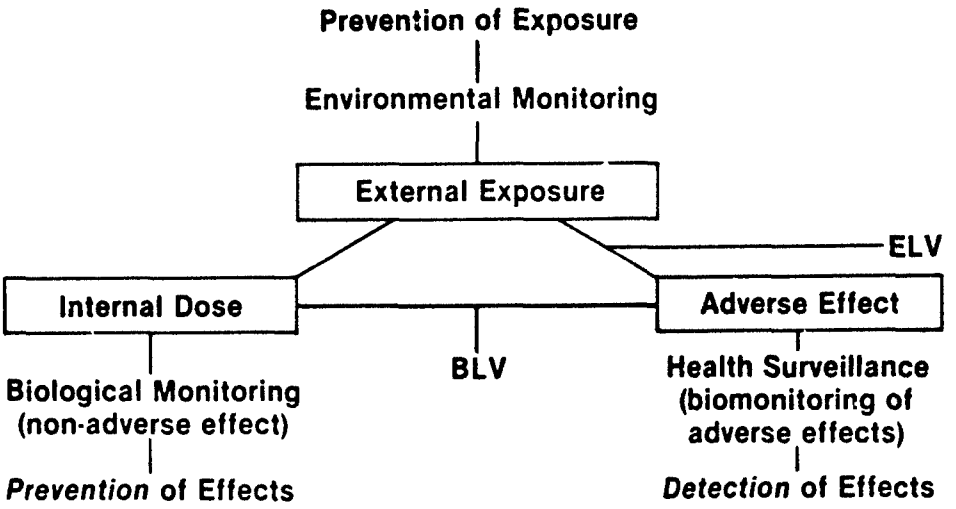


Figure 1. Bernard and Lauwerys' concept for determining BLVs and ELVs.

Usage and Limitations of BLVs

There are distinct advantages to using biological monitoring to assess human exposure to chemicals in the workplace. Unlike environmental monitoring, biological monitoring can assess exposure by all routes, not just by the inhalation route. Biological monitoring is particularly useful for situations in which there has been possible exposure by oral or dermal routes. Biological monitoring can assess the actual *uptake* of the workplace chemical by the worker. For example, it is often assumed that compliance with an airborne concentration limit will protect workers from adverse effects of airborne chemicals. However, the environmental concentration does not take into account breathing rate (minute volume) or duration of exposure. Thus, two workers may be exposed to the same concentration of an airborne chemical in the same breathing zone for the same length of time, but one may be doing heavy work (minute volume 30 l/min) while the other may be doing light work (minute volume 15 l/min). The first worker will absorb about twice as much airborne chemical as the second, hence the importance of uptake measurements as assessed by biological monitoring. The proper role of biological monitoring is as a complement to environmental monitoring. Both techniques are valuable, and each offers independent data that can help in the overall assessment of exposure and potential health effects.

There are significant limitations to using BLVs to assess workplace exposure. Because these measurements are estimates of uptake, the results do not distinguish between occupational and nonoccupational exposures. There is often wide interindividual variation due to exposure to other chemicals in the workplace and to life-style. While genetics can be a potential source of variation in response of the individual, diet, alcohol consumption, smoking, and drug use can also significantly

affect the levels of chemical or metabolite in a biological specimen and make interpretation of the results difficult.

Other practical limitations are the lack of sensitive methods and the lack of information about the pharmacokinetics of specific chemicals in humans. Knowledge of factors that affect the interpretation of biological monitoring results are known for only a few chemicals. Another serious limitation is the lack of published guidelines or biological limit values.

The ACGIH Biological Exposure Index

The BEI is defined as an index chemical that appears in a biological fluid or in exhaled air following an exposure to a workplace chemical and that serves as a warning of exposure by (a) the appearance of the chemical or its metabolite, or (b) the appearance of a biological response indicative of exposure (4). The BEI is primarily an index of exposure and not an indicator of some health effect that may have been produced from exposure to a workplace chemical.

BEI Documentation

The biological exposure index (BEI) documentations (5) review human workplace and experimental studies as well as the general pharmacology of the chemical agent. Factors such as absorption, metabolism, elimination, and kinetics are considered. The selected BEIs are further reviewed to address sampling instructions, methods of analysis, and storage stability. The pharmacokinetic properties of the BEI determinant are evaluated, including factors, both metabolic and workplace related, that could affect interpretation of the BEI. Finally, the specific basis of the BEI is documented along with the recommendations. An extensive reference section supports the final document (6).

Interpretation of BEIs

Notations that aid in the selection of the appropriate BEI and that provide guidance in the interpretation of BEIs are included in the documentations. Included is information on populations not protected by the BEI, and those BEI determinants that are not specific to a single chemical but that exhibit a good dose-response relationship. Other notations identify specific determinants that do not show an acceptable dose-response relationship but can be used to confirm exposure. Finally, those determinants that are normally present without exposure are identified, as well as those that show very wide interindividual variability (5).

Intended Usage of BEIs

The ACGIH BEIs are intended to be used as guidelines to assess total exposure (uptake by the worker). The introduction to the BEIs states that "Workers are not expected to suffer any ill effects as long as the prescribed measurement of the determinants are maintained within limits of the

recommended BEIs" (4). It must be emphasized that compliance with the BEIs is not a substitute for controlling the workplace environment. Environmental monitoring is still the preferred method to characterize worker exposure. Indeed, Occupational Safety and Health Administration (OSHA) regulations require environmental monitoring, but do not, with the exception of blood lead, require biological monitoring. Therefore, prudent practice would be to use environmental monitoring to assess the workplace levels of chemicals and thus comply with OSHA regulations; to use biological monitoring to more accurately assess uptake of workplace chemicals by the employee; and to use BLVs as a guideline to ensure worker health protection.

What should be the response by the responsible authority in the workplace to an individual or group of individuals with some elevated biological monitoring test above a recommended BLV? The ACGIH suggests repetition of the test and follow-up industrial hygiene measurements to determine the source of exposure that may have led to the elevated levels (4).

It is critically important that those who plan to use BEIs to assist in monitoring worker exposure consult the documentation published and updated each year (5). It is very important to know which BEI is most appropriate, when the sample should be collected, how the sample should be stored and analyzed, and what factors might affect interpretation of the results.

APPLICATIONS

The utility of biological monitoring is best demonstrated by a review of several National Institute for Occupational Safety and Health (NIOSH) studies in which biological monitoring played an important role. Some of these examples are taken from the NIOSH Health Hazard Evaluation Program for which work-site investigations were performed to evaluate the potential health effects of chemicals found in the workplace. Other examples include human laboratory studies to evaluate the utility of biological monitoring tests under controlled laboratory conditions.

Health Hazard Evaluation: Carbon Monoxide Exposure in a Warehouse

This NIOSH Health Hazard Evaluation (7) was conducted during the height of the so-called energy crisis, when concern for energy conservation was high. The warehouse used, located in the midwest, was typical of many and was used as a distribution center. Propane-fueled industrial trucks (forklift trucks) were used to move pallets. The study was conducted in the winter, with warehouse doors and windows tightly shut. Work classifications included industrial truck operators, storekeepers who kept records of inventory, and receivers who checked incoming and outgoing shipments. Some of the workers were cigarette smokers.

The work environment was sampled using area and personal breathing zone samples. Air samples were collected in Mylar bags and analyzed on site using an infrared analyzer. Health records

were examined and a questionnaire administered to determine smoking history, possible work-related health effects, and other sources of exposure to carbon monoxide. Pre- and postshift blood samples were collected for carboxyhemoglobin (COHb) analysis. These analyses were performed using a CO-Oximeter.

Eight-hour time-weighted average (TWA) values for carbon monoxide showed exposure to carbon monoxide near the NIOSH recommended limit of 35 ppm (Table 1). Area samples ranged from 38 to 60 ppm (n = 4). The medical data showed no work-related health effects. Table 2 shows the COHb data obtained from workers in the warehouse, including nonwarehouse controls with no occupational exposure to carbon monoxide. All postshift blood samples showed levels of COHb above the NIOSH recommended limit of 5% for nonsmokers except the NIOSH field investigator. Smokers' postshift samples exceeded 10% COHb. (Note: The BEI for carbon monoxide, established after this study was completed is "less than 8% COHb," with a notation indicating that the BEI may not be protective for all workers.) The data clearly show that smoking has a significant impact on COHb levels since both pre- and postshift COHb levels in smokers are elevated over nonsmoking controls. However, workplace exposure to carbon monoxide is also apparent since postshift COHb levels are higher than preshift levels, both in smokers and in nonsmokers.

TABLE 1

CARBON MONOXIDE LEVELS IN PERSONAL BREATHING ZONE[a] SAMPLES OF WAREHOUSE WORKERS

Worker	Carbon Monoxide (ppm)[a]	8-h TWA CO (ppm)
ITO I	52 - 55	31 - 33
ITO II	59 - 62	34 - 37
ITO III	62 - 66	40 - 43
Receiver	65 - 68	38 - 41

ITO = industrial truck operator

[a] Values shown represent the range of hourly samples collected throughout the work shift.

Examination of the net change in COHb levels in the three industrial truck operators who were nonsmokers (Subjects 2, 3, and 5) showed a mean increase of 5.9% COHb (range = 5.3 to 6.4%), which reflects exposure to carbon monoxide during the workday. The results clearly showed an increase in COHb during the workday, both in smokers and in nonsmokers. The medical officer concluded in his report that "There appears to be excessive exposure of workers to carbon monoxide from industrial truck emissions" (7).

TABLE 2

CARBOXYHEMOGLOBIN LEVELS IN WAREHOUSE EMPLOYEES

Worker ID and Job Classification	Sample Collected[a]	Cigarettes Smoked before Sample	COHb (%)
1 - Receiver	AM	none	2.2
2 - ITO[b]	AM	none	2.8
	PM	none	8.1
3 - ITO	AM	none	2.3
	PM	none	8.7
4 - Storekeeper	AM	none	2.0
5 - ITO	AM	none	2.2
	PM	none	8.2
6 - Storekeeper	AM	3 – 4	7.6
	PM	15 – 20	11.2
7 - Storekeeper	AM	none	2.0
8 - Receiver	AM	5	8.4
9 - Storekeeper	PM	none	6.8
10 - ITO	PM	15 – 20	10.8
11 - NIOSH[c]	PM	none	4.0
12 - Control	AM	none	1.5
13 - Control	AM	10 – 12	5.0

[a] AM samples collected between 8:00 and 9:00 am. PM samples collected between 2:54 and 3:15 pm.

[b] ITO = industrial truck operator.

[c] NIOSH = NIOSH field investigator.

Health Hazard Evaluation: Trichloroethylene in a Degreasing Operation

A NIOSH Health Hazard Evaluation was conducted at a compressor manufacturing company that used a trichloroethylene vapor degreaser to clean the grease off of the parts before assembly (8, 9). The employees who operated the degreaser did not use personal protective equipment.

Environmental personal breathing zone samples were collected using charcoal tubes. The tubes were capped and sent to the NIOSH laboratory where they were analyzed by gas chromatography after desorption with carbon disulfide.

Exposure to trichloroethylene results in the excretion of trichloroacetic acid (TCA) and trichloroethanol (TCE). Pre- and postshift urine samples were collected from each worker and shipped to the laboratory for analysis. TCA and TCE were analyzed as free TCA and as total trichloro

compounds minus free TCA (TCE) using the Fujiwara reaction (9). Urine data were corrected for dilution by expressing the results as TCA or TCE excreted per gram of creatinine.

Eight-hour TWA values ranged from 200 to 420 mg/m^3, compared to the NIOSH recommended limit of 270 mg/m^3. The urine metabolite data are shown in Table 3. The controls were employees working in other areas of the plant who were not exposed to trichloroethylene. The additional controls were the two NIOSH investigators who conducted the study. Note that NIOSH Worker B spent about 5 h in the exposure area, while NIOSH Worker A was only present in the exposure area for about 2 h. There were no recommended BLVs for the two trichloroethylene metabolites at the time the study was conducted.

TABLE 3

TRICHLOROETHYLENE METABOLITES FOUND IN THE URINE OF DEGREASERS USING TRICHLOROETHYLENE

Group (n)	TCA (mg/g Creatinine)	TCE (mg/g Creatinine)
Controls		
AM (9)	ND	ND
PM (9)	ND	ND
Exposed		
AM (19)	48 ± 7	34 ± 5[a]
PM (19)	43 ± 10	145 ± 33[a]
NIOSH		
AM (2)	ND	ND
PM (a) (1)	ND	25
PM (b) (1)	ND	60

ND = not detected. Detection limits were defined as 2 mg metabolite/g creatinine

[a] Statistically different at $p<0.01$

Interpretation of the metabolite data requires some knowledge of the pharmacokinetics of metabolite excretion in humans. The reported half-time for elimination of TCA following human exposure ranges from 48 to 96 h, whereas the corresponding half-time for TCE has been reported to be between 3 and 5 h. Use of these two metabolite assays on urine specimens collected before and after the workshift then allows one to estimate the exposure of the worker the previous week (TCA) and during the workshift (TCE). Indeed, the exposed workers do not show statistically different pre- or postshift TCA levels, indicating exposure on previous days. However, there are statistically different post-TCE levels compared to preshift levels, indicating exposure during the workshift.

(Note: The BEIs for trichloroethylene, established many years after this study was completed, are 100 mg of TCA per liter of urine, with the specimen collected at the end of the workweek; and 300 mg of TCA plus TCE [total trichloro compounds] per liter of urine, with the sample collected at the end of the shift and at the end of the workweek.)

The NIOSH Health Hazard Evaluation based on environmental and biological monitoring data concluded that there was excessive exposure to trichloroethylene in the degreasing operation (8). Note that the conclusions from the report, prepared in 1973, are not consistent with the current BEIs, which indicate that exposures were within the acceptable levels. These types of situations, where biological monitoring and environmental data do not agree, do occur. Proper interpretation of these data requires careful consideration of all the data. In addition, one must have an understanding of the limitations of the data and the methods used to collect and analyze the specimens. In this study, the biological monitoring data provided additional information that workers had been exposed to trichloroethylene on previous days.

Exposure to the Glycol Ether, 2-Ethoxyethanol, by Shipyard Painters

Thirty-five workers from a shipyard painting operation who applied paint containing the glycol ether, 2-ethoxyethanol (EE), were evaluated for exposure to EE. Work practices varied considerably between brush and spray painters. Some work environments were in confined spaces below deck, while others were in the open. The study was done in the winter, and the temperatures varied greatly depending on the painters' work areas.

Environmental breathing zone samples were collected for each worker for three consecutive days. Self-reported work practices were collected on a questionnaire and included the number of hours spent painting, the type of paint used, the location worked, and the use of respirators and gloves. TWAs were calculated, but may not be of real value in assessing exposure because of the relatively low volatility of EE. Although the control of air levels can reduce exposure, consistent or repeated dermal contact with EE can result in significant exposure (10).

Once absorbed, EE is metabolized to 2-ethoxyacetic acid (EAA), which is excreted in the urine (11). Spot urine samples were collected in 100-ml polyethylene wide-mouth bottles every day for one week, at the beginning and end of each workday. EAA in urine was measured using a recently validated procedure. EAA was removed from urine as an ion pair using tetrabutyl ammonium hydrogen sulfate, and quantitated by gas chromatography with flame ionization detection after derivatization with pentafluorobenzyl bromide (12,13).

Table 4 shows the maximum levels of EAA, expressed as milligrams EAA per gram creatinine, found in unexposed shipyard workers, painters not using paints containing EE, and painters using paints containing EE. The mean values show marked differences in maximum EAA values. The low

levels of EAA seen in workers not using paints containing EE probably reflects usage of EE-containing paints by other workers in the work area. The wide range of EAA levels seen in painters using EE-containing paints is probably due to considerable variation in work assignments, such as spray versus brush painting, location of work area, and use of personal protective equipment.

TABLE 4

URINARY ETHOXYACETIC ACID (EAA) IN WORKERS EXPOSED TO 2-ETHOXYETHANOL (EE)

Group (n)	Maximum EAA (mg/g Creatinine)[a]
Controls, shipyard workers (20)	Not Detected
Painters, not using EE (5)	6.6 ± 3.91
Painters, using EE (27)	25.0 ± 20.7

[a] Mean of the maximum levels of 2-ethoxyacetic acid seen in (n) workers + 2 standard deviations. The limit of detection is 5 mg/l corresponding to about 3 mg/g creatinine.

The relationship between urinary excretion of EAA and exposure has not been established. Because environmental measurements do not assess the total uptake of the solvent by the worker, TWA levels may not be useful indicators of a painter's exposure to EE because of observed skin contact with the paint.

This study has not yet gone through extensive evaluation to determine the importance of the many variables on the levels of EAA seen in the urine. The only statement that can be made now is that there appears to be a relationship between the excretion of EAA in the urine and the use of paints containing EE. At this point, biological monitoring for EAA seems to be the only clear means by which to assess exposure to EE. There are no published BLVs for glycol ethers.

Experimental Exposure to Paint Spray Solvents

Many common paint spray solvents produce neurobehavioral effects. A research study designed to evaluate early decrements in neurobehavioral performance provides the means to evaluate blood and expired air as biological monitoring tests for acetone and methylethyl ketone (MEK) (14). Human volunteers were exposed for 4 h while at rest to either 250 ppm acetone, 200 ppm MEK, or 125 ppm acetone plus 100 ppm MEK in a controlled environmental chamber. Environmental concentrations of acetone and MEK were monitored continuously using an infrared analyzer, and quality control was performed using a gas chromatograph equipped with gas sampling valves. During the exposure period, subjects performed a variety of behavioral tests.

Venous blood and end-exhaled air samples were collected at intervals during and after exposure. Blood samples were analyzed for acetone, MEK, and ethanol using the head-space gas chromatographic technique. End-exhaled air samples were collected in Mylar bags and analyzed by gas chromatography.

Average blood and expired air levels of acetone found at various collection times are shown in Figure 2. Corresponding MEK values are shown in Figure 3. The figures show that average blood levels of acetone and MEK continue to rise after a 4-h exposure and that the increase in acetone levels between 2 and 4 h is greater than that for MEK. Expired air samples appeared to reach equilibrium sooner than the corresponding solvent levels in blood. The estimated half-times of elimination for acetone and MEK were 3.9 h and 49 min, respectively, if first-order elimination is presumed. It might then be reasonable to expect that workers exposed to these solvents for 6 – 8 h will accumulate acetone over the workweek, while MEK would be totally eliminated before the next day. Blood levels of acetone and MEK appear to be additive; single exposures produce approximately twice the blood levels than when the solvents are used in combination at one-half the high concentration.

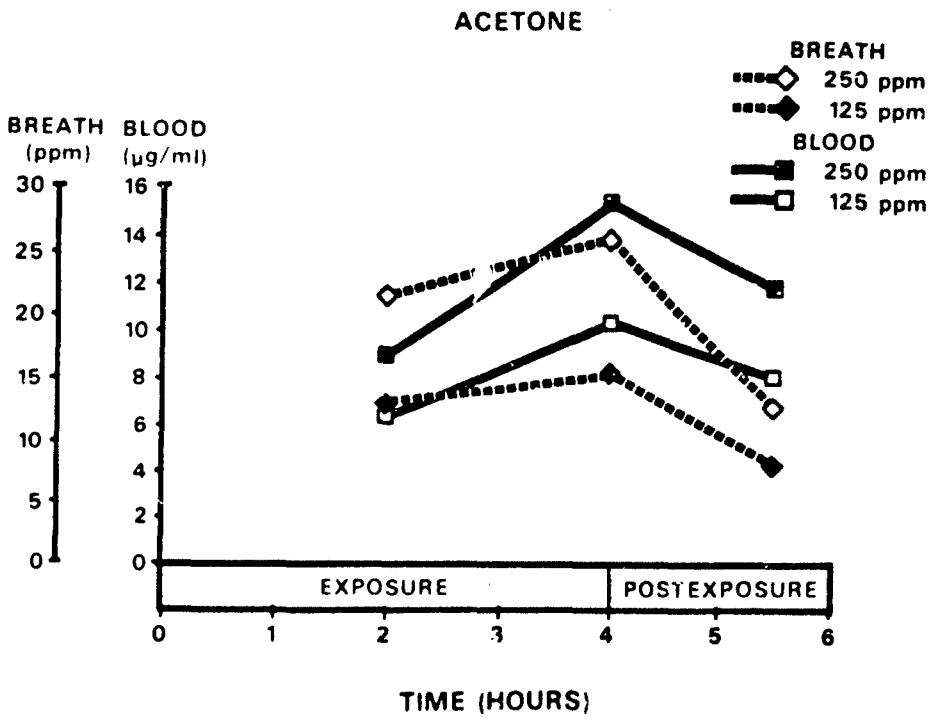


Figure 2. Average blood and expired air levels of acetone collected at intervals during and after exposure.

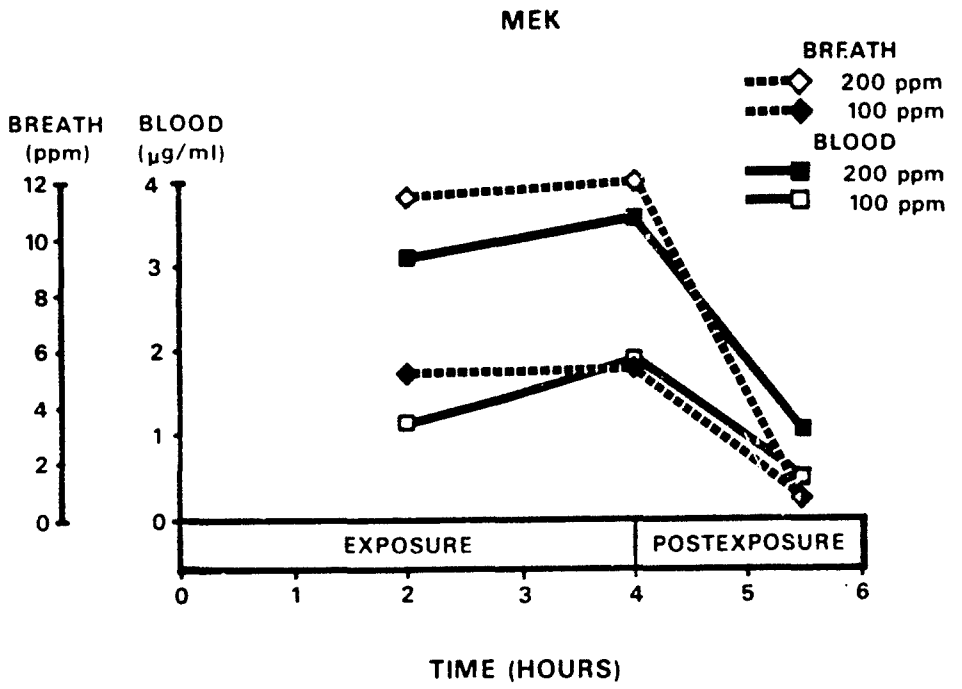


Figure 3. Average blood and expired air levels of MEK collected at intervals during and after exposure.

A similar study was done using 100 ppm toluene alone and at 50 ppm in combination with MEK (15). There appears to be no interaction of these solvents regarding blood and expired air levels. The elimination half-time of toluene appears to be between that of acetone and MEK, with little toluene expected in blood or expired air the day following exposure.

These experimental studies are valuable because they allow biological monitoring to be done under controlled conditions and under defined physiological workload. In addition, multiple-sample collection allows a better understanding of the elimination kinetics of the solvents and shows the need to understand these kinetics when collecting samples from humans in the workplace. Combined exposures are also important to study because most workplaces do not involve exposure to a single pure chemical.

FUTURE DIRECTIONS

The Future of ACGIH BEIs

The ACGIH BEI committee plans to concentrate its efforts on refining the definition of BEIs and developing a more comprehensive introduction to explain the intent, use, and interpretation of BEI data. Chemicals that are absorbed primarily by the dermal route will be the subject of future BEIs.

Future Trends in Biological Monitoring Research

Future research in the evolving field of biological monitoring can be classified into five major areas.

1. Development of improved methods of analysis, particularly the development of immunochemical methods to assess chemicals or their metabolites in biological fluids. Many methods have been developed for therapeutic drug monitoring, but virtually none have been developed for assessing industrial chemicals in biological fluids.

2. Development of new approaches to aid in the assessment of uptake/exposure in mixed exposures, such as is seen in many industries and in hazardous waste sites. The complicated interactions of mixtures of chemicals are not well understood.

3. Performance of more integrated studies to better understand the relationships between external exposure, health effects, and internal dose. This type of research could be conducted in the workplace on a chemical that has demonstrated neurobehavioral effects as an early indicator of potential health effects. It would involve the collection of environmental data, biological monitoring data, and behavioral effects data on exposure of the worker.

4. Research into the many factors (e.g., physiological, kinetic, metabolic) that can affect the interpretation of biological monitoring data. This type of research would have to be conducted using human volunteers, and would, of course, be limited to relatively innocuous chemicals. An example of this type of research would be the investigation of factors that could affect the dermal absorption of insect repellants. Methods would be validated for measuring the chemical in blood and urine, and for assessing the skin absorption of the selected insect repellant under a variety of conditions, such as site of application, dose, and formulation applied. The effects of heat, humidity, and exercise on absorption could also be investigated.

5. Research on the utility of biomarkers, such as hemoglobin adducts, to assess human exposure or uptake of chemicals found in the workplace. For example, aromatic amines form adducts with both hemoglobin and DNA in animals. The relationship between these two adducts and dose could be investigated through animals. If a quantitative relationship between these two adducts were found in animals, then aromatic amine hemoglobin adducts might be used to assess workplace exposures to aromatic amines.

There are many other potential areas of research that do not depend on technological developments, but on the application and acceptance of biological monitoring by workers and by employers as a cost-effective tool to assess exposure of employees.

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QUESTION AND ANSWER SESSION

DR. YANG (NIEHS): I have a comment, looking at your last few slides, particularly the neurobehavioral study with acetone on methylethyl ketone (MEK). This brings back Mel Andersen's research work. I think in this research work he uses a gas uptake technique for rodents. This technique is a very neat little technique in that it's noninvasive. They are monitoring the chamber concentration of a chemical and from there they deduce whatever happened in the animal. It seems to me that the same principle could be applicable to human studies in terms of acetone and MEK. You expose humans for four hours, and in your case you only have two sampling points. But those two points could be used to validate the gas uptake technique which could generate an infinite number of points. I think there might be some real utility to apply that type of technique on humans and thereby generate more results. I think it fits beautifully into the extrapolation aspect from rodent animal to human.

DR. LOWRY: I agree totally. And as I said before, this study was not designed as a biological monitoring study. It was designed as a neurobehavioral study. If you go in and perturb the subjects too much you will destroy the behavioral aspects of the study. You are measuring the behavioral response to the person with the needle or the blood sample, rather than exposure to the solvent. But certainly that's an excellent idea, and I thank you for the comment.

PANEL DISCUSSION II

Major William Keller, D.V.M., USAF, BSC – Rapporteur

Brooks Air Force Base

MAJ KELLER: It certainly is an honor and a pleasure to have the opportunity to wrap up this session this afternoon on occupational toxicology. Although somewhat of a challenge, the papers were excellent. As you know, they are somewhat diverse. So I bring you the proverbial good news and bad news. The bad news is that when I was contacted about doing this I was affiliated with the Air Force Occupational and Environmental Health Laboratory as a consultant/toxicologist and it seemed like a really good idea at the time. A month or so ago I moved over to the headquarters, Aerospace Medical Division, and joined the staff. When you become a staff officer you tend to want to philosophize, generalize – and some of you became aware of that this morning. So I have an overwhelming urge to do that and I will do that. The good news is that I have only been there a month and I think I can get it over in about three minutes.

I want to do two things this afternoon. One is, as I said, make some personal observations in the nature of an overview, rather than trying to find a common question for our speakers. Perhaps that will stimulate some questions. Then, secondly, with your permission, as a way of wrapping up the afternoon, I want to introduce a bit of humor in the form of a short anthology of toxicology, risk assessment cartoons, and then we will have the discussion period.

In regard to composite materials, the discussion we received this afternoon provided us with a timely overview of the emerging technology which is driving serious health and safety issues. Of particular concern to me is the dynamic nature of the matrix and resin selection which makes decisions about what agents to evaluate for toxicity particularly difficult. It seems to me that this is very much a dynamic moving target. Hydrazine, on the other hand, is associated with an older, more stable aerospace technology, as evidenced by Major McDougal's paper. We have sufficient understanding of hydrazine's occupational hazards, however, to stimulate evaluation of such complex issues as dermal absorption of vapors. Of course this is an absolutely critical issue when dealing with a carcinogen that is a known skin penetrator with a TLV of 100 parts per billion. Clearly, the determination of a mechanism of action is fundamental to the establishment of the most sensitive toxicity end point for a given material. The examination of tri-*n*-butyltin's effect on the red blood cell membrane is an example of an innovative use of four different microscopy techniques to study a potential mechanism of action for tri-*n*-butyltin. Related to this is the question that I so adroitly dodged earlier in the afternoon regarding the determination of end points for hydrazine. I was glad Dr. Olson was in the audience, believe me. Biological monitoring and the development of the biological exposure index presupposes a significant knowledge base regarding the mechanism of

action of a toxic agent. It represents an ambitious but much needed evolution in our efforts to maintain a healthy work environment. Biological monitoring is, of course, not new. Complementary use of air sampling and physical exams to identify and control workplace hazards has a long history. However, new efforts, including the use of the biological exposure index and toxicokinetic modeling using computers to predict body and tissue burdens, offer a significant and needed step up in our capability to assess both potential and real worker exposure. These potential improvements in applied occupational toxicology are demanded by an increasing need for new chemical technology which also produces potential worker exposures and by a society that recognizes that, for instance, a hitter in baseball who hits 3 out of 10 times successfully or a basketball player who is successful in 50% of his attempts certainly is considered a star, but a toxicologist who is only successful 90% of the time is an abysmal failure. Thus the need for enhanced predictive capability for occupational exposures. I hope I have stimulated a few questions. I was going to say it is difficult to find a toxicologist this dedicated to his work, even an Air Force toxicologist, but I have heard of several studies today using human volunteers, so I don't know. Nevertheless, we principally depend upon animal models. At this point we will move into our discussion period.

DR. YANG (NIEHS): I have a question for LCDR Gray. Is there any knowledge of mutagenicity or oncogenicity of tri-*n*-butyltin?

LCDR GRAY (NAVAL DENTAL SCHOOL): There have been some studies done, and one government study claimed that it was not mutagenic. Of course tri-*n*-butyltin in the body is going to be degraded to di-*n*-butyltin, and I think there have been some positive studies with that in cell culture. The problem with the tri-*n*-butyltin, though, is that it is degraded to dibutyl and monobutyl by the liver before being eliminated . So you can't really tell which one is having the effect.

DR. YANG: Are you suggesting that the dibutyl and monobutyl are less toxic?

LCDR GRAY: That's been demonstrated with acute toxicity.

DR. YANG: So the structure has a bearing on toxicity.

LCDR GRAY: Yes, the three alkyl chains are more toxic than two or one.

MAJ KELLER: I have a follow-up question to that question on tri-*n*-butyltin. First, did you look at any of the other alkyltin compounds in your model? And second, given the effect that you found and the fact that organotins attack the central nervous system, can you make any comments about what you found and relate that to the central nervous system?

LCDR GRAY: The answer to the first part of your question is yes. In fact, Marty and I and Carlyle Flemming and Mrs. Lee are going to have another paper in which we look at various compounds – tetrabutyltin, tri-*n*-butyltin, tripropyl, triethyl, and trimethyl. Four of them, tributyl,

tripropyl, tetrabutyl and triethyltin compounds, all form the aggregates. The others didn't, up to five millimolar. Now the second part of your question was the central nervous system toxicity. It's been widely known for a number of years that with trimethyltin compounds you get hippocampal lesions. Triethyltin compounds give you the edema; with tripropyl, less central nervous system toxicity. By the time you get to tributyl, there doesn't seem to be central nervous system toxicity in the LD_{100} range. Animals that are intoxicated with tributyltin become apathetic. If you have done gavage, they start to salivate and they die. Mechanism, well we can't find very good evidence for why they die except the heart stopped beating. That's not good enough. You'd like to know a lot more about it.

MAJ KELLER: Thank you. I've got one for Dr. Lowry. Given that BEIs are dependent on analytical capability, what is your assessment of the medical analytical laboratory technology base? Do you think it can support these? Obviously there are some places that can, but I'm talking about across the country.

DR. LOWRY (NIOSH): I don't think your basic, general-purpose clinical laboratory can support these types of studies. There is not enough demand for the tests for them to develop them specifically. There are a few selected laboratories that will deal with industrial clients, and I think they are becoming more and more concerned as the demand increases from looking at these types of deaths. So that is a definite problem; and we have run into that problem trying to find laboratories to do some of the work for us that we can't do ourselves and we have had some horror stories because of that. If there is money involved, people say yes we can do. You give them the sample, and then the answer is no. So it's an important factor. That's a good question to ask.

LCDR PORVAZNIK (NMRI/TD): This is directed to Dr. Lowry. Concerning the glycol ether-exposed workers, did you look at any, say, white blood cell effects, depressions, suppression of any particular white blood cells as biological exposure in this study?

DR. LOWRY: No, we didn't specifically. I should say that this study was kind of a complex administrative disaster in that there were two studies conducted. One was the one I talked about this afternoon, which was strictly an environmental biological monitoring assessment. In conjunction with that study, another study was conducted, basically via Yale University, on reproductive assessment. There were a variety of biological clinical tests run in addition to collection of semen samples for semen quality. All of the people haven't gotten together yet. There may very well have been some of those types of analyses run. I have not seen them. One of the problems you have in relating a level of a metabolite in urine to semen quality or potential reproductive things is you are talking about a different time span, and that's a problem we have to deal with.

MAJ KELLER: I have a question for Maj McDougal. The problem of condensation of hydrazine vapor at high levels brings me to ask, did you observe any skin lesions on these rats at the high concentration ?

MAJ McDOUGAL (EOARD, London): No we didn't. I didn't get a chance to mention this other than in conversation with somebody afterwards, but hydrazine does go into the skin. In some cases, two or three days after an exposure, if you swab the animal with an alcoholic solution of orthochlorobenzaldehyde you get a colorimetric reaction. I don't ever remember seeing any lesions, although this colorimetric reaction was telling us that hydrazine was still there quite awhile after the exposure. So although I think we can be fairly confident that not enough hydrazine vapor goes through the skin to cause systemic problems, one of the things that needs to be investigated is to determine whether or not there really is a depot for hydrazine in the skin, and if there is, does that have any ramifications in toxicology? That's something that should be looked at.

MR. PATNODE (INDUSTRIAL COMMISSION OF OHIO): I have a quick question about the tri-*n*-butyltin. Did you investigate what happens to the mercury portion of those molecules and might data account for any? What was the Hg on there? That wasn't mercury?

LCDR GRAY: No, No. That must have been a poor slide. There is no mercury involved. It's tri-*n*-butyltin. It's *n*-butyltin.

MAJ KELLER: I have a question for Ms. Beck. Back to what I mentioned earlier. It's very difficult, at least in my impression, to get a handle on the matrix and resins of these compounds, because the people in the materials labs have changed their idea about what the composite of the future is every two or three years. Do you have a feel for when this technology is going to mature and when we can know what matrix we are using? As sort of an applied follow-up to that, in the Air Force we have things called technical orders that tell us how to do procedures, and safety is one of the things that is in these technical orders. Does Northrop contribute to the technical orders on these composites?

MS. BECK (NORTHROP CORP.): Well, my answer to the first question is that the technology will probably never be mature. If you look at the Forecast II initiative, it's calling for increased technology in the field of composites. All new groups of composites to be made. The newest and latest and greatest composite matrix materials are what they are calling ordered polymers, molecular polymers where they are thinking about going the whole wing span, molecule by molecule on the aircraft. So when you are thinking about things like that you can easily say that technology will never be mature in our near future. What we are going by as far as guidelines on safety, we get in with our prepreg material, material safety data sheets. Now those are supposed to key out dangerous or partially harmful or potentially harmful chemicals in the prepreg material. Sometimes the safety data sheets from the suppliers are not complete. So the next thing that we at

Northrop do is take a look at the information that is supplied. We handle it in the lab environment first. We check and note if there is anything that we see that should be watched when it goes into the production environment, and then from that point we do write our own guidelines. At this time, most of the composite material that the defense agencies are handling out on bases is completely cured.

LCDR WYMAN (NMRI/TD): I would like Ms. Beck to follow up on something we discussed briefly at the break. When utilizing composites, what happens when you have burned them? If you would characterize some of the concerns that may exist, and if you have knowledge of the material, how it decomposes and along that line?

MS. BECK: Again, that's an area in which there has not been a whole lot of work done. You do have a combustion of the organic matrix and then you will have the normal combustion-type products, carbon dioxide, carbon monoxide, pitch, tar formation-type products. The carbon fibers themselves are very light materials. They will fly and float away. In order to do anything with the glass material you would have to get to pretty high temperatures; same with boron. So glass and boron fiber-containing matrix composite materials are probably, in that respect, maybe a little bit safer than the graphite, but again the graphite is the one that is being most used.

CDR MACYS (PACIFIC FLEET HEADQUARTERS): If I could follow up on that a little further. Can you address the fibers themselves? Have there been any data presented regarding fiber toxicity or even mechanical data as far as the friability of the fibers – what size particulate we are likely to encounter?

MS. BECK: Diameters of carbon fibers that are used right now are between four and seven microns. They are long, continuous-type fibers, and I know that in discussions with people during the break that some people are starting to do some work on toxicity of fibers. I don't think there are any results that people can talk about at this point. So, no, there really hasn't been much work done.

LCDR GRAY: Can I ask a question? Is there any way to make those fibers longer so that they might not be inhaled?

MS. BECK: Well, the fibers that are used in the matrix in making a composite part are extremely long. We are talking of fibers in length on the order of feet, but the diameter is what is so small.

SESSION III

FRONTIERS IN PREDICTIVE TOXICOLOGY: A FORUM

INTRODUCTORY REMARKS

Major Gerry M. Henningsen, USAF, BSC – Chairman

Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base

Good Morning, Ladies and Gentlemen. I would like to extend a warm welcome to everyone to the second day and the third session of the 16th Conference on Toxicology. This morning's session is entitled "Frontiers in Predictive Toxicology: A Forum." Because it is a forum, we have set up this particular session a little differently from the other four in that it is meant to be a tutorial. We will not be having any discussion panel or rapporteur after the final speaker. Rather, five prominent scientists will be discussing various topics within their fields of expertise. The conference organizers selected these five topics based on foreseeable toxicological research needs for the Department of Defense. We are privileged this morning to have a group of very distinguished and accomplished scientists who will be describing recent developments and implications in toxicology in their particular subject areas.

This morning we will hear from Dr. Ray Tennant from the National Institute of Environmental Health Sciences, who will speak on valid genotoxic assays. He will be followed by Dr. Gerald Fisher from Battelle Columbus Laboratories, who will discuss some *in vitro* techniques used in assessing pulmonary toxicity. We will then hear from Dr. William Scott from the Children's Research Hospital Foundation in Cincinnati, who will describe some of the principles and methodologies for looking at the effects of chemicals on the developing embryo. After a short break we will reconvene and hear from Dr. John Frazier from Johns Hopkins University, who will speak to us about some valid alternatives to animal toxicity testing. We will conclude with a presentation by Dr. Loren Koller, from Oregon State University, on recent developments in immunotoxicology.

SELECTION AND USE OF VALID GENETIC TOXICITY ASSAYS

Raymond Tennant, Ph.D.

Manuscript Not Submitted

IN VITRO MODELS OF LUNG TOXICITY

Gerald L. Fisher, Ph.D., and Michael E. Placke, Ph.D.

Toxicology and Pharmacology Department, Battelle Columbus Division, 505 King Avenue, Columbus, Ohio 43201

SUMMARY

In vitro assays that emphasize cellular components critical to the host defense system have been developed to evaluate pulmonary toxicity and define deleterious changes in parenchymal cell populations. Assays that employ pulmonary alveolar macrophages (PAM) have demonstrated good correlation between macrophage toxicity and pulmonary fibrogenicity for many inorganic compounds. The PAM assays provide simple and inexpensive screens of potential respiratory tract toxicity. Many investigators screen chemicals for their ability to alter the mucosal epithelium of the conducting airways by performing tracheal organ cultures. The tracheal assays have also provided useful screens for Vitamin A analogues required for epithelial cell differentiation. Most recently, *in vitro* respiratory tract models have been extended to include whole-lung explants, an approach that allows for development of fibrosis and epithelial cell toxicity after *in vitro* exposure to inorganic and organic fibrogens. The whole-lung explant system appears to duplicate the *in vivo* response to a variety of lung toxins, including bleomycin, silica, and crocidolite asbestos. Together, these assays provide a description of potential toxicity to key components of the lung, emphasizing the pulmonary macrophage, conducting airways, and alveolar septae. It is expected that continued research in these models will enhance their predictive abilities and utility in risk assessment.

INTRODUCTION

In recent years a number of *in vitro* approaches have been developed for evaluating pulmonary toxicity. Although the models were initially developed to define mechanisms of toxicity, their ability to predict toxic responses in whole animals has resulted in their development for use in multiple-tiered approaches to safety evaluation. The currently used cell and organ culture techniques provide a battery of assays to be used as screens for potential chemical toxicity as well as research tools to define mechanisms of action.

Although *in vitro* macrophage assays may be conducted with cells from a variety of species, we have emphasized the use of bovine PAM (1-3). There are several advantages to selecting this animal model. Sufficient cells (10^8 PAM) may be harvested from a single lobe of a bovine lung without the extended effort and individual animal heterogeneity of cellular lavage from multiple rodents. In this respect, one typically harvests 10^5 cells from a mouse lung and 10^6 cells from a rat, whereas an *in vitro* macrophage assay may well utilize 10^7 to 10^8 PAM. Furthermore, derivation of cells from a single

donor obviates concerns relating to interanimal variability and potential histocompatibility differences. Also noteworthy, as described below, is that bovine macrophages behave similarly to human PAM in culture. Of interest to the biochemist is the availability of reagents, sera, and biochemicals (2,4) derived from the bovine that may be used, without crossing species immunological barriers, to elucidate PAM mechanisms and function. Finally, the bovine cells are derived from lungs of healthy cattle slaughtered for food production, thereby minimizing the use of laboratory animals.

Historically, tracheal tissue was one of the first organs successfully cultured and maintained as an intact tissue, the first trials of which were described more than 20 years ago (5). The mucosal lining of the trachea and connecting major airways are critical for the maintenance of pulmonary function. *In vitro*, tracheal organ cultures retain their anatomical and functional integrity and are capable of responding to numerous toxic agents in a manner similar to tissues from whole animals. Tracheal organ cultures are currently being used by Government and industrial laboratories to assay analogues of essential biomolecules.

The tracheal organ culture model is suited for long-term culture and is well characterized in its morphologic and metabolic responses to a variety of toxic and carcinogenic substances (6,7). Previous studies have shown that tracheal explants exposed to polycyclic aromatic hydrocarbons (PAHs) or asbestos fibers produce mucosal lesions characteristic of those preneoplastic lesions in animals and humans following exposure to similar materials (8,9). This report briefly describes our studies (10) on the effect of age on the *in vitro* response of tracheal mucosal epithelium to organic and mineral carcinogens. The importance of chemical form as a determinant of biological response is also reviewed (11).

We recently developed an *in vitro* model to maintain long-term cultures of adult peripheral lung derived from hamster, rat, human, and bovine (12,13). By combining several techniques described in other cell culture systems and using a synthetic surgical material as a supportive base, the researcher can maintain the tissue's normal morphologic features and the biochemically functioning lung for four to six weeks in culture (14). Optimal culture conditions have been identified for tissue from different species, because particular laboratory animal species are better suited to the study of specific pulmonary diseases. For example, hamsters develop respiratory tumors similar to those observed in humans, whereas the rat is generally considered to be a better choice for studying pulmonary fibrosis.

Using a variety of pulmonary toxicants, we have produced, *in vitro*, tissue lesions that correspond closely to those observed in whole animals and humans following inhalation exposure of each compound (13). In initial experiments we examined the *in vitro* response to asbestos, silica,

paraquat, and bleomycin exposure; treatment with each of these agents resulted in the development of pulmonary interstitial fibrosis. The progression of the lesions and associated tissue changes varied between test materials, results which are similar to those described for whole animals. The ability to conduct correlative studies in human lung tissue provides a link between animal and human responses to toxic insult and makes the lung organ culture system a promising model for future use in respiratory toxicology.

MATERIALS AND METHODS

Bovine alveolar macrophages (BAM) (Figure 1) were harvested from the cardiac lung lobe of a healthy adult steer immediately after slaughter (1). The lung lobes are obtained from a local slaughterhouse and maintained on ice for one to two hours prior to use.

A sterile, blunt 15-gauge needle was secured with hemostats in the bronchi of the lung lobe, and the lung was repeatedly lavaged with 50-ml aliquots of ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS), until a total of 250 ml of lavage fluid had been collected. The cell pellet obtained after low-speed centrifugation (300 g, 30 min), was resuspended in minimum Eagle's essential medium (MEM). Aliquots of the cell suspension were removed for assessment of viability (trypan blue exclusion), total cell yield (hemacytometer counting), and differential cell counts. Cell suspensions were used only when the viability and number of macrophages exceeded 90%.

The cellular composition of lung washouts was usually greater than 90% BAM, and the remainder comprised lymphocytes with an occasional neutrophil. On rare occasions, a ciliated epithelial cell was also observed. Viability of BAM exceeded 95%. After attachment to glass slides in the initial incubation period, cultures were almost exclusively BAM (>97%).

Two-hundred-thousand viable macrophages were added to each Leighton tube containing complete media composed of 79% MEM containing a 1% mixture of penicillin, streptomycin, and fungizone (PSF); 1% L-glutamine; and 20% heat-inactivated fetal bovine serum. Macrophages were attached (1 h, 37°C) to glass coverslips in Leighton tubes to obtain a homogeneous, adherent cell population. The cells were then exposed to the test materials in the media and incubated for time periods of up to 20 h. At the end of the incubation, viability was evaluated for two coverslips from each group, and cells challenged with inert, carbonized latex microspheres at a 20:1 particle-to-cell ratio, and reincubated for 30 min. At the end of 30 min, coverslips were rinsed with PBS, air-dried, and stained with Wright-Giemsa. Cells losing adherence were collected and counted from the individual tubes after the initial 20-h exposure period and the 30-min challenge with microspheres. Coverslips were then stained with Wright-Giemsa, mounted on slides, and scored, 200 cells per coverslip (50 per quadrant) for phagocytic uptake of the inert microspheres. The phagocytic index

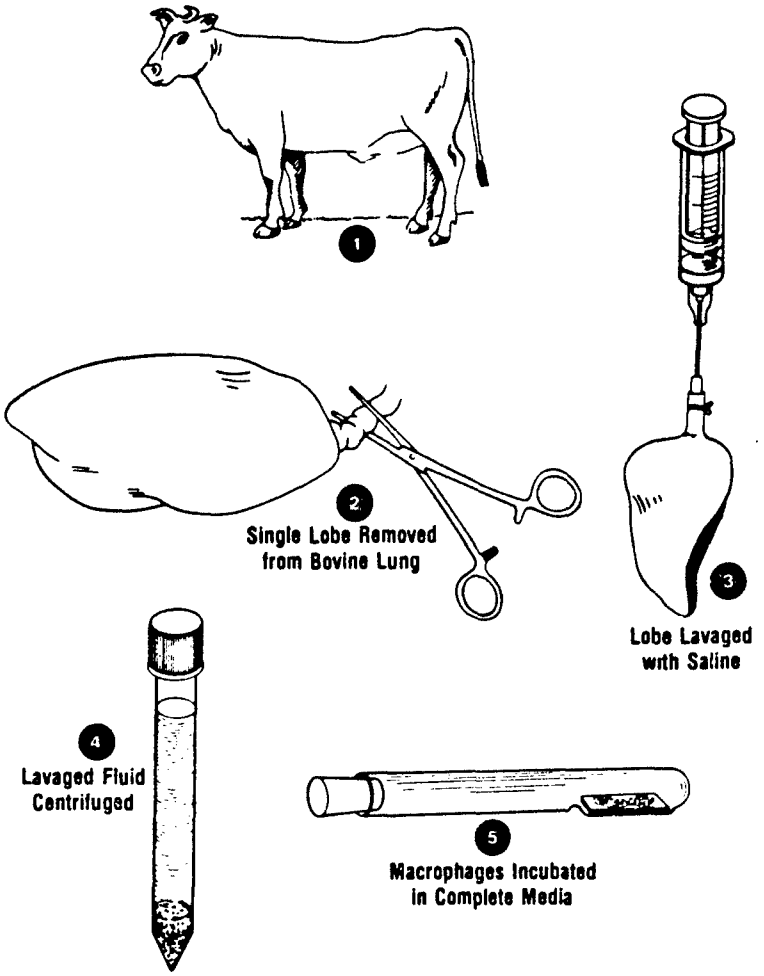


Figure 1. Summary of method for culture of bovine pulmonary alveolar macrophages. The cardiac lung lobe is obtained from cattle at slaughter, lavaged with calcium- and magnesium-free PBS, centrifuged, and cultured at 37°C.

was defined as the fraction of cells that contained one or more microspheres that were at least 50% encapsulated within the cell (15).

TRACHEAL EXPLANT CULTURE

Tracheal explants were obtained from female golden Syrian hamsters utilizing a method similar to Mossman and Craighead (16). Hamsters were anesthetized with sodium pentobarbital, and tracheas were aseptically removed distal to the larynx and proximal to the initial bifurcation of the primary bronchi (Figure 2). The tracheas were placed in sterile Petri dishes, longitudinally opened along the cartilaginous discontinuity, and washed with PBS containing 1% PSF. The tracheas were then halved longitudinally and transversely divided at every second or third cartilage ring,

yielding 2-mm x 2-mm explants. These were plated, serosal side down, onto scored culture dishes. The explants were maintained in MEM (Gibco Laboratories) with Earle's salts, 1.5 times the standard MEM concentration of amino acids and vitamins, and further supplemented with 1.0 µg/ml insulin, 0.1 µg/ml retinyl acetate, 0.1 µg/ml hydrocortisone hemiacetate, 0.1% PSF, and 100 µg/ml gentamicin at 37°C in 95% air and 5% CO_2. The explants were permitted to acclimate for four to five days in culture prior to treatment.

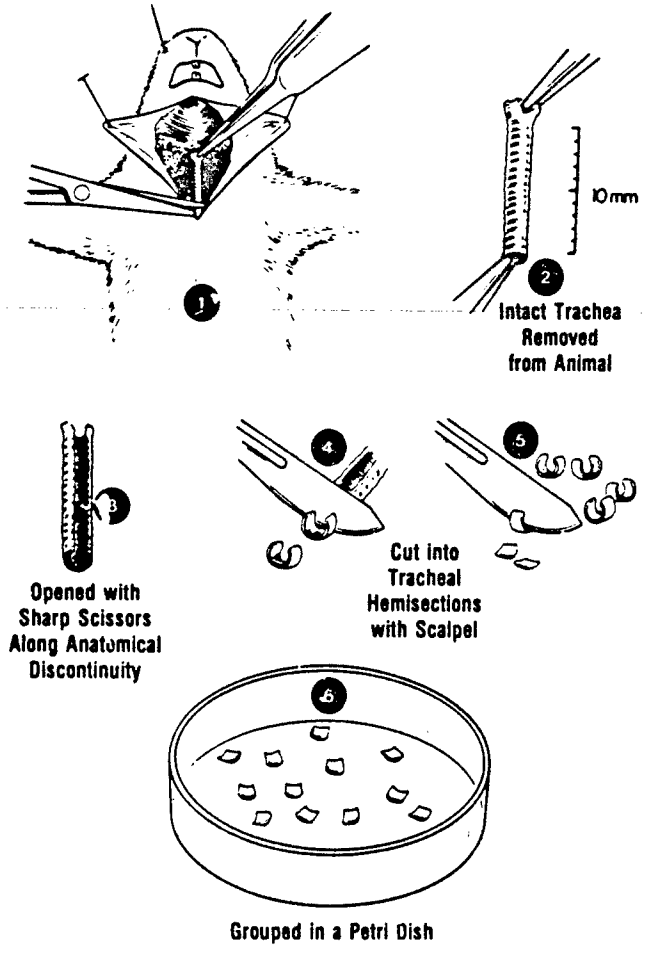


Figure 2. Method for tracheal explant culture. The trachea is aseptically removed, opened logitudinally, cut at every second cartilage ring, halved, and cultured (Figure courtesy of B.T. Mossman).

Tissues from each treatment group were collected one or three weeks after a two-hour exposure to the test article. Explants were immersed in a solution of 2% formaldehyde and 2% gluteraldehyde in 0.1 M phosphate buffer and processed for light microscopy by paraffin

embedding. Sections (3 to 5 µm) were stained with either hematoxylin and eosin (H&E) or Alcian Blue periodic acid-Schiff (PAS) at pH 2.5 for acidic mucopolysaccharides. At least three serial sections were used for each staining procedure. Explants prepared for scanning electron microscopy (SEM) were critical point dried, sputter-coated with gold, and examined with a Joel 100B STEM microscope.

WHOLE-LUNG EXPLANT CULTURE

Female golden Syrian hamsters were anesthetized with sodium pentobarbital and killed by exsanguination through arterial puncture of the abdominal aorta. The trachea was cannulated *in situ* with microtubing, and the trachea, heart, and lungs dissected from the body (Figure 3). The dissected tissue was then rinsed with PBS containing 1% PSF. The cannula was attached to a gravity perfusion apparatus, and the lungs were infused at a constant hydrostatic pressure of 20 cm of H_20 with heated (40°C) 0.5% agarose dissolved in tissue culture media. The concentration of agarose allowed it to remain in a soft, solidified state for an extended time at 37°C. The cannula was clamped and the lungs cooled to 4°C for 15 to 30 min to allow the agarose to solidify. After the lung had cooled, the heart, trachea, and all remaining fascia were removed. Complete transverse serial sections, approximately 1 to 2 mm thick, were cut from each lobe with a pair of scalpels, yielding 20 to 25 lung slices. Depending upon the distance from the primary bronchus serving each lobe, the sections measured 20 to 50 mm^2 (most 5 x 10 mm). Three sections were placed on 20- x 20- x 3-mm squares of sterile, gelatin sponge in 35-mm culture plates. Media was added to saturate the sponge and cover the bottom of each well, but was not permitted to cover the top of the gelatin support. The selection of appropriate media for peripheral lung culture is discussed in detail in the Results and Discussion Section of this paper. Sections were immediately incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and media was changed every other day.

Tissues were harvested for analysis up to 28 days after initial treatment. Three replicate wells (each containing three sections) per test article per time point, were included in the study design. Tissues were collected in 10% buffered formalin, paraffin-embedded, and serially sectioned at 5 µm. Separate sets of tissue sections from each group were stained for routine cellular differentiation with H&E; with PAS at pH 2.5 for polysaccharides and carboxylated and/or sulfated acidic mucosubstances; with periodic acid-methenamine silver for basement membrane and reticular connective fibers; with Masson stain for differentiation of connective and muscle tissue; and by the Armed Forces Institute of Pathology (AFIP) method for demonstration of lipofuchsin pigment.

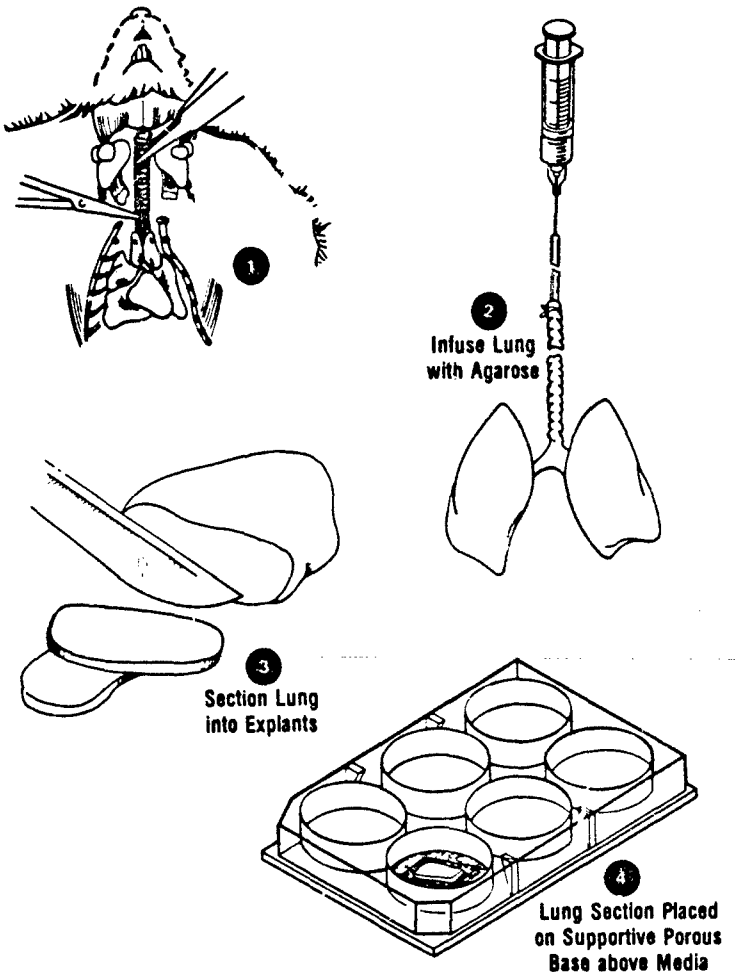


Figure 3. Summary of method for whole-lung explant culture. The trachea is cannulated, lungs infused at 40°C, cooled to 4°C for sectioning, and cultured on gelatin sponges.

RESULTS AND DISCUSSION

Pulmonary Alveolar Macrophages

Effect of Exogenous Factors. Studies were performed with macrophages derived from a variety of animal models to identify species-specific environmental conditions for cell culture of macrophages derived from murine, bovine, and human sources. For all species, the rate of test microsphere phagocytosis was affected by time in culture and by serum source. Detailed investigation of the effect of the bovine serum source on *in vitro* phagocytosis was performed using murine macrophages. When compared to cells cultured in fetal, mature, or adult bovine serum, cells cultured in newborn calf serum were most phagocytically active (Figure 4). In contrast with phagocytosis, no effect of serum source on initiation of glass attachment, maintenance of

adherence, or cellular viability was observed. Also, as indicated in Figure 4, extended preincubation of cells prior to test article challenge enhanced phagocytic rates. Subsequent studies with human (17) and bovine macrophages (unpublished results) also demonstrated a nearly twofold enhancement in phagocytosis when fetal bovine serum was replaced with newborn bovine serum.

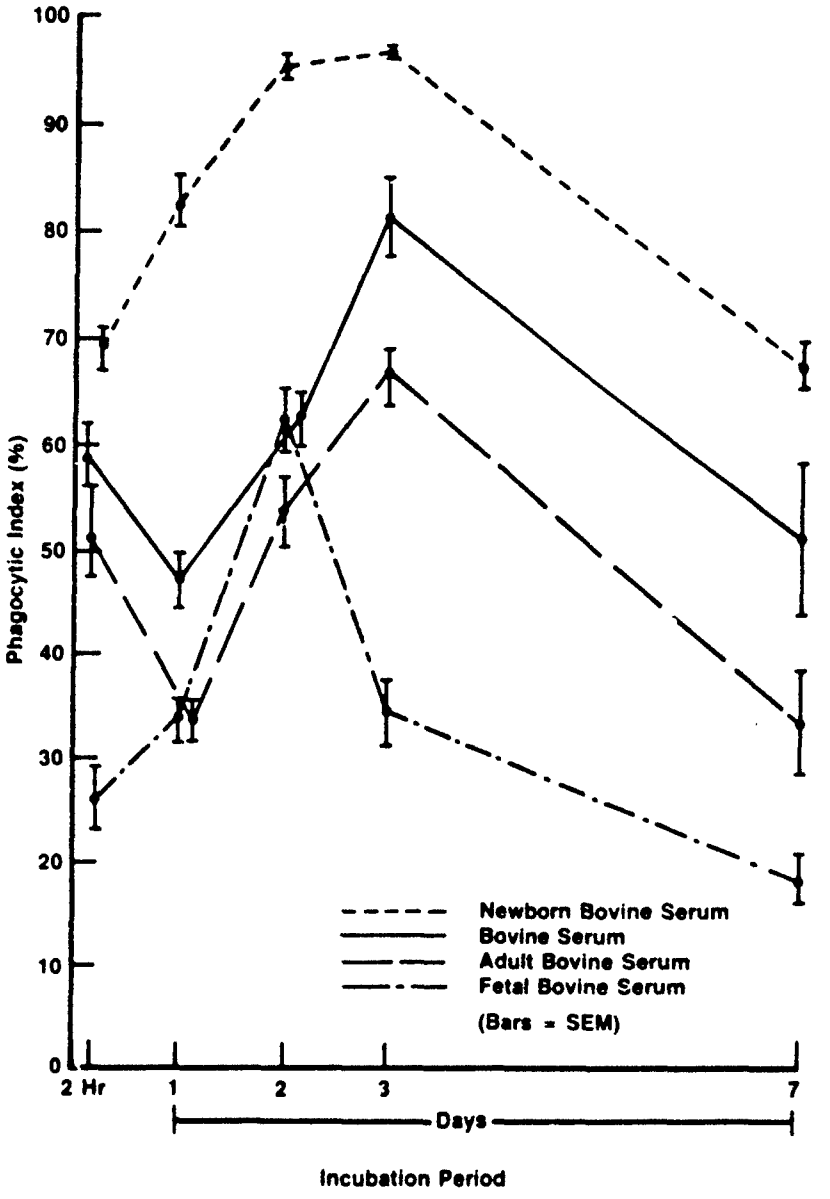


Figure 4. Murine pulmonary alveolar macrophage phagocytosis after culture with different sources of bovine serum for varying lengths of time.

Performance of cellular *in vitro* assays requires a careful definition of all parameters that may affect *in vitro* response. Regarding pulmonary macrophage assays, it is critical to understand the importance of the serum source (17), present- and post-challenge incubation periods (3), and the test particle-to-cell ratio (15). Clearly, assays should be designed to allow evaluation of either enhanced or diminished phagocytic activity after test article challenge.

Trace Element Studies. We have studied a variety of pulmonary toxicants using the bovine macrophage model. In one study we evaluated the dose-response relationship for soluble and insoluble inorganic compounds (18). The effect of concentration on reduction of phagocytosis to 50% of control activity (EC_{50}) differed by a factor of approximately 1,000 for the compounds studied. Vanadium was the most toxic element, whereas sodium selenite and zinc oxide were relatively nontoxic.

Trace element interaction studies were performed (18) using six trace elements (nickel, manganese, arsenic, vanadium, selenium, and zinc) in a pair-wise evaluation scheme. Interestingly, synergistic or additive relationships were not identified; however, three antagonistic relationships were clearly demonstrated. Selenite antagonized the cytotoxicity of arsenite and vanadium pentoxide, and zinc was weakly antagonistic to nickel. These interactions may be due to competitive anion interactions. The most complete inhibition of cytotoxicity was the reaction of selenite with vanadium. To evaluate whether the response of these two compounds was due to the chemical structure of selenite as a divalent anion or perhaps due to its oxidation-reduction potential, sodium sulfite, a compound of similar chemical structure and redox potential, was used as a comparator. We found no indication that sodium sulfite behaved as sodium selenite. These findings suggest a unique role for selenium in inhibiting the macrophage cytotoxicity induced by vanadium.

Particulate Exposure. In our BAM assays we generally utilize a positive and a negative control particle to provide historical reference data and to allow comparison of unknown test articles with biologically active and inactive particles. The glass beads (GB) are 1 to 3 μm in diameter and tend to induce a nonspecific (nontoxic), minimal inhibition of phagocytosis at concentrations of 3 mg/ml (1). In contrast, silica (1 to 3 μm) at 0.1 mg/ml reduces phagocytosis to 20% of control value. Thus, the evaluation of unknown test particle toxicity is performed relative to historical control particles that have extensive whole-animal data bases.

The macrophage assay has also been useful in identifying toxic components of complex mixtures. In a study of fossil-fuel-derived respirable particles it was found that oil fly ash was approximately 30 times more toxic than coal fly ash or GB, and 3 to 10 times more toxic than silica (19). Analysis of the oil fly ash indicated that the toxic components were primarily water soluble. Extraction of the oil fly ash in culture media reduced particulate toxicity to levels approaching that of

GB, whereas the soluble fraction maintained a significant toxicity. Elemental analysis of two sequential soluble fractions indicated vanadium concentrations of 20 and 17 µg/ml, respectively, and nickel concentrations of 267 and 13 µg/ml, respectively. As indicated earlier, macrophages are extremely sensitive to vanadium, with an EC_{50} of approximately 0.4 µg/ml (18). Because vanadium is approximately 25-fold more toxic than nickel to bovine macrophages, our studies are consistent with the hypothesis that the unique toxicity of oil fly ash relative to coal fly ash was due to the high vanadium concentrations.

In a collaborative research effort to evaluate the mechanisms of cytotoxicity of chrysotile asbestos, comparative studies were performed using heated and irradiated asbestos samples (20). Biological evaluations included studies of bovine serum albumin and DNA binding to asbestos, human skin fibroblast toxicity, tracheal organ culture membrane stability, and pulmonary macrophage viability and phagocytosis. All assays demonstrated similar responses consistent with the hypothesis that chrysotile asbestos toxicity is mediated through electron transfer mechanisms. Initial biochemical and cell membrane interactions may be initiated through electrostatic binding with hydroxyl groups on asbestos surfaces with subsequent electron transfer from trapped electrons within the chrysotile crystal matrix. In such mechanistic studies it is often important to employ a variety of *in vitro* systems to elucidate potential mechanisms of action.

Morphology. Morphological descriptions of macrophage disruption after exposure to toxic agents often include cell rounding, bleb formation, membrane smoothing and/or breakup, and complete cell lysis. After PAM were exposed *in vitro* to Ni_3S_2, TiO_2, and GB (Figure 5), both external and internal particles were observed using SEM and whole cell mount transmission electron microscopy (TEM) (21,22). There was a significant association between Ni_3S_2 content and the PAM damage markers consisting of focal bleb clusters, membrane mottling, and degranulation (Figure 6), whereas the negative controls, GB, exerted no such effects. Similarly, Ni_3S_2 and TiO_2 internalization was significantly associated with cell death, and GB was apparently nontoxic. Ni_3S_2 is a potent lung toxicant that induces inflammation and fibrosis after intratracheal instillation. In contrast, TiO_2 is widely considered to be a nontoxic, nuisance dust. The reason for the biological activity of TiO_2 is unclear, but may relate to the jagged TiO_2 particle morphology, resulting from grinding and comminution to obtain specifically sized particles.

Tracheal Organ Culture

In vitro studies have been used extensively to evaluate mechanisms of respiratory carcinogenesis. Oftentimes, these studies provide a bridge between clinical observations in humans and whole-animal investigations by examining *in vitro* responses of specific target tissues. Rat tracheal organ cultures and primary mucosal monolayers are tools widely used to examine

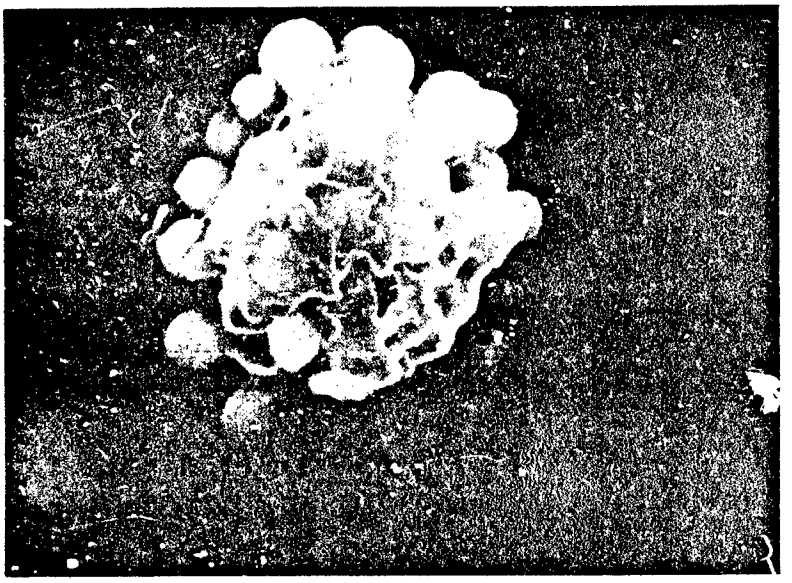


Figure 5. Scanning electron micrograph of a bovine pulmonary macrophage exposed to glass beads, demonstrating typical cell surface processes and membrane attachment. (Micrograph courtesy of G.L. Finch; 5040 ×)



Figure 6. Scanning electron micrograph of a bovine macrophage exposed to nickel subsulfide, demonstrating loss of surface features, membrane disruption, and bleb formation. (Micrograph courtesy of G.L. Finch; 5040 ×)

carcinogen effects on respiratory epithelium. Such studies have examined morphologic changes in organ cultures, cellular metabolism of organic carcinogens, damage and repair of DNA, progression to malignant tumors within carcinogen-exposed grafted tissues, and quantitative evaluations of the multistage transformation processes that occur in rat tracheal epithelium after carcinogen exposure (23-25). Similar, albeit fewer, studies have been conducted using hamster (26) and human tissues (27).

Exogenous Factors. In studies by Mossman and Craighead (28), the concentrations of nutrients and other constituents of media used to support tracheal organ cultures were systematically explored. Explants in test media were harvested and examined histologically (28), by autoradiography (28), and by SEM (G.L. Finch, personal communication). A striking proliferation of epithelial elements was observed in organ cultures maintained in a complex medium containing serum, whereas the columnar structure of the normal tracheal epithelium was maintained with other, less complex media or in minimum essential media containing serum. In contrast, disorganized epithelial changes resembling squamous metaplasia were observed in cultures maintained in the absence of serum. With some media these changes were focal, whereas with other media they were extensive and the normal respiratory mucosa was largely replaced by squamous cells. Subsequent studies have shown that adding Vitamin A to medium will reverse these alterations (29). Trachea are presently cultured without sufficient Vitamin A, as a model to screen for new retinoid analogues.

Chemical Carcinogens. Tracheal culture studies have been widely employed to delineate selected aspects of neoplastic progression following PAH exposure. We assessed the occurrence and histopathologic appearance of preneoplastic alterations in the mucosa of hamster tracheal organ cultures (30). Explants were singly or repeatedly exposed to a range of concentrations of benzo(*a*)pyrene (B[*a*]P). A spectrum of changes (e.g., focal hyperplasia, multifocal anaplastic proliferations, and diffuse cytotoxic damage) developed.

Generally, the lesions were enlarged nodular structures comprising undifferentiated epithelium lying in unordered arrangements. The frequency and extent of anaplasia increased with higher cumulative B(*a*)P concentrations. The lesions developed from single or small groups of cells with altered genetic expression of growth and maturation characteristics related to the B(*a*)P dose. It was noted that the most severe dysplasias with expansive masses or downward epithelial growth met many of the morphologic criteria for carcinoma *in situ* (Figure 7). The marked pleomorphism, hyperchromatic nuclei, increased nuclear-to-cytoplasmic ratios, and variation of cytoplasmic organelle populations were indicative of a functional shift from cellular metabolism to reproduction of immature cell forms (31). Hamster morphological lesions were more prolific and disordered than those reported for rat tracheas exposed to B(*a*)P (32). The increased susceptibility to B(*a*)P

respiratory carcinogenesis in hamsters may be related to differences in metabolism or to an inherent genetic predisposition of hamster respiratory epithelium to convert to a phenotype that is consistent with benign neoplasia.



Figure 7. Tracheal explant exposed to B(*a*)P and cultured for four weeks. A large focus of anaplastic proliferating epithelia arising from the mucosa has obliterated normal mucosal architecture. The mass comprises highly undifferentiated cells that share many morphologic criteria of a carcinoma *in situ*.

Age Sensitivity. To evaluate the importance of organ-donor age, a comparative study using tissues from 30-, 100-, and 180-day-old hamsters was undertaken (10). This study revealed an age-dependent sensitivity of hamster tracheal epithelium to the toxic effects of *in vitro* asbestos or B(*a*)P exposure. The most severe changes were observed in tissues taken from the 30-day-old animals compared to tissues from 100-day-old animals. Lesion frequency, severity, and area of involvement generally correlated with the dedifferentiated nature of the lesions. Based on the proliferative and dedifferentiated nature of the lesions, the changes noted may be due, in part, to the tumorigenic properties of asbestos and B(*a*)P.

Cellular Repair. Studies of the regenerative properties of tracheal respiratory epithelium were performed using hamster trachea exposed *in vitro* to Ni_3S_2. The predominant tissue change observed in exposed explants was epithelial degeneration and necrosis of the tracheal mucosa. The incidence and severity of mucosal damage was concentration-dependent for each nickel compound. The relative potential of nickel compounds to cause cytotoxic damage was ranked as follows:

$$\alpha\text{-}N_3S_2 > NiS > Ni\ powder > NiCl_2.$$

Ni_3S_2 caused early and severe degeneration and necrosis. Explants exposed to 1.5 µg/ml and 15.0 µg/ml and collected immediately after a one-hour exposure period had widespread cytomegaly and clear vacuolization of the columnar epithelial layer (Figure 8A,B,C). Multifocal cell necrosis and single-cell sloughing were also evident in the group collected immediately after exposure. Explants exposed to two lower concentrations of Ni_3S_2 (0.15 mg/ml and 0.015 µg/ml) showed focal, mild to moderate degeneration of the lumenal epithelium.

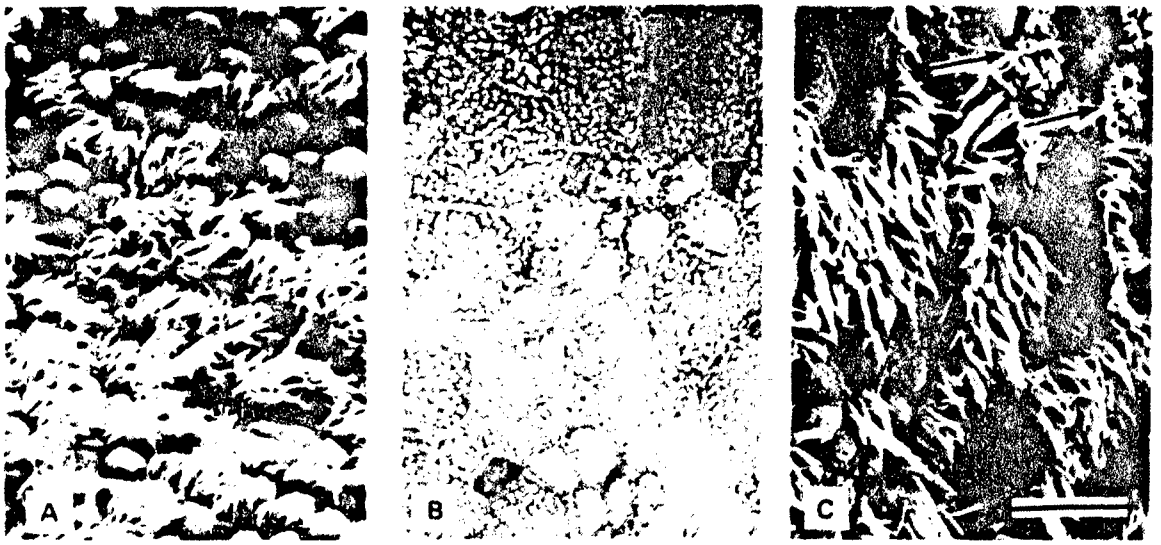


Figure 8. Scanning electron micrograph of cultured tracheal explant. After 7 days, the control culture (8A) demonstrates a normal incidence of ciliated and nonciliated cells; shortly after exposure to Ni_3S_2, marked desquamation and loss of ciliated cells is observed (8B); three days after Ni_3S_2 treatment, essentially normal ciliated and nonciliated cells are present (8C). (Micrographs courtesy of G.L. Finch)

Explants exposed to 15.0 µg/ml and 1.5 µg/ml of Ni_3S_2 showed no evidence of epithelial regeneration during the subsequent four weeks of culture. A thin layer of connective tissue covered both the serosal and lumenal surfaces of the partial ring of supporting cartilage, which were the only remaining viable tissues visible after four weeks. There were, however, focal areas of epithelial regeneration in explants exposed to 0.15 µg/ml and 0.015 µg/ml of Ni_3S_2. Frequent hyperplastic nodules and regions of squamous metaplasia were also observed. Two- to four-week cultures from the other nickel compounds did not demonstrate significant morphological changes compared to control cultures.

SEM studies were performed to further evaluate the epithelial regeneration in cultures taken from 30-day-old hamsters and exposed to Ni_3S_2 (11). The surface of control cultures appeared similar throughout the seven-day time course. The lumenal surface comprised ciliated and nonciliated cells

occurring with variable frequency (Figure 8A). Ni_3S_2-exposed explants displayed a marked pattern of cell sloughing and regeneration of epithelium. The epithelium appeared discontinuous immediately postexposure and had both ciliated and nonciliated cells sloughed from the surface, which revealed an underlying network of basal cells (Figure 8B). Desquamated areas contained epithelia with holes between cells and sloughing cells at the surface. After 20 h to several days, recovery advanced to a point at which the epithelium was either squamous in appearance, with flattened polygonal cells sharing raised cell borders, or essentially normal, with ciliated and nonciliated cells present (Figure 8C). Thus, it appeared that both desquamation of the normal columnar epithelium and basal cell replacement occurred rapidly, the latter beginning 20 h after Ni_3S_2 exposure.

Peripheral Lung Culture

Exogenous Factors. We have recently developed a system that allows long-term culture of peripheral lung sections (12-14). Airways and alveolar spaces were initially infused with media containing dilute agarose, which appeared to enhance nutrient and gas exchange by increasing surface area and providing a supportive, diffusable matrix. The agarose mixture persisted over a four- to six-week period, maintaining the parenchyma in an inflated state and thus preventing atelectasis and subsequent necrosis. The use of a gelatin sponge provided a gas-liquid interface that allowed media addition without submerging the tissue. This interface allowed ambient air to be used, avoiding the toxicity associated with the use of increased O_2 concentrations.

Biochemical determinations of hamster lung cultures indicated that two of the eight media evaluated supported *in vitro* protein and DNA syntheses as indicated by a moderate increase in protein and stable DNA concentrations through four weeks of culture. Thus, it appeared that the lung cultures maintained essential anabolic and catabolic processes. Morphometric analyses indicated that those cultures maintaining protein and DNA syntheses demonstrated normal septal thickness in contrast to the metabolically affected tissues. It appeared that the differences in the ability of the media to sustain lung viability is a reflection of the requirements for essential amino acids. Subsequent studies with rat, bovine, and human lung explants also demonstrated the advantages of media containing MEM or M199.

Morphological changes in adult peripheral lung explants were evaluated following *in vitro* exposure to a variety of classic fibrogens. In each case, exposure to either silica, asbestos, paraquat, or bleomycin resulted in the progressive development of interstitial fibrosis in lung explants during a four-week period after exposure. The histopathological characteristics of the primary tissue lesions shared many general features, but were uniquely specific to the fibrogen to which the tissue was exposed.

The distribution and compound-specific distinctions of each lesion were similar to pulmonary changes reported in whole animals following inhalation exposure of the corresponding test material. The single significant difference between *in vivo* and explant lesion formation was the absence of peripheral inflammatory response in lung organ cultures.

Fibrogen Exposure. Lung explants exposed to a fibrogenic substance develop fibrotic lesions in a time course and degree similar to that observed in whole animals exposed to the same material. Fibrogen-treated explants were compared with lung cultures maintained in media alone and tissues exposed to the nontoxic control particle, GB Exposing lung explants through the major airways to 5 mg/ml of GB resulted in no significant lesions of bronchioles, alveoli, or interstitium (Figure 9). There was a visible response by resident pulmonary alveolar macrophages to ingest the foreign particle. However, there was no syncytial cell or granuloma formation.

Explants exposed to finely divided silica developed diffuse interstitial thickening after two weeks in culture. The thickening of the alveolar walls was primarily due to increased cellularity and the presence of an eosinophilic amorphous material. Trichrome stains indicated that there was minimal increased collagen deposition. Four weeks following silica exposure, explant cultures had diffuse interstitial fibrosis with hypercellularity and increased collagen deposition in alveolar septae (Figure 10). The severity and, to a lesser extent, distribution of lung fibrosis were directly correlated to the silica concentration, until the concentrations were sufficiently elevated to cause irreversible cytotoxic damage.

Lung organ cultures exposed to crocidolite asbestos developed interstitial fibrosis in a time frame similar to silica. Significant differences in the response of cultured lung tissue to asbestos were a multifocal to regional distribution of the fibrotic lesion and the induction of squamous metaplasia in airway mucosa along respiratory bronchioles and alveolar ducts. Alveolar histocytosis involving resident macrophages was also a feature of *in vitro* asbestos exposure.

Lung explants exposed to paraquat or bleomycin had a slightly different pathogenesis of fibrotic lesion formation. Paraquat caused a concentration-dependent necrosis of alveolar lining epithelia. Concentrations at which the necrosis was mild to moderate within one week of culture often caused diffuse interstitial fibrosis in similarly treated explants cultured for four weeks. Bleomycin is an antineoplastic drug that causes secondary pulmonary fibrosis, which often limits its clinical use. Peripheral lung explants exposed to concentrations of bleomycin ranging from 1 µg/ml to 1000 µg/ml developed focal to multifocal septal thickening caused by Type II pneumonocyte hyperplasia. The hyperplasia appeared in a dose-related manner during the first one to two weeks following treatment. The epithelial hyperplasia persisted but was accompanied by fibroplasia and collagen formation during Weeks 3 and 4 of tissue culture.

SUMMARY AND CONCLUSIONS

A number of cell and organ culture assays are available for the *in vitro* evaluation of pulmonary toxicants. The bovine macrophage assay is perhaps the simplest and the best characterized of these short-term assays. The macrophage assay provides an initial screen for the evaluation of cytotoxic and cytolytic chemicals. We have found that evaluations that use a homologous series of chemicals or insoluble particles of similar physical properties are generally predictive of relative animal toxicities. The bovine macrophage system has been used for a variety of soluble and insoluble inorganic compounds and is well defined relative to the effects of culture conditions on the assay outcome.

Macrophage cell culture has also been used to identify potentially toxic components of complex mixtures. Thus, this relatively simple cellular assay provides for biological activity-directed chemical separation and hence may be used as a tool to support chemical analysis of biologically active complex mixtures.

The use of bovine cells provides the advantage of a large homogeneous cell population. Furthermore, because the cells are obtained from cattle slaughtered for food production, use of the bovine macrophage system helps to minimize the use of laboratory animals.

Tracheal explant culture provides a well-defined system for evaluating epithelial changes associated with chemical exposure. Morphological end points are evaluated in this system, with an emphasis on damage and loss of ciliated epithelium, basal cell hyperplasia, squamous metaplasia, and a variety of anaplastic changes. The tracheal explant system also has reparative capacity. Epithelial cell desquamation may occur after exposure to toxic chemicals. Often this lesion is followed by basal cell hyperplasia and, on occasion, repopulation by ciliated epithelial cells.

Cellular differentiation and maturation of ciliated epithelial cells requires Vitamin A or Vitamin A analogues. Tracheal organ culture provides a convenient approach to the evaluation of retinoids and other biomolecules as differentiating agents.

The ability to maintain peripheral lung tissue in culture for extended periods provides a unique opportunity to evaluate chronic obstructive pulmonary diseases. Furthermore, it appears that the explant system's response to a variety of fibrogens mimics whole-animal responses. Not only is increased collagen deposition observed, but, for particular fibrogens, the observed fibrosis is accompanied by other unique morphological changes including Type II cell hyperplasia, squamous metaplasia of the airways, respiratory bronchioles, and alveolar ducts, as well as interstitial wall thickening. Most interesting is the observation that after chemical exposure the pulmonary macrophages demonstrate an increase in numbers and occasional giant cell formation. The lack of

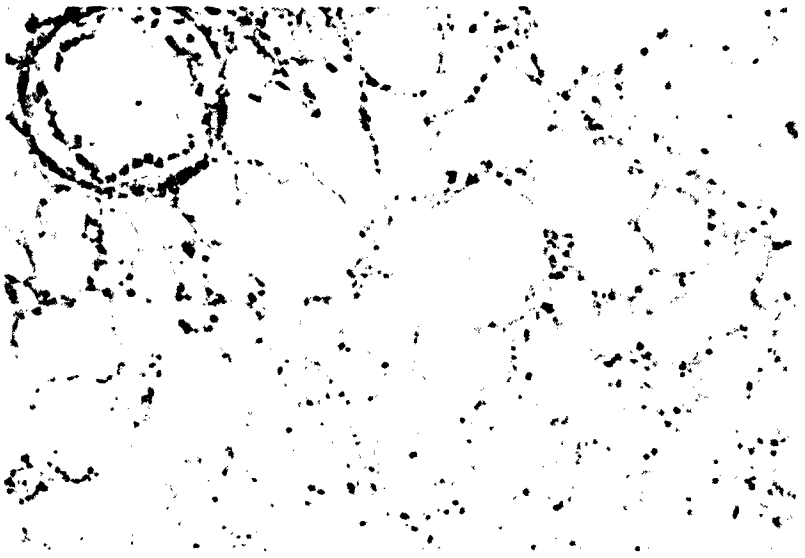


Figure 9. Peripheral rat lung explant exposed to glass beads via the airways and cultured for four weeks. The lung section appears normal, with no apparent reaction to the foreign particles. There is no septal thickening or increased amount of collagen.

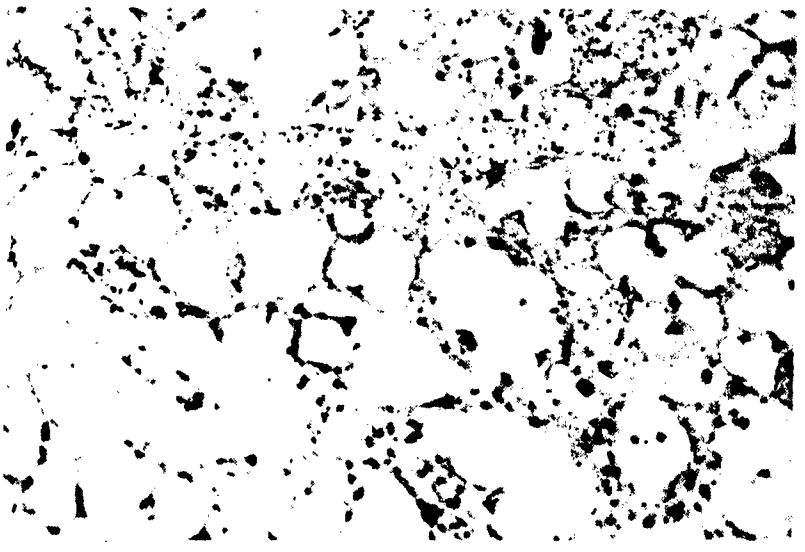


Figure 10. Peripheral rat lung explant exposed to silica quartz and cultured for four weeks. Micrograph shows diffuse interstitial fibrosis characterized by septal thickening due to pneumonocyte cytomegaly and mild to moderate hyperplasia with interstitial collagen deposition.

hematogenous recruitment indicates that the macrophages are derived from *in situ* progenitors, perhaps within the pulmonary interstitium.

With the ability to maintain adult lung tissue in culture for exended periods of time, it is possible that the culture techniques may be extended to include maintenance of lung explants for potential organ transplantation. Transplantation of lung lobes could extend the quality and duration of life for many people suffering from debilitating and degenerative chronic lung diseases.

In summary, a number of *in vitro* assays exist for screening of pulmonary toxicants. These assays may be tiered to provide information on the relative toxicity of environmental toxicants as well as new chemicals and drugs. The assay systems may also be used to complement standard animal evaluations to provide further insight into the pathogenesis of a variety of lung lesions.

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QUESTION AND ANSWER SESSION

DR. GARDNER (NORTHROP SERVICES, INC.): In doing phagocytic studies, one of the major conditions that you should maintain is a ratio of phagocytic spheres (or whatever you're using to be phagocytized) to the viable cells. In the condition with metals, such as with the vanadium studies, you had a 50% reduction in viability. How did you maintain that ratio or handle those data when you referred back to the phagocytic index?

DR. FISHER: I guess there are two issues here. First of all, we find that the human and bovine studies work well at an approximate 100 to 1 particle-to-cell ratio. These are 1- to 3-micron galvanized polystyrene beads that we are using. For the murine systems we often may work at a 200 to 1 particle-to-cell ratio. We do not correct for viability in terms of phagocytosis. The issue that Don is raising is, if one adjusted for the number of dead cells, would that change in ratio account for lethality? We chose not to do that but to look at the correlation between the two and when, indeed, we see inhibition of phagocytosis that is equivalent to loss of viability, then one can say that the decrement in viability was the cause of inhibited phagocytosis. On the other hand, as I demonstrated with arsenic, we saw that there wasn't any change in viability, yet phagocytosis was inhibited. Basically we look at both parameters but we don't correct phagocytosis for lethality.

PRINCIPLES AND METHODS OF PREDICTING DEVELOPMENTAL TOXICITY

William J. Scott, Jr., D.V.M., Ph.D.

Division of Basic Science Research, Children's Hospital Research Foundation, Cincinnati, Ohio 45229

SUMMARY

The detection of potential human teratogens is based on administration of test chemicals to pregnant laboratory mammals during organogenesis. Subsequently, usually just prior to the expected time of parturition, the fetuses are recovered by cesarean section, and the offspring are examined for structural malformations. These results are then extrapolated to assess the potential risk of exposure to the test agent during human pregnancy. Because of the expense and uncertainty associated with these procedures, a variety of new approaches, mostly *in vitro*, have been devised and are currently being scrutinized as a means for selecting the most appropriate agents for whole-animal testing. Some of these methods will be described briefly, and their advantages and disadvantages will be discussed.

BACKGROUND

In 1966, the U.S. Food and Drug Administration (FDA) proposed specific guidelines for testing the reproductive effects of exposure to various chemical agents. These guidelines have been incorporated into a testing scheme which, since 1966, has been used almost to the exclusion of all other test methods (Figure 1). Of the three segments shown in Figure 1, Segment II is the only one that was designed for the specific purpose of identifying potential teratogens. In this design, pregnant females are treated with test agent throughout the period of organogenesis. Days 6 through 15 constitute the so-called critical period for the rat, the animal species most commonly used in these studies. The animal's pregnancy is allowed to continue until just before parturition, at which time the rat is sacrificed and the fetuses are removed and subsequently examined for external, skeletal, and visceral malformations. Preparing and examining these fetuses consumes a modest amount of time because each pregnant female has approximately 12 or 13 fetuses. There are usually 20 pregnant females per dosage group; three treated and one control group comprise this segment of the teratology study. Sometimes a second species is also tested, which results in the expenditure of a tremendous amount of time, effort, and money. In light of this background and the fact that the number of environmentally relevant chemicals is extremely high, we can see how the need for *in vitro* screening for potential teratogens has arisen.

Species-specific differences in the teratologic response to chemical exposure are extremely common and prevalent during study, and, therefore, extrapolating the results of animal studies to human exposures is a method fraught with uncertainty. Yet many members of the scientific

I. **Fertility and General Reproduction**
   - **Males given minimal toxic dose (m.t.d.) for 60 days before mating**
   - **Females given m.t.d. for 2 weeks before and during mating, pregnancy, and lactation**
   - **Young examined at 13 days gestation, term, and nursing**

II. **Teratology Study**
   - **Pregnant females treated Days 6 through 15**
   - **Young examined 1-2 days before term**

III. **Perinatal and Postnatal Study**
   - **Dam treated last third of pregnancy and throughout lactation**
   - **Young evaluated for survival and growth**

Figure 1. Testing scheme for reproductive efforts of exposure of pregnant laboratory mammals to various chemical agents.

community believe that extrapolation from animal studies is the best method of those currently available. So, it is against this background that one must evaluate the *in vitro* screening method proposed here.

Teratologic Assays for Use in Screening Environmentally Relevant Chemicals

A great deal of experimentation and ingenuity have gone into the development and selection of the various systems for detecting reproductive effects of chemical exposure. Figure 2 lists some of the most pertinent screening systems in use today. These systems, which typically use either rats or mice, can be classified into those that depict the whole animal, specific organs, and whole-embryo cultures. For detailed information regarding screening methodologies, please refer to the excellent review from a Consensus Workshop on *In Vitro* Teratogenesis Testing (1). This publication also clearly delineates the significant issues regarding the usefulness of *in vitro* systems in predicting risk.

Validation Studies

Since 1982, many researchers have been trying to validate the various teratology assays by comparing the results of *in vitro* and *in vivo* systems using chemicals that have been classified as either teratogenic or nonteratogenic. The process by which the chemicals are selected is an important issue. A group of well-respected teratologists promulgated a list of teratogens and nonteratogens (2). This list has come to be known as the Smith List, after the senior author of the publication.

This paper will describe two *in vitro* systems, both of which were selected, developed, and validated by the National Toxicology Program -- the human embryonic palatal mesenchyme (HEPM)

Whole Animal

- Chick embryo
- Frog embryo
- Zebra fish embryo
- Drosophila embryo
- Planarian regeneration
- Hydra regeneration

Cell Culture

- Human embryonic palatal mesenchyme
- Neural crest cells
- Limb bud mesenchyme
- Drosophila embryo cells
- Mouse ovarian tumor cells
- Embryonal carcinoma cells

Organ Culture

- Whole embryo culture
- Limb bud culture

Virus

- Pox virion production

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Figure 2. Systems currently in use for teratology screening.

assay and the mouse ovarian tumor (MOT) assay (3). An extensive amount of work has gone into the studies of these two systems, both of which have just recently been finished.

The MOT assay was developed by Dr. Andrew Braun from the Massaschusetts Institute of Technology (4). The scientific rationale for this assay is that cell-to-cell and cell-to-matrix interactions are important events during fetal development, the interference of which would lead to subsequent teratogenesis. In this system, the MOT cells are labeled with tritiated thymidine and placed in a dish coated with Concanavalin A. The tumor cells then attach themselves to the Concanavalin A-coated plastic discs. The degree of attachment to the dish is measured at various concentrations of test chemicals added to the dish to evaluate their potential teratogenicity.

The HEPM assay was developed by Dr. Robert Pratt of the National Institute of Environmental Health Sciences (5). The HEPM cell line was derived from the palatal shelf of a single human embryo prior to palatal shelf evaluation. The rationale for using this cell line is that if an agent interferes with the growth of these cells, then it will likely also cause cleft palate, another teratogenic effect. These two systems, the MOT and the HEPM, were chosen, in part, for their complementary characteristics. It is apparent that antiproliferative agents are strong teratogens and have little

effect on cell attachment. In fact, they are very easily identified by the HEPM system because this system looks essentially at cell growth.

Results from Studies with Dilantin and with Ethanol

This discussion will review the results of studies in which two chemicals were tested using the HEPM assays. The first chemical is diphenylhydantoin, or dilantin or phenytoin, an anticonvulsant used in the treatment of epilepsy and often implicated for the small rise in the frequency of malformations in children born of epileptic mothers. The 50% inhibitory concentrations (IC_{50}) for cell attachment in the MOT assay were 6.6 mM and 2.13 mM. Two values are reported because two laboratories studied the chemicals simultaneously to investigate the issue of interlaboratory variation. The IC_{50} values for the inhibition of growth in HEPM were 0.61 and 0.49 mM. I provide these values because, as far as teratological evaluations are concerned, there is a major problem with *in vitro* screens. Teratologic effects are highly dose dependent, and, while it has not been satisfactorily demonstrated, the production of malformation is probably a threshold phenomenon. Because there is a dosage level below which the embryo will not be affected, it is not sufficient to state that, in an *in vitro* system, a chemical has an inhibitory concentration. There must also be some relationship between the inhibitory concentration and the *in vivo* potency of an agent. Most teratologists believe in another phenomenon called Karnofsky's Law (6), which states that all chemicals are, when employed at specific times of development and at specific dosage levels, teratogenic. If the appropriate amount is given to the appropriate species at the appropriate stage of development, developmental effects will occur. Given this, the only nonteratogens are those chemicals that are more toxic to the pregnant mother than they are to the embryo. The dilemma is how to determine whether a particular value identified in an *in vitro* system is indicative of a chemical's potential teratogenicity. For example, in one of the two laboratories, the researchers arbitrarily chose to use 0.5 mM as an effective concentration. Agents that were effective below that concentration were considered positive teratogens, and agents that were effective only at higher concentrations were considered negative teratogens. The 0.5 mM concentration was chosen because, when compared to *in vivo* studies, it resulted in the fewest number of false negative and false positive results. In three of the four tests done on dilantin, the results would have indicated that this agent is a nonteratogen. Only one of the four tests would have indicated that this agent is a teratogen, and yet dilantin is a known human teratogen.

The second human teratogen studied, ethanol, is perhaps the most important chemical teratogen of our time. Neither laboratory was able to find a concentration at which ethanol inhibited cell proliferation in the HEPM assay or cell attachment in the MOT assay. In one laboratory, the highest concentration tested was 54 mM, which seems like an overwhelming concentration until you consider that circulating in the blood of a female chronic alcoholic are concentrations in excess

of 100 mM. Thus, in these systems, ethanol also was labeled negative even though it clearly is a positive teratogenic agent for humans; this is also probably due to the dosage level utilized in these studies.

Discussion of Validation Findings

Another factor that needs to be considered when conducting *in vitro* teratogenicity assays is the ability of the test cells to metabolize the chemical agent being tested. Lack of such metabolism could be the reason why dilantin and ethanol were found to be nonteratogenic in the studies conducted. In the literature there is evidence that suggests that metabolism is required for dilantin and ethanol to be teratogens (7,8). It is interesting that the most notorious chemical teratogen, thalidomide, was also tested in these screening procedures, and that the results were negative in every screen. This result also was thought to be a reflection of the lack of metabolic activity. The screening procedures discussed here do have provisions for the inclusion of cytochrome P_{450} activity, which is certainly a step in the right direction toward perfecting these *in vitro* assays. Unfortunately, no provisions have been made for other enzymes, such as alcohol dehydrogenase, which may or may not be important in the teratogenesis of ethanol but is clearly implicated in the developmental toxicology of the glycol ethers. Although metabolism can be addressed in some respects, there is certainly still no *in vitro* system that can mimic the complete maternal system.

Another factor that needs to be considered when conducting these assays is that the mammalian embryo is not sensitive to every passing insult; that is, not every chemical agent that a woman might be exposed to is necessarily teratogenic. By replacing cells that have been lost or injured, the embryo has a tremendous capacity to repair or recuperate from a wide variety of damages. It does this by replacing the cells that have been destroyed or injured, and there is even some biochemical repair of the resulting lesions within a molecule. Figure 3 illustrates an example of cellular repair. On the right side of the figure are limb buds from a developing rat embryo, photographed a few hours after maternal administration of hydroxyurea, an anticancer agent; on the left side is a control limb bud. There is a tremendous loss of cellularity in the treated limb; the areas stained dark are the cells killed by the hydroxyurea. We have estimated that approximately 50% of the cells in this limb were lost due to treatment with the hydroxyurea, yet were this embryo allowed to develop until term, most of the buds would have essentially normal limbs. While a small percentage of the rat pups may be missing a single finger, most of the embryos will, from a loss of 50% of their cells, fully recuperate by increasing the proliferation of the remaining cells. This recuperative mechanism is not available in most of the *in vitro* screening procedures. Some of the whole-animal screens, the chick embryo for example, have just as great a capacity for repair as the one depicted in Figure 3.

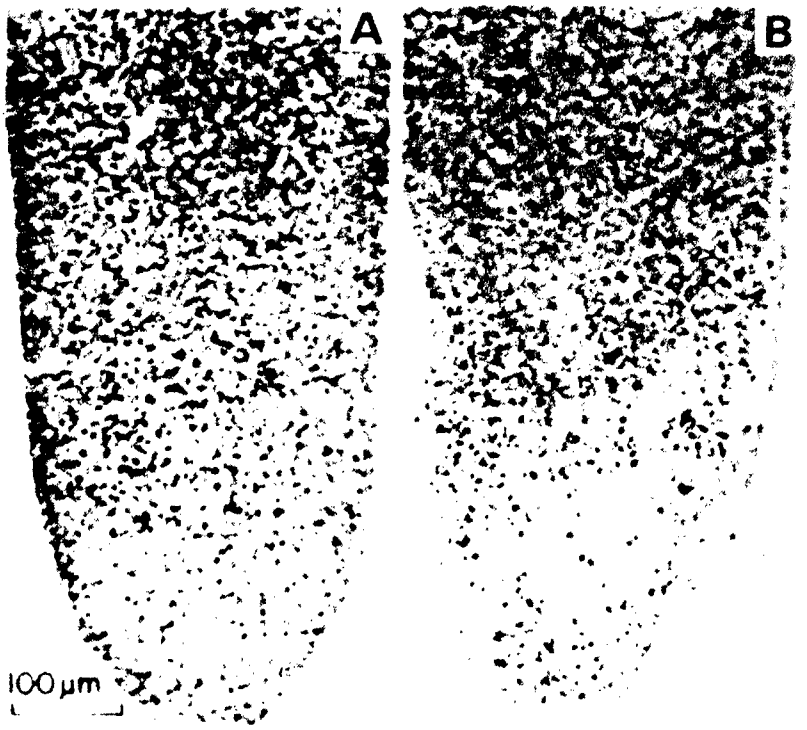


Figure 3. Cellular repair in the limb buds of a developing rat embryo after maternal administration of hydroxyurea.

CONCLUSION

Because of the multitude of chemicals that need to be evaluated, and because of the various problems associated with the existing methodologies, it would be very difficult to recommend any single *in vitro* assay. However, a simple *in vivo* screening assay might be the best course to take. The Chernoff-Kavlock assay (9) employs pregnant mice and a single dosage level; to ensure that a sufficient dose will reach the embryo, the dosage level must be maternally toxic. Dosing takes place during fetal days 8 through 12, the major phase of organogenesis in the mouse. The mother is then allowed to deliver the litter about one week later. The end points measured are the number of live fetuses born and the weights of the pups. The study continues until three days after birth, during which time the mother is allowed to suckle the offspring. The pups are again weighed and examined for external malformations. After the third day the study is terminated. The *in vivo* assay is less labor-intensive than the *in vitro* designs discussed earlier; it has another advantage in that it is conducted on a developing mammalian system, with full maternal metabolic capacity. At this time, this is, in my opinion, the most reasonable alternative to the phase FDA Segment II study.

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DR. NEWELL (ELECTRIC POWER RESEARCH INSTITUTE): I have a question related to the overall Segment II type approach – it may be a difficult one to answer. With the various teratogens that have been identified in that system, how many of those might be considered, say, false-positive teratogens in relation to what we might know would happen in the human being?

DR. SCOTT: That's a good question. It is a difficult one to answer. Superficially there would be a large number of false positives in that system. I think that's because we can push the dose very hard in those studies, which certainly isn't done clinically. So that I think agents such as aspirin are probably not human teratogens but can be made to look so in an animal system.

VIABLE ALTERNATIVES TO ANIMAL TOXICITY TESTING

John Frazier

Manuscript Not Submitted

IMMUNOTOXICOLOGY: EMERGENCE OF SENSITIVE AND PREDICTIVE ASSAYS OF CHEMICAL TOXICITY

Loren D. Koller, D.V.M, Ph.D.

College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331

SUMMARY

Immunotoxicology has undergone considerable growth and expansion since its inception in the early 1970s. The discipline was essentially founded by combining knowledge from the areas of immunology and toxicology and by ascertaining the effect xenobiotics exert on the functioning immune system. The field has progressed through a series of stages, including initial identification of immunotoxic chemicals, development of sensitive, quantitative assays to assess chemically induced immunomodulation, and determination of the mechanism by which xenobiotics compromise immune function. Immunoassays have been successfully developed and modified to characterize the immunotoxic properties of xenobiotics. Acceptable immunotoxicological procedures are available to assess modulation of humoral immunity, cell-mediated immunity, macrophage function, natural killer cell cytotoxicity, and cytokine activity. These procedures, coupled with recent developments in monoclonal antibodies and advances in cell culture techniques and immunology, are available to immunotoxicologists to objectively probe the biomolecular actions of drugs and chemicals. The wealth of immunotoxicological data accumulated in animals, combined with experimental modeling, establishes a basis for extrapolation to humans. Immunotoxicologists will continue to refine the classic immunoassays, develop and/or adapt new bioassays, and facilitate assessment of the mechanisms by which xenobiotics react with cell membranes, receptors, or internal structures, and interfere with other regulatory systems in the cascade of immune events. This will include not only applying such methods as flow cytometry and immunocytochemistry but also investigation of the putative interrelationships of the immune, endocrine, and central nervous systems.

IMMUNE SYSTEM

The immune system is extremely complex and sophisticated. It is composed of a delicate network of organs, tissues, cells, and cellular products that act in unison to protect the living organism against invasion by infectious and neoplastic agents. This system, which is dispersed throughout the body of living organisms, regulates defense mechanisms by recognizing and subsequently responding to altered antigenic composition of the body. Therefore, the regulatory events of the immune system identify foreign "non-self" antigens, such as microorganisms and neoplastic cells, to successfully destroy and eliminate them from the body. A delicate balance must be maintained for optimum performance.

The immune system is regulated by multiple chemical signals that operate at different levels. Antigens trigger local chemical signals which, in turn, incite an intricate network of responding cells throughout the body. A typical cascade of immunological events following antigenic stimulation would be initial expression of Ia molecules on the surface of macrophages, which activates the production of interleukin 1 (IL1). This event activates T lymphocytes to produce interleukin 2 (IL2), which is responsible for the proliferation of helper T (HT) lymphocytes that stimulate B lymphocytes to proliferate and differentiate into antibody-producing plasma cells or memory B lymphocytes. Thus, the overall responsiveness of the system is controlled by modulations of immunocytes and their secretory products.

Four major classes of immunocytes have been identified to date, including B and T lymphocytes, macrophages, and natural killer cells. The B lymphocytes are the primary effector cells for humoral immunity, that is, antibody production. They generally require HT cells and macrophages for optimum response to most antigens. T lymphocytes are composed of both effector and regulator subsets, which can be divided into at least two subpopulations based on function and phenotype. These subpopulations are the cytotoxic and delayed-type hypersensitive reactive T lymphocytes. Effector T cells are responsible for cell-mediated immunity, and act via cell-to-cell contact. Regulatory T lymphocytes are separated into HT and suppressor T (ST) subsets based on phenotype and regulatory activity. $L3T_4$ and $Lyt1^+$ are the phenotype surface markers for the T cells responsible for helper activity, mixed lymphocyte reactions, and cutaneous hypersensitivity in mice (T4 in humans) whereas $Lyt23^+$ (T8 in humans) is the surface molecule expressed on ST and cytotoxic T lymphocytes. T lymphocytes also synthesize and secrete numerous lymphokines which act as immunohormones. Two of the most prominent lymphokines are interferon (IFN) and IL2.

Macrophages, which are also composed of both effector and regulator subpopulations, phagocytize, process, and transfer antigenic information to other immunocytes, particularly T lymphocytes. Macrophages also produce and secrete numerous monokines. Two of the most reactive cytokines are IL1 and prostaglandin E_2 (PGE_2). The cytokines act on activated effector immunocytes in an interrelated manner to regulate the magnitude of the ongoing immune response.

Natural killer (NK) cells are morphologically classified as large granular lymphocytes but, unlike other lymphocytes, can react immediately without prior antigenic sensitization. Therefore, NK cells serve as a first line of defense against infectious and neoplastic agents. They also produce and secrete lymphokines, such as IFN and IL2, which have been suggested as a feedback mechanism to turn off antibody production of B lymphocytes (1).

The immune system is extremely sophisticated and appears to be both intraregulated and interregulated. Immunocytes orchestrated by their secretory products act in a network fashion for optimum performance. Although for decades the immune system was considered to be autonomous in both regulation and action, recent data suggest that a significant reciprocal interaction occurs between the nervous, endocrine, and immune systems (2-5). For instance, some classical neuroendocrine hormones and neurotransmitters possess immunomodulatory activity, whereas immunocytokine hormones share common receptors with the central nervous system and function as endocrine glands (4,5). These interregulatory patterns confirm the existence of a nervous-endocrine-immune axis, which complicates the researcher's attempt to duplicate whole-body system responses *in vitro*. Therefore, intact animal systems are essential for an accurate assessment of the immunotoxic potential of xenobiotics.

IMMUNOTOXICOLOGY

Immunotoxicology originated in the early 1970s when immunologists and/or toxicologists began investigating the immunotoxic potential of prominent environmental chemicals such as the polychlorinated biphenyls and lead (6,7). From these early studies it became apparent that chemicals known to be ubiquitous in the environment could compromise immunity in animals. Thus, the stage was set for a new discipline, presently known as immunotoxicology. The adverse immune effects exerted by chemicals were confirmed when exposure to these agents resulted in increased susceptibility of the host to infectious agents. These initial discoveries provided the foundation for a series of events which today have established immunotoxicology as a prominent discipline shared by immunopharmacology.

After it was recognized that chemicals could modulate immunity, conventional immunoassays were adopted to assess chemical-induced immune dysfunction. These studies generally included evaluation of humoral- and cell-mediated immune response as well as macrophage activity. In addition, animals exposed to chemicals for prolonged periods were frequently challenged with an LD_{50} dose of an infectious agent to assess for natural resistance to infections.

During the past decade, a number of immunoassays have been developed and adapted to test for immunotoxicity of chemical agents. Some of these procedures include responses to mitogens, antibody plaque-forming cells (PFC), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, delayed-type hypersensitivity (DTH), mixed lymphocyte reaction, lymphocyte cytotoxicity, helper T/suppressor T cell ratios, NK cell cytotoxicity, bone marrow progenitor cells, cytokine activity, and several host-resistant assays. The discovery of monoclonal antibodies has stimulated renewed interest in flow cytometry and immunocytochemistry. Many of these

procedures have been validated as acceptable assays to assess chemical-induced immune dysfunction.

Immunotoxicology gained credibility when it was confirmed that some chemicals, such as pentachlorophenol (8), lead (9), toxaphene (10), and polychlorinated biphenyls (9), produce immunosuppression at dosages lower than those that altered other known and commonly used toxicological indices. This feature revealed that the immune system was indeed a sensitive indicator to detect perturbation by chemicals and that the ensuing immunosuppression could not only render an animal susceptible to infectious agents, but also could cause an increased risk of cancer. There is substantial evidence that immunocompromised individuals are highly susceptible to some forms of cancer. This is exemplified by Kaposis' sarcoma, which occurs in patients with Acquired Immune Deficiency Syndrome (11).

Most chemicals target a specific organ within the body. Toxicologists, pathologists, and clinicians have used this characteristic to diagnose, treat, and ascertain the biological mechanism by which a chemical produces toxicosis. Thus, the symptoms and pathology associated with a chemical that is primarily hepatotoxic are expressed in those parameters used to detect injury to the liver. The immune system, on the other hand, is distributed throughout the body in cells, tissues, organs, and the circulating blood and lymph. Therefore, different components of the immune system can be exposed to the parent chemical and its metabolite(s) systemically at different sites throughout the body. This factor in itself acknowledges the immune system as a highly sensitive organ for detecting toxicosis. Compromised immune function could be life-threatening, as are many other manifestations of toxicosis.

The impact of toxic substances on human immune function is virtually unknown. There is, however, documented evidence that exposure to chemicals such as polychlorinated and polybrominated biphenyls and lead suppress immune responses in man (12-16). The continued development of new clinical immunological technology to assess immune function in man will facilitate accumulation of data needed to evaluate chemical-induced immune dysfunction in man.

MECHANISMS OF IMMUNE TOXICOLOGY

Immunotoxicology is a science that explores the effects of physical and chemical agents and other toxic substances on the immune system. The founding years of the discipline were devoted primarily to identifying immunotoxic chemicals and to developing a battery of sensitive, quantitative immunoassays. However, much of the research during the past few years has concentrated on investigating the mechanisms by which immunotoxic chemicals compromise immune function.

Because optimum performance of the immune system is dependent upon a cascade of immune events, disruption of any one of the components of this circuit can alter the immune response. Interference in the early stages of the immune sequence would be suspected to result in a generalized effect on immune function. However, occasionally a chemical will elicit a more selective response within this chain of events. This can be attributed to the chemical exerting a more specific effect directly on an immunocyte or a particular subset of cells. Thus, the response may range from highly selective to generalized. These features account, at least in part, for the lack of a typical linear dose response normally observed in other standard toxicological procedures.

The mechanisms of immunity are extremely complex. A complete immunological paradigm would include assessment of several compartments of the immune response which are inclusive of humoral immunity, cell-mediated immunity, macrophage activity, NK cell cytotoxicity, and cytokine production/activity. Chemicals can compromise one or several reactive sites within the immune network. The actual mechanistic effect, however, may occur by altering internal cell structures, membranes, surface antigens, and/or a variety of receptors. Chemicals may alter the composition of these structures, bind to or block their activity, or interfere with numerous putative non-immune regulators required for activation, differentiation, proliferation, and normal development of immune responsiveness.

ASSESSMENT OF IMMUNOTOXICITY

A myriad of immunoassays has been developed to assess the integrity of the immune system both *in vivo* and *in vitro*. Most immunotoxicologists agree that a panel of immunoassays should include procedures to assess humoral immunity, cell-mediated immunity (CMI), macrophage function, pathotoxicologic examination of lymphoid tissues, and host resistance to an infectious and/or oncogenic agent (List 1). More recently, this panel has been expanded to include NK cell cytotoxicity and production/activity of numerous regulatory cytokines.

LIST 1

STANDARD PROCEDURES USED TO ASSESS IMMUNOTOXICITY OF XENOBIOTICS

- Humoral immunity
- Cell-mediated immunity
- Macrophage function
- NK cell cytotoxicity
- Cytokines
- Host resistance
- Pathotoxicity of lymphoid

Several laboratories have organized a multitiered approach to evaluate the immunotoxic potential of xenobiotics. The initial tier generally includes nonspecific but quantitative procedures to assess the major compartments of the immune system. Other tiers are designed to specifically evaluate in greater detail those compartments of the immune response which, in tier one, were identified as being affected by the test chemical. Thus, a comprehensive immune profile can be characterized for each agent tested.

Humoral immunity can be enumerated by several techniques that assess B-lymphocyte activity and/or antibody production. Assays commonly utilized to evaluate humoral immunity are listed in Table 1. The availability of immunoassays and ease of assessing humoral immunity have contributed to the numerous chemicals tested for compromising this compartment of the immune response. Some of the procedures (e.g., PFC and ELISA) test for immune function whereas others are more subjective and essentially measure nonfunctional parameters (e.g., mitogen and immunoglobulin levels). The humoral immune response to T-dependent antigens is a particularly useful tool for assessing the immunotoxic potential of xenobiotics because the humoral system is regulated by other components (e.g., T lymphocytes and macrophages) of the immune network.

TABLE 1

HUMORAL IMMUNOASSAYS COMMONLY USED IN IMMUNOTOXICOLOGY

Assay	Indices
Immunoglobulin	IgM, IgG, IgA, IgE
Enzyme linked immunosorbant assay	Antibody, circulating
Radioimmunoassay	Antibody, circulating
Hemagglutination	Antibody, circulating
Serum neutralization	Antibody, circulating
Hemolytic antibody isotope release	Antibody synthesis
Plaque-forming cell	Antibody synthesis
Mitogen, LPS	B cell division
Surface markers	Lyb5$^-$, LyB5$^+$ Ly1$^-$, Ly1$^+$

Cell-mediated immunity can be assessed by several immunoassays (Table 2). This compartment of the immune system is composed of several subsets, helper, suppressor and cytotoxic T cells, as well as those subpopulations responsible for systemic delayed and localized contact hypersensitivity. These cells can be identified by specific surface markers. T lymphocytes also secrete numerous lymphokines that regulate the intensity of the immune response. Tests considered most representative of CMI are various modifications of DTH assays. The DTH procedures have been

adapted for various species of animals and have proven to be a sensitive as well as quantitative method for assessing chemical-mediated immune dysfunction of CMI.

TABLE 2

CELL-MEDIATED IMMUNOASSAYS COMMONLY USED IN IMMUNOTOXICOLOGY

Assay	Indices
Delayed type hypersensitivity	Helper, Suppressor, Cytotoxic T
Mixed lymphocyte reaction	Helper, Suppressor, Cytotoxic T
Graft vs. Host Reaction	Helper, Suppressor, Cytotoxic T
Mitogens, Con A/PHA	T Cell Division
Surface markers	Helper T – Lyt 1 + Suppressor T – Lyt 23 + Cytotoxic T – Lyt 23 +
Lymphokines	Interferon Interleukin 2

The phagocytic index of macrophages was used extensively in the early stages of immunotoxicology. However, this procedure appears to be a relatively insensitive method for assessing immunotoxicity of xenobiotics because macrophages can compensate for considerable insult before marked effects in phagocytosis can be detected. Macrophages can also be examined for their ability to kill and destroy microorganisms. Macrophages, like lymphocytes, secrete numerous products called monokines, which regulate at various levels of several immune events. Monokine production/activity appears to be a relatively sensitive indicator of macrophage function. Assays used to evaluate macrophage activity in immunotoxicology are listed in Table 3.

TABLE 3

MACROPHAGE IMMUNOASSAYS COMMONLY USED IN IMMUNOTOXICOLOGY

Assay	Indices
Phagocytosis	Ingestion
Microorganism cytotoxicity	Digestion
Macrophage cytotoxicity	Tumor cell lysis
Chemiluminescence	Oxidative metabolism
Monokines	Interleukin 1 Prostaglandin E_2

A miscellaneous group of immunoassays has also been used to test for chemical-induced immune dysfunction (Table 4). The NK cell, which has been recognized as an important effector cell in immune surveillance of both virus-infected and neoplastic cells, has proven to be extremely sensitive to chemical-induced immunomodulation. This assay has been used extensively during the past few years by immunotoxicologists. Another procedure, antibody-dependent cell cytotoxicity, is mediated by the K cell, which is attached to tumor cells by a surface antibody resulting in lysis of the tumor target. Flow cytometry and immunocytochemistry are two procedures that have gained prominence because of the availability of monoclonal antibodies. These two procedures can easily identify specific cell subpopulations in solution (flow cytometry) or tissues (immunocytochemistry) not only for diagnostic purposes but also to identify the underlying mechanism by which xenobiotics mediate immune dysregulation.

TABLE 4

MISCELLANEOUS IMMUNOASSAYS COMMONLY USED IN IMMUNOTOXICOLOGY

Assay	Indices
NK cell cytotoxicity	Spontaneous cell lysis
Antibody-dependent cell cytotoxicity	Antibody cell lysis
Flow cytometry	Surface markers (solution)
Immunocytochemistry	Surface markers (tissue)

Many laboratories have utilized host-resistance models to ascertain whether xenobiotics alter the pathogenicity of a variety of microbial organisms. These protocols have used bacteria, viruses, fungal agents, protozoan, parasites, and tumor cells to assess host susceptibility to these agents following periods of exposure to chemicals. Impaired ability of the host to combat infection or neoplasia is indicative of immune dysregulation, but specific immune responses must be performed to determine the direct immunotoxicological properties of a xenobiotic.

A pathotoxicology profile can be initiated at necropsy by collecting blood for hematology, weighing lymphoid organs, preparing bone marrow smears, and preserving tissues for histopathology. The lymphoid organs of particular interest are the spleen, thymus, representative lymph nodes, and bone marrow. The spleen and thymus are quite easy to evaluate and are occasionally affected by exposure to xenobiotics at relatively low dosages of the test agent. Some of the most common immunoassays used today to assess chemical-induced immune dysfunction are the PFC, ELISA, DTH, mixed lymphocyte reaction, NK cell cytotoxicity, IFN, IL1 and IL2, flow cytometry, and immunocytochemistry. Pathotoxicity is included in almost all protocols, and host-resistant

procedures are used fairly extensively. These procedures have been validated and are considered to be acceptable for assessing xenobiotic-induced immune dysfunction.

MODELS OF IMMUNOTOXICITY

Unlike most target organs that are confined anatomically within a host, the immune system is dispersed throughout the body and can be exposed to xenobiotics at many locations and during various stages of metabolism. The immune system promises to be one of the most sensitive biological systems for detecting adverse reactions resulting from exposure to drugs and toxic substances. The difficulty in testing for chemically induced immune dysfunction in the past has limited the knowledge available as to the immunotoxic potential of a multitude of environmental pollutants. Nevertheless, a wealth of information is available from animal studies to suggest that many chemicals displaced in the environment could be putative immunotoxins for humans.

Advances in biotechnology and animal models have improved the process of interpreting and extrapolating data from laboratory animals to man. Many of the immune procedures utilized for immunotoxicology are reproducible, quantitative, and highly sensitive in detecting chemical-induced immune dysregulation. It is universally accepted that the immune systems of some animals and man are comparable, that animal models are available to objectively assess immune dysfunction, that positive immunosuppressants such as cyclophosphamide and corticosteroids are used to validate assays, and that data obtained in animals have been verified in man. Although the principles and phenomena in man and animals are basically similar and comparable, it is recognized that variations in these responses can occur between various species.

Animal models have been used successfully during this century to develop methods of diagnosis, prevention, and treatment of human disease. Two laboratory animals, the mouse (17) and the rat (18,19) have been proposed as animal models for assessing xenobiotic-induced immunotoxicity. The primary advantages of the mouse are that the immune system has been described in great detail and many inbred strains are available. Multiple immunoassays can be performed using a single mouse, but the small size of the animal limits the number of tests and amounts of samples that can be collected per animal.

The rat model can be used to evaluate multiple, concomitant immune compartments within an individual animal (18,19). The multiple parameters of immunity assessed in each rat include humoral immunity (ELISA), cell-mediated immunity (DTH), NK cell cytotoxicity, and the production of three potent immune regulatory immunocytokines: macrophage-derived IL1, PGE2, and lymphocyte-derived IL2. The multi-assay single-animal approach represents an economical, versatile, sensitive, and relatively comprehensive paradigm for assessing the immunotoxicological properties of xenobiotics. The model minimizes animal-to-animal and day-to-day variation, reduces experimental

and comparative error, is sensitive to detect immunosuppression as well as immunoenhancement, can simultaneously test the major types of immune response/immunocyte populations and immunoregulatory pathways, and is economical. Another advantage of utilizing the rat is that almost all pharmacological/toxicological data are amassed for this species, permitting comparison of systems to ascertain the relative sensitivity of different body functions to the test agent. Considerable basic information has been acquired for the rat immune system during the past decade.

One must remember, however, that the effective dose response of a chemical may not directly translate from the animal model to humans, although similar or even identical biological responses are emulated. It is known that polychlorinated biphenyls (12), polybrominated biphenyls (13,14), lead (15,16), selenium (20), nickel (21,22), and cobalt (22) have resulted in immune dysfunction in man. However, not until considerable epidemiological and experimental data on humans have been obtained can the impact of immunotoxicosis in man be fully known or appreciated.

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MR. FISHER (MIAMI UNIVERSITY): I have one question concerning the use of immunotoxicology by the legal profession. Would you care to comment on that?

DR. KOLLER (OREGON STATE UNIVERSITY): That depends on how you're asking your question. Yes, I will comment on that. There have been some eccentrics, and the idea is that a patient will be presented with an array of symptoms that cannot be diagnosed with some known diagnosis. There are some physicians who will take these patients and claim that they have been compromised by toxic substances. In most cases they are being defeated, fortunately. I've been involved in several of these cases. Let me give you an example. The physician actually had assessed, not the immune functions, but immune parameters; that is, helper T cells/suppressor T cells key ratios, etc., 14 months after an exposure to a chemical. That exposure was only for five minutes. In the meantime, that patient had been exposed to 26 different drugs, and the drugs that that patient was exposed to were much more immunotoxic than the chemical itself. We are seeing more and more of this, but in most instances it is being defeated – which is great in my opinion. It is going to cause a tremendous burden on both our drug and chemical industries if this exploitation takes over.

LUNCH ADDRESS

Thomas R. Dashiell

Director, Environmental and Life Sciences

Office of the Under Secretary of Defense for Research and Engineering, Pentagon

It is a distinct pleasure for me to be here today. I have enjoyed your conference very much. I am unsure how I received this honor since I am not a toxicologist; my training is in bacteriology and chemical engineering. However, having served in a technical position and policy-making role as the Director of Environmental and Life Sciences, I am aware of several flaws in today's risk assessment process which I would like to call to your attention.

We are faced with the almost impossible task of establishing standards for pollutants or toxic substances for which so-called safe levels do not exist. Many of these substances cannot be eliminated from the environment or from our operational scenarios because of the very large expenditures this would require or because adequate substitutes to perform the mission do not exist. An additional problem, one which I'd like to focus on today, is how to develop methodologies and technologies to both assess the risk and support the decision-making process that is inherent in the management of that risk.

A necessary and vital part of this decision-making process involves the science of toxicology that you and your colleagues have discussed at this conference and initiated through various industrial and academic contracts. Toxicological research is very expensive – to gather acute, subchronic, chronic, mutagenic, teratogenic, and carcinogenic toxicology data for one compound may take years and several million dollars. Our intent at the Department of Defense (DOD) is to use available funds to their best advantage, to improve the scientific side of this debate, that is, to determine the allowable limits, the effect on human health, and the impact on military operations, among other considerations. At the same time, we must present our information to the public in simple and understandable terms. A computerized risk analysis is only a lot of paper to most people; it is not easily understood or believed by most of the public. The basic issue is to develop a system that will put the risk, which is defined by the toxicologist, into a perspective that everyone can understand.

In a recent issue of the EPRI (Electric Power Research Institute) Journal, a very useful chart was presented. The paper presented the risk of dying in a single year as a result of various activities. Most activities with a risk above 1 in 1000 are both voluntary yet generally unacceptable to the public. For example, occupations for which the risks are 1 in 1000, such as stuntman and race car driver, are not the norm. However, smoking one pack of cigarettes a day also has a 1 in 1000 risk –

one which many people accept. Similarly, life-styles that include heavy drinking ensure risks of 1 in 10,000, while light drinking poses a risk of 1 in a million (1 in 10^{-5}). There is less risk involved in living downstream of a dam or having diagnostic X rays or natural background radiation, which are generally acceptable at 1 in a million. Eating a charcoal-broiled steak once a week, a risk that is normally very acceptable to us, has a risk of 1 in 10 million. A large variety of natural disasters, such as being caught in a tornado or a hurricane or being hit by lightning on the local golf course produces this same relative risk. My intent in reviewing these cases is to note that these risks are known with reasonable accuracy, since thay are based on actuarial data.

Looking at this dilemma from the DOD's standpoint, we are continually confronted with problems that require filing an environmental impact assessment or statement – which again is a risk/benefit process. At the present time, we are demilitarizing our stockpile of obsolete chemical munitions, which is a truly hazardous task. However, the technological options available to perform this task in the required time frame are limited, mostly to available incineration techniques, since we want to be sure to protect human health and the environment. However, this action has already drawn and will continue to draw severe criticism.

The offshore incineration of chlorinated solvents – Polychlorinated biphenyls and others – has already been the subject of intense debate and has been delayed by the U.S. Environmental Protection Agency, while at the same time the Office of Technology Assessment has recently supported this disposal method, at least as an interim solution. At DOD we are concerned with a large variety of issues – trichloroethylene in groundwater, disposal of explosives, herbicide residues in soil, tributyl tin coating of ship hulls for antifoulant purposes, and others almost too numerous to mention. On one hand we need a system that can separate the scientific analysis of risk assessment in relation to the political and economic conclusion, from how it should be handled or disposed of on the other hand.

Generally speaking, the risk assessment process attempts to measure or determine the magnitude or the seriousness of a health hazard. Even the Office of Management and Budget (OMB) ran afoul of Congress on this issue when a House Subcommittee deleted 5.4 million dollars from OMB operating funds because they felt that OMB had been influencing the regulatory agencies to downplay human cancer risks. Regardless of the merits of the case, I must generally agree with the head of that office, Mrs. Gramm, when she says that risk estimates must include an easy-to-understand summary of the so-called conservatisms. For example, a risk assessment for drinking water might point out that it is based on the assumption that a person will drink two quarts of water from the worst well in the neighborhood every day for 20 years. This would be in lieu of the outmoded thesis that anything in any quantity that causes cancer in any animal would be prohibited. This thinking is outmoded because our analytical techniques have improved so dramatically in recent

years that we can now measure substances at far lower levels than we could a few years ago. Using a GC/mass spectrophotometer (MS) or an MS/MS, we can now detect and quantify toxic substances such as dioxin to 10^{-12} g, when in 1969 only two laboratories were capable of detecting it at 10^{-6} g. I do not believe that simply because we can now measure and quantify a substance that it is necessarily bad at that lowest detectable level – although I have been faced with that argument by some regulatory officials.

When I review your conference program, I see a variety of substances of great interest to DOD – hydrazine, composite materials, tributyl tin, thermal degradation products, and others. These are indeed critical to the maintenance of our DOD programs.

Let me now address these problems. In an increasingly complex world, the fulfillment of U.S. national interests requires military strength because the international order we envision cannot be guaranteed in the face of the numerically superior forces of our adversaries. Major developments in Soviet military capabilities pose new challenges to our defense posture and policy. In recent years, substantial progress has been made toward ensuring that our military capabilities are strong enough to fulfill their critical missions and purposes. We have current programs to build more ready conventional forces, more modern nuclear forces, more modern command and control systems, well-equipped tactical ground and air forces, and greatly improved maritime forces. The technology that has provided the options for this progress was developed over the past two decades by the DOD Science and Technology program that's currently being performed in both the in-house laboratories and in industry and academia. This is especially true in the toxicology community.

The theme of this year's conference, "Predictive Toxicology," is particularly fitting in this area. Technology, however, is a perishable commodity. DOD's task is to maintain progress in order to ensure the availability of technological options to provide future commanders and troops with the tools to accomplish difficult and uncertain tasks in many potential combat environments. However, the time it takes for ideas and scientific data to be implemented as military hardware is usually very long. Therefore, it is important that we conduct a strong and vigorous Science and Technology program now as an investment in the future well-being of the country's security.

The Soviets also recognize the importance of technologically superior weapons and have given their research, development, testing, and evaluation program high funding priority. This heightens the challenge to the United States in that we depend heavily on qualitatively superior weapons to provide an edge over numerically superior adversaries.

In the technology race, we do have an advantage not available to the Soviets. In addition to the efforts undertaken by the DOD program, the U.S. possesses a strong, viable, and innovative private sector that is available to do sound research and development both on its own behalf and in

support of the defense sector. The combined efforts of these participants have provided us the lead in most militarily significant technologies. It is important to continue a large and vigorous Science and Technology program to preclude progressive erosion of our position. The resulting technological edge is the most viable of our options, and we must increase our investment to maintain our technological lead. We plan to continue to make full use of the in-house laboratories and the private sector. In addition, we will continue to strengthen our relationship with the university community by allocating to them a significant portion of the basic research program, by continuing our program of upgrading university research instrumentation, and by supporting efforts to increase scientific and engineering education in areas of interest to DOD.

Our nation relies heavily upon technological excellence for the margins of military superiority essential to national security and to our well-being. Through technology, we strive to endow a few with the strength of many as the key to national survival. This dependence upon technology demands sophistication and wisdom to employ technology effectively. The need for sophistication and wisdom as a prerequisite to apply technology continues to drive the systems designer, the military planner, and the tactician to the edge of understanding for everyday solutions and the margins of advantage necessary for continued military superiority.

Against this backdrop, the fundamental researcher, the applied scientists, and, in fact, the collective body of the entire community are increasingly important as necessary ingredients in national defense.

Because of these factors, substantial effort is being made in both the public and private sectors to improve risk assessment and risk management. A critical part of this requires closing the gap in scientific knowledge, which is your primary task in toxicology. This is being accomplished through a variety of tools – advanced biological modeling and pharmacokinetics for example. These new tools can help dramatically in the risk assessment process because pharmacokinetic studies can enable scientists to better predict a reliable dose-response relationship for humans exposed to toxic substances. Pharmacokinetics can also indicate which species of animal most closely mimics man and is therefore most appropriate for use as an experimental model.

Such tools can substantially improve the dialogue between government, industry, regulators, and the public by providing better estimates of the probability of harm. This must still be coupled with research on how people judge values related to risk and why perceptions of risk often differ widely from objective calculations of risk probabilities.

A necessary and vital part of our Science and Technology effort is a risk assessment program designed to meet the DOD goals of new and better systems realistically presented to regulators, Congress, and the public. This program can provide a valid prediction of potential health risks to the

public and to our military personnel – the system operators. Although this need is widely recognized, substantial problems must be solved before this approach can be fully recognized. This is probably not your task as toxicologists, but you can recognize that the data you generate are only one part of the total problem of an adequate, valid risk assessment methodology.

SESSION IV

STATE-OF-THE-SCIENCE IN CHAMBER DESIGN AND EXPERIMENTATION FOR INHALATION STUDIES

INTRODUCTORY REMARKS

Donald E. Gardner, Ph.D. – Chairman

Northrop Services, Inc. – Environmental Sciences

This afternoon we will be looking at some of the problems, and hopefully some of the solutions, that are involved in inhalation exposure of laboratory animals to various airborne chemicals, and in various state-of-the-art techniques that are presently being used in designing chambers for inhalation toxicology. A major principle in toxicology testing is that the route of exposure employed in the laboratory should mimic the route of exposure by which man is exposed to the chemical. The concentrations that are used in testing should reflect those in the exposure environment, and the test chemical being used should not be altered during the exposure process. This is true whether we are dealing with inhalation exposures, dermal exposures, or any other route of exposure. The respiratory system is a primary site of the deposition of airborne gases and particles. Once these airborne chemicals are deposited in the lung, the chemicals or their metabolites may be transported systemically to various other parts of the body. The lung has a very large surface area, much larger than that of the skin. The surface area of the lung amounts to about the size of a tennis court. Therefore, with every breath you take, environmental chemicals can enter the respiratory system and, with the proper chemical and physical properties these airborne substances, can be deposited in the respiratory tract.

To conduct valid inhalation studies one must be able to generate the atmosphere. It is not that we need only to mimic the concentration to be assured that the concentration we are exposing animals to is the one we want – we also have to make sure that the chemical and physical forms of that test atmosphere are what we expect them to be. We must have the capability to monitor the atmosphere. Ideally, we would like to know the amount of the dose that is getting to the target site. At minimum, we must know what the concentration is at the breathing zone of the animal.

Finally, we should maintain the animals in a condition that causes minimal stress, so that any effect that is measured can be attributed to the test agent. Today's presentations address many of these conditions. The next speakers are going to present the state-of-the-art in inhalation toxicology.

TOXICANT DISTRIBUTION IN THE THOMAS DOMES

Robert L. Carpenter, Ph.D., Edgar C. Kimmel, Ph.D., Carlyle D. Flemming, M.A., and Charles R. Doarn

Northrop Services Inc., THRU, 101 Woodman Dr. Suite 12, Dayton. Ohio 45431

SUMMARY

Measurement of test article concentration distribution for light gases have been made in the Thomas Dome inhalation chambers at Wright-Patterson Air Force Base, using propane as a test agent. The method used to analyze for inhomogeneities in test article spatial distribution deliberately varies the dome operational parameters rather than requiring extreme operational stability. The variation in test article concentration is analyzed by regression to determine which operational parameters most influence the test agent distribution. Unaccounted concentration variability is assumed to be the inherent spatial variation of the test article in the dome. The propane studies indicated that the spatial variation within the dome was 6.4% of the mean and that room air temperature at the top of the dome, propane analyzer baseline stability, and dome pressure were (listed in order of decreasing importance) the variables influencing the test article distribution.

INTRODUCTION

When conducting inhalation toxicology studies, it is desirable to expose all the animals in a chamber to the same concentration of test agent. If the test agent concentration and physical properties are uniform in the exposure apparatus, then the variation in inhaled test agent dose will depend only on the variation in the anatomy and physiology of the individual animals; this should result in a more uniformly deposited dose among the animals. Evidence that uniform exposure concentrations will reduce variability in inhaled dose can be found in a paper by Hemmenway et al.; in this study animals were carefully permuted through selected positions in an inhalation chamber during an exposure. The animals rotated through the chamber positions had significantly lower variations in inhaled dose than the animals that remained stationary (1). A knowledge of chamber distribution is also necessary to allow selection of a chamber sampling point that is representative of the average chamber concentration for the purpose of measuring exposure concentration during an inhalation exposure. For these reasons, modern inhalation toxicology practice includes characterization of test agent concentration and other relevant properties of test agents prior to conducting inhalation exposures.

The Toxic Hazards Division of the Harry G. Armstrong Aerospace Medical Research Laboratory has eight large exposure chambers known as Thomas Domes (Figure 1). The domes can be used to expose animals to potential toxicants under environmental conditions simulating those of high

altitude and space flight. To understand how the Thomas Dome operates, researchers have conducted test agent distribution studies within these exposure systems. We have analyzed the time required for test agent concentration to reach equilibrium when the test agent is introduced into the airflow through the Thomas Domes. Additionally, the time taken for the test article concentration to fall to zero upon shutdown of the dome has been measured. The degree of mixing between the air in the dome and newly introduced air can be calculated from data on the rise and fall characteristics of the dome exhaust concentration (2,3).

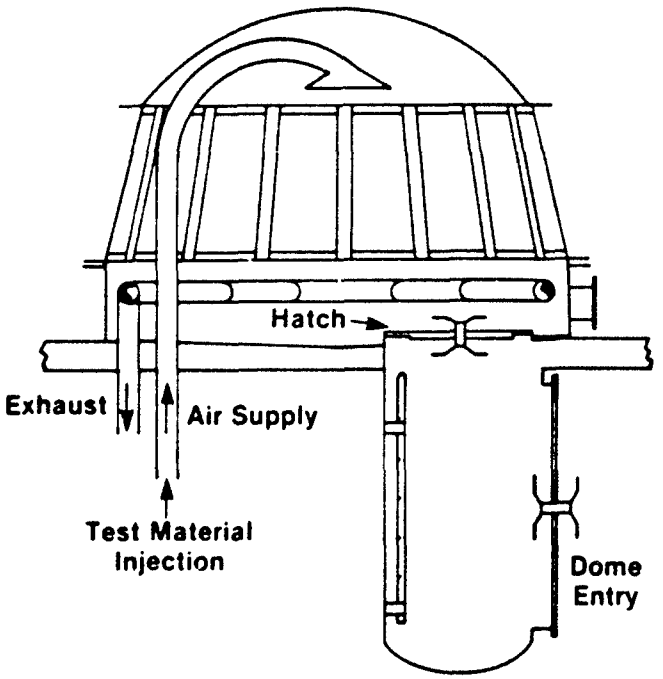


Figure 1. Cross section of Thomas Dome.

The spatial distribution of the test article introduced into the dome has been determined for gaseous test articles. We have taken advantage of the fact that the hydrocarbon analyzers available to us will obtain data at a much higher rate than available aerosol concentration measurement equipment. This high data-aquisition rate has allowed us to measure the propane concentration at sets of 50 points in a grid of 250 points. Analysis of the data obtained can be used to determine how many measurement points are needed to characterize the test article distribution within the dome. Finally, leak rate measurement methodology has been applied to operation of the dome to determine how much room air leaks into the dome during operation.

To directly determine the variations in test article concentration from point to point in an exposure chamber, one must measure the test article concentration at a number of locations within

the exposure chamber. Most efforts to characterize chamber distributions attempt to obtain a snapshot of chamber distribution by obtaining as many measurements as possible in a short period of time and analyzing the observed variation in test article concentrations (4-6). For larger chambers, it becomes difficult to measure enough points in a sufficiently short time that the observed concentrations can be said to instantaneously represent the chamber. The 23-m^3 Thomas Domes are difficult to characterize using conventional methods for measuring test article concentration. Measurement of test article distribution in other inhalation chambers indicates that, in general, the test article distribution within the chamber depends on both the design of the chamber and the design of the air/test article delivery system (6). For this reason, test article distribution studies should be done as a function of chamber operating conditions, further increasing the measurement burden in these studies. Therefore, we are investigating methods to characterize toxicant distributions in large exposure chambers as part of this measurement effort.

Moss has suggested a method for reducing the requirements for rapid sampling (7). Sequential measurements of test article concentration are made from a central reference point in the chamber. These data are a measurement of the variation of the test article concentration with time. Reference point measurements are interleaved with measurements at different points within the exposure chamber to determine the total (time and spatial) variation of test article concentration. Statistical analysis is used to determine the spatial variation of the test article concentration (8). This method appears to work as long as the variation of test article with time is not greater than the spatial variation within the exposure chamber.

To apply spatial distribution measurements to the Thomas Dome, we combined the two approaches described above by measuring test article concentration at a greater number of points and deliberately varying the dome operating parameters in a random manner. The measured concentrations were analyzed using factorial analysis of covariance. The results of this analysis provide a measure of spatial variation within the dome and also provide an indication of the operational parameters that most strongly influence this variation. The relative importance of the operational parameters can be used to determine what changes or repairs need to be made in the operation of the dome if the measured spatial variation is too large.

METHODS

For vapor-phase test articles, the major mechanism by which the test article can move across airflow patterns is Brownian diffusion. To study distribution characteristics of gaseous materials in the Thomas Dome, two different materials will be used in two measurement phases. In the first phase, propane, a gas with a low molecular weight and a high diffusion constant, was used. In the

second phase, dodecane, a much heavier hydrocarbon with one-half the diffusion constant of propane, will be used.

Test Article Generation

A tank of pure propane equipped with a flash arrestor was connected to a rotameter which metered the propane into the dome air supply at low concentrations. To ensure thorough mixing, a counterflowing jet was used to introduce the propane into the main chamber airflow. Morgan and Brinkworth (8) demonstrated that mixing of a turbulent jet into a counterflowing turbulent flow occurred in less than 10 pipe diameters if the flow Reynolds numbers were above 1000 for the main flow and 10,000 for the jet (8). To obtain these high Reynolds numbers in the counterflowing jet, we mixed propane with metered dilution air. The mixture enters the dome through the contaminant introduction system, which automatically switches to bypass if there is no airflow through the dome. Figure 2 illustrates the propane generation system.

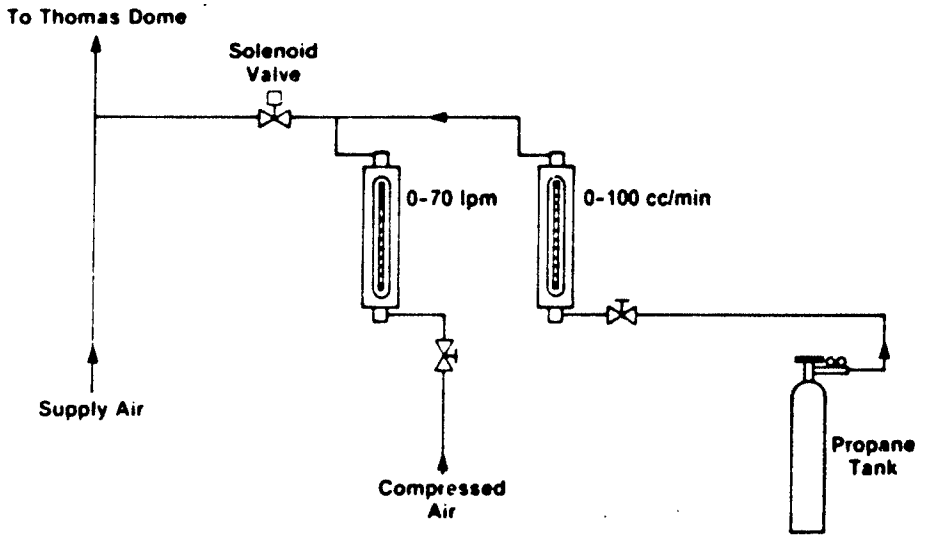


Figure 2. Test gas injection system.

In the second-phase generation system, dodecane will be metered into a heated evaporation tower. Metered air will flow up through the tower and into the dome contaminant introduction system. Temperature, both upstream and downstream of the tower, will be monitored to prevent overheating. The airflow and flow of the compound into the tower will determine the concentration.

Analytical Method

Hydrocarbon vapors or gases can be detected using a hydrocarbon analyzer based on the flame ionization detector (FID). Six Beckman Model 400 hydrocarbon analyzers were used to monitor concentration at various points within the dome. One analyzer monitored concentration at the diffuser ring (inlet) and the exhaust line (outlet). The remaining five analyzers sampled from concentrations at locations within the dome. Each analyzer is situated between two windows. Five stainless steel probes, 3/8" OD and 76" long, were placed at five different levels in each of the ten dome windows. These probes were mounted so they could be moved radially to allow sampling from all points in a sampling plane. The five vertical levels occupied by the sampling probes are shown in Figure 3A. The radial sampling probe locations are shown in Figure 3B. The probes were connected to a manifold system of solenoids controlled by an International Business Machines personal computer (IBM PC). The manifold outlet was connected to a hydrocarbon analyzer, which had a vacuum pump in line before it. Sample flow rate through the analyzer was measured with a rotameter on the exhaust side of the instrument. Flow rate was set at 2.0 l/min. All six analyzers were equipped with pop-off valves to prevent any damage to the instruments in the event of a sudden surge of pressure. Compressed air, in combination with 100% pure hydrogen, was used as a FID fuel mixture. The analyzers were calibrated with a standard calibration gas of 33 ppm propane. Figure 4 illustrates the experimental setup. The dome input air was used as baseline for all analyzers.

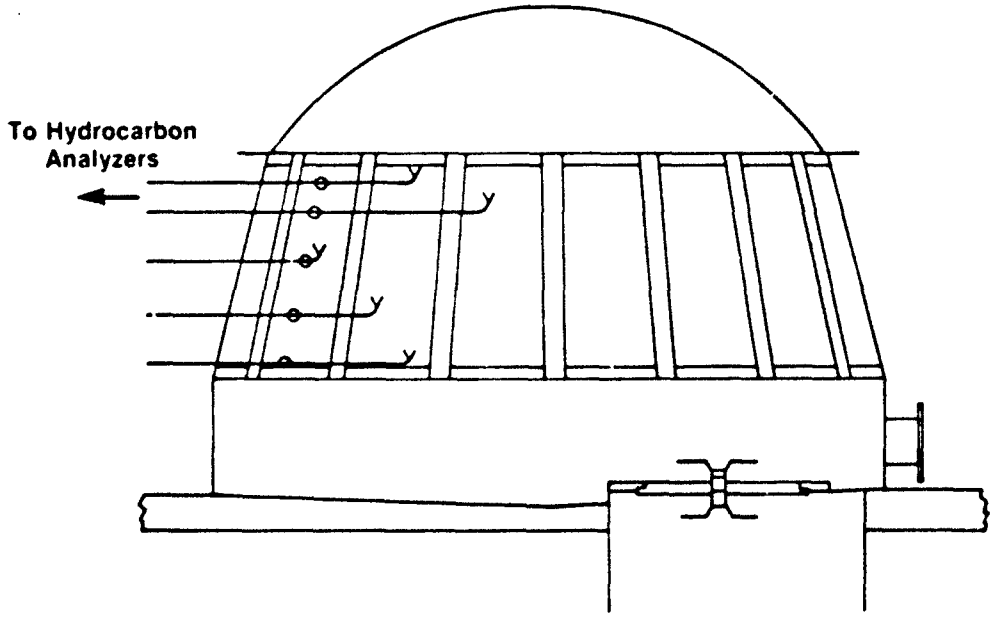


Figure 3A. Sampling probe levels

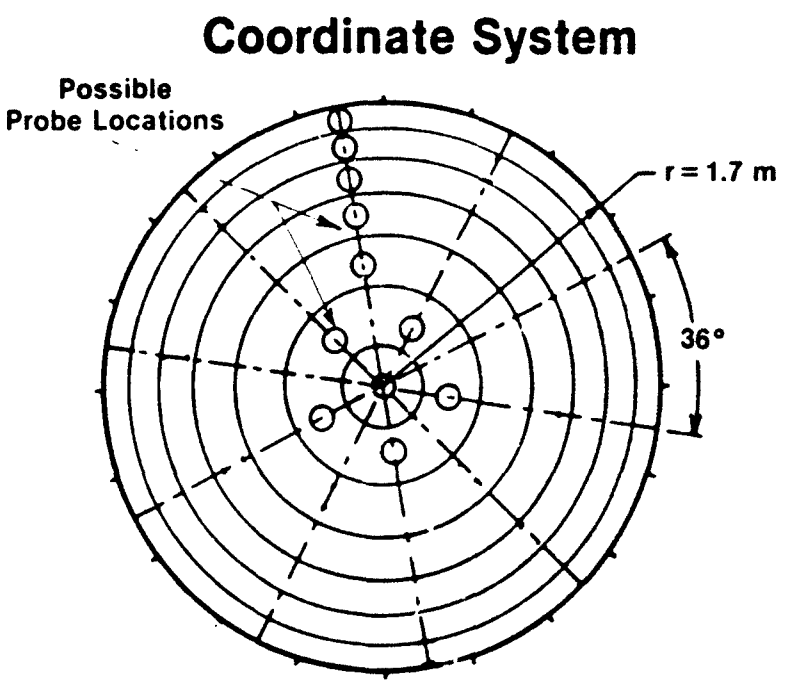


Figure 3B. Sampling probe radial positions.

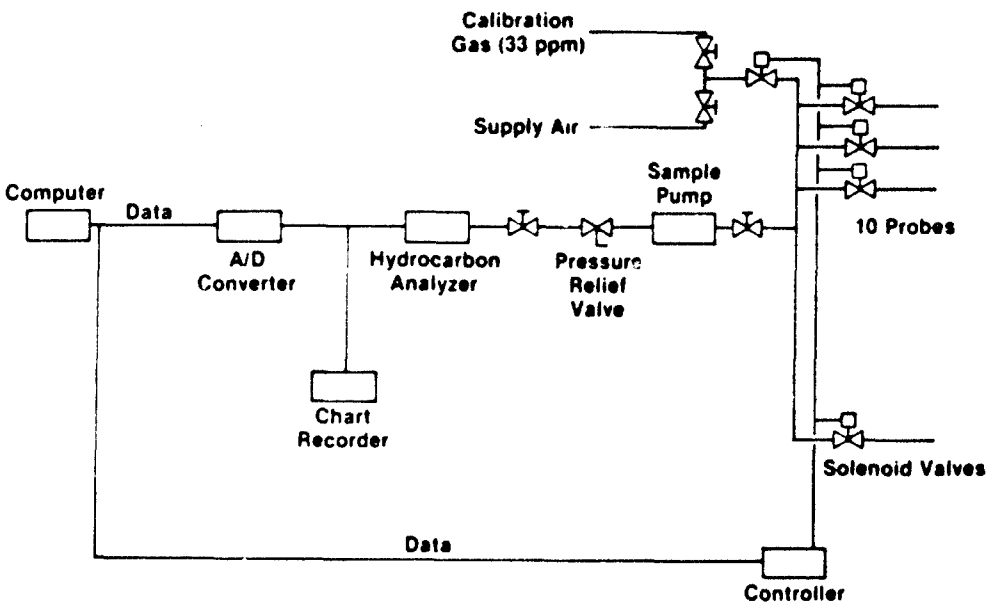


Figure 4. Sampling control and data acquisition.

Data from each analyzer output were collected by a microcomputer data acquisition system based on the IBM PC. This program also operated the electrical valves, sequentially connecting the analyzers to each probe. To allow operators to view the data in real time, analyzer output was also connected to strip chart recorders.

Probe locations were randomly selected at the beginning of each day. The dome was operated at eight different flow rates, ranging from 25 cfm to 95 cfm, and at eight different pressure settings. The combinations of these two parameters were randomly chosen in a manner that ensured that each preselected parameter value was equally represented. For each of the five analyzers that sampled the dome volume, data were collected in sets of ten concentration values from two windows. Before and after each data collection set, a baseline reading was taken from the chamber inlet air supply. The sixth analyzer monitored concentration at the inlet and the exhaust as well as baseline, recording rise time and fall time in the dome. The hydrocarbon analyzers were calibrated prior to distribution measurements using dilutions of the 33-ppm standard gas prepared in Teflon® sampling bags. Single concentrations of standard gas were measured three times daily to monitor the drift of each hydrocarbon analyzer. These data were used to determine when an analyzer had drifted outside the calibration confidence interval.

Statistical Methods

To determine the relationship between test article concentration and dome operating parameters (flow, pressure, external temperature, internal temperature, analyzer drift, sampling point position, and time) a regression procedure (RS1/FIT MULTIPLE), with concentration as a dependent variable, was used. A factorial analysis of covariance was performed to find differences in position and time with covariances of flow, pressure, internal temperature, external temperature, and analyzer drift (BMDP2V). Because position and sample are categorical variables, a log-linear model procedure was done with the above-mentioned variables and concentration. A check of normality of concentration was done using a Kolmogorov-Smirnov Test (9). Because this analysis was exploratory, all of the above analyses were required.

Leak Rate Measurements

Dome leak rate was measured by the method of Mokler and White (10). The pressure in the Thomas Dome under study was lowered to two inches of water, and the dome was isolated from the air supply and exhaust system. A standard pressure gauge was used to monitor the increase in dome pressure due to air leakage into the dome. The time for the pressure to raise one inch of water was recorded. From this data the fractional leak rate of the dome can be calculated

RESULTS

Dome Leak Rate

Chamber leak rates were measured during dome operation, but no extraordinary efforts were made to seal small leaks prior to data analysis. Initial leakage rates were high, indicating that excessive air was infiltrating the dome and/or the air delivery system. An investigation revealed that the manual shut-off valves present in the air supply system did not seat completely. It was also found that cycling the dome pressure served to seat the dome cap. Chamber leak rate decreased after dome pressure was lowered and returned to ambient a few times. The initial leak rate was 26.4% of the chamber flow rate. After sealing the dome cap, the leak rate was found to be 15.3%. The measured equilibrium concentrations of the chamber inlet and outlet differed by 1.3 ppm, or 5.4% of the average propane concentration. Statistical analysis of the data demonstrated that the majority of this difference was correlated with changes in the analyzer baseline. When these effects were mathematically removed, there was no significant difference between inlet and outlet concentrations.

Dome Mixing Characteristics

Analysis of the rising propane concentration after starting the propane flow into the chamber indicated that the Thomas Dome behaves as a well-mixed system for gaseous test articles. Table 1 shows the calculated and measured rise times obtained during these measurements. The propane concentration decreased with time after shutting off the propane flow. This decrease can be analyzed by the method of Cholette and Cloutier (3) to yield information concerning the fraction of the chamber volume that is poorly mixed. Figure 5 is a plot of these data, obtained at a flow rate of 95 cfm. The best least-squares fit to the data resulted in the equation in Figure 5. This equation corresponds to the flow conditions for which 88-95% of the chamber is well mixed with the incoming flow, and some of the exhaust flow is generated by piston flow of the air within the chamber.

Measurement System Characteristics

The propane metering system produced an average dome propane concentration of 23.6 ± 0.96 ppm (mean ± standard deviation). Dome inlet and outlet concentrations showed a 7% fluctuation around the mean. The measured propane concentration showed trends with time, but there was no persistent concentration pattern within the dome itself. Propane distributed uniformly throughout the dome, and the fluctuations about the mean at any point were random. The propane distribution data were not normally distributed by the Kolmogorov normality test (Figure 6), and showed an excessive number of data points well removed from the mean. Regression analysis

TABLE 1

COMPARISON OF DOME RISE TIME WITH THEORETICAL RISE TIME FOR PERFECT MIXING

Flow Rate (cfm)	Pressure (mmHg)	Flow/Volume	
		Observed	Calculated
	10	0.040	0.0354
25	18	0.0382	
	24	0.0475	
	36	0.0396	
95	Mean ± SD	0.0413 ± 0.004	
	5	0.153	0.135
	10	0.147	
	18	0.085	
	24	0.156	
	36	0.163	
	Mean ± SD	0.141 ± 0.032	

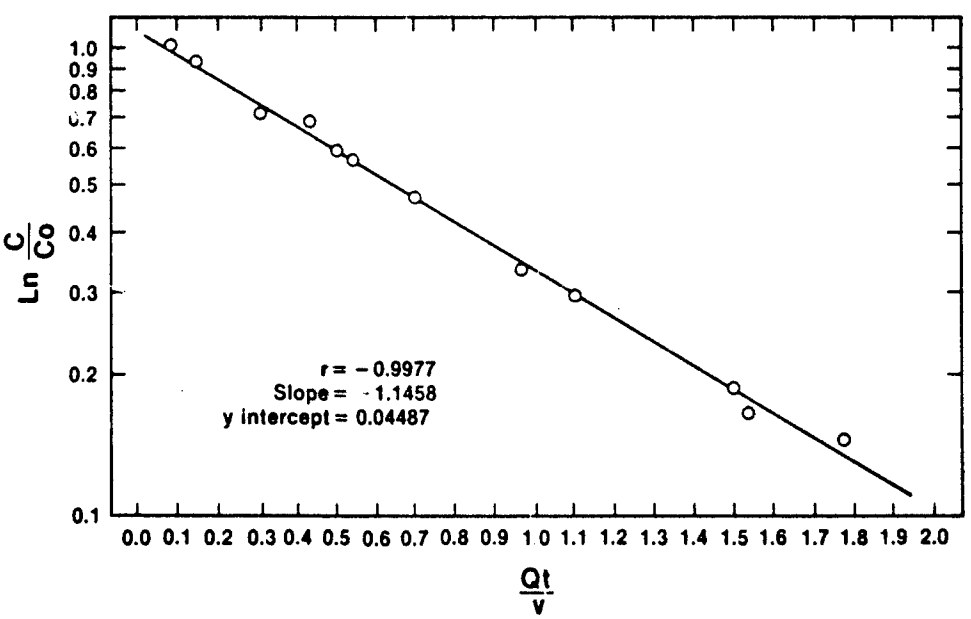


Figure 5. Graphical analysis of dome mixing characteristics. Q = Dome flow rate (cubic feet/min); t = Time (minutes); V = Dome volume; C_0 = Steady-state gas concentration; C = Gas concentration at time t.

showed that the temperature at the top of the dome was the most significant factor influencing the concentration, followed by analyzer baseline drift and dome pressure (Table 2). Removing the variability attributable to these parameters reduced the data range by eliminating low analyzer readings (Figure 7).

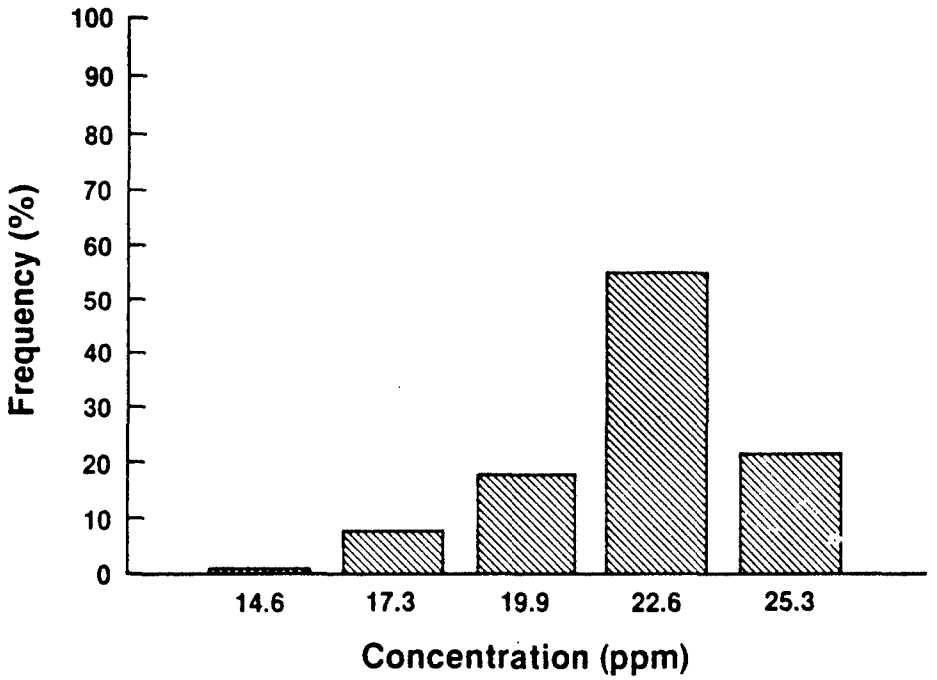


Figure 6. Distribution of hydrocarbon analyzer measurements.

TABLE 2

ANALYSIS OF VARIANCE FOR DOME TEST ARTICLE COMBINATION DATA

Significant Variable	Multiple R^2
Top dome temperature	31%
Analyzer baseline	20%
Dome pressure	12%

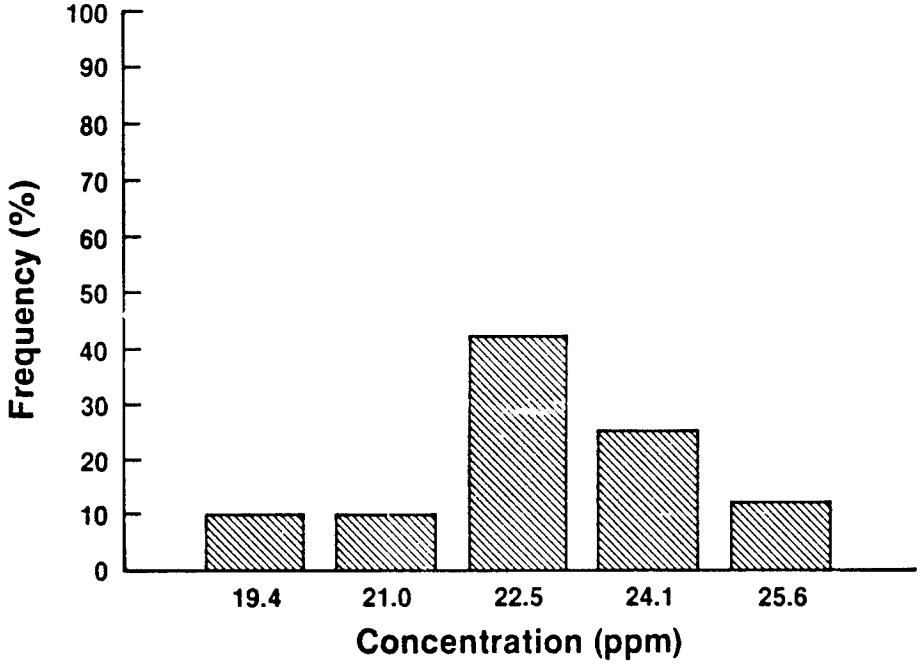


Figure 7. Distribution of calculated hydrocarbon analyzer concentration measurement after removing explained variability.

The hydrocarbon analyzers exhibited good short-term stability; however, their calibration shifted by detectable amounts from day to day. Calibration curves were developed each day to assess the analyzer stability. The parameters from a least-squares analysis of the daily calibration data are shown in Table 3. The individual daily calibration curves for each analyzer were used to convert the analyzer voltage levels to parts-per-million units of propane.

Dome Spatial Distribution

The individual propane concentration measurements were compiled and analyzed using factorial analysis of covariance. The distribution of the data was sufficiently normal such that statistical tests could be applied under the assumption that the data were normally distributed. The relative standard deviation of the measured propane concentration was 9%. When the effects of the operating parameters were removed, the relative standard deviation of the spatial distribution in the dome was 6.4%. This value is taken to be the inherent spatial variation of the Thomas Dome when operated with a light gaseous test agent. Changes in operating parameters were found to account for the remaining variation in the measured concentrations. In order of decreasing effect, local temperature fluctuations, analyzer initial zero, and dome internal pressure were seen to affect test article distribution.

TABLE 3

HYDROCARBON ANALYZER CALIBRATION DATA

	Analyzer 1		Analyzer 2		Analyzer 3	
	Slope	Intercept	Slope	Intercept	Slope	Intercept
Day 1	2.42	0.434	2.01	0.520	2.01	−0.191
Day 2	2.43	−0.088	2.02	−0.039	2.08	−0.452
Day 3	2.43	−0.088	2.02	−0.039	1.99	−0.610
	Analyzer 4		**Analyzer 5**		**Analyzer 6**	
	Slope	Intercept	Slope	Intercept	Slope	Intercept
Day 1	2.06	0.0273	2.03	1.06	2.01	−0.088
Day 2	2.08	−0.453	1.82	0.744	1.95	−0.793
Day 3	2.08	−0.453	1.82	0.744	1.95	−0.793

DISCUSSION

When the dome is operated at a pressure of 1 mmHg below ambient, there are no noticeable leaks. As pressure decreases, leaks become detectable. The average overall leak rate calculated from the inlet and outlet concentration data was 5.4%, less than the 15.3% observed during leak measurements. Because the apparent difference between dome inlet and outlet concentration correlates with changes in analyzer baseline, we assume that the measured leakage is due to the inability to completely close the manual inlet and exhaust valves.

The Thomas Dome appears to behave in a manner described by a well-stirred tank model, indicating that for light hydrocarbons, such as propane, the inlet test article flow is mixed into the entire chamber volume with little loss or delay. The fraction of the dome volume that mixes poorly with the rest of the chamber (dead volume) is between 5 and 12% of the total. There appears to be no shunting of material from the inlet to the outlet of the chamber, which indicates that the material supplied to the chamber reaches the animals without significant loss. Some of the airflow in the chamber appears to exit the chamber via displacement by the incoming air, much as if the incoming air behaved like a piston. Cholette and Cloutier (3) show that the time required for the outlet concentration to fall is equal to the time required to displace the unmixed volume of the chamber. This displacement must arise from a piston-like behavior of the inlet flow since there is no exchange of material between the dead volume and the active volume of the chamber. Therefore, the fraction of the inlet flow that acts like a piston can be calculated from the flow rate, the time for

displacement, and the size of the dead volume. For the Thomas Dome, the fraction of inlet flow acting like a piston is 5.3%.

The spatial distribution for light hydrocarbons in the Thomas Dome varies by only 6.4%, suggesting that very uniform doses are delivered to animals exposed in the chambers. In spite of frequent calibrations, experimental parameters related to analyzer stability were important contributors to the observed concentration variations in this study. This emphasizes the fact that one's perception of how test material is distributed within an exposure chamber is limited by the stability of the instruments used in making the concentration measurements. One can anticipate that the stability of instruments used in making concentration measurements of particulate test agents will be much worse than that observed in this study. Therefore, an understanding of aerosol distribution within inhalation chambers will be even more restricted by measurement errors. The variation in dome spatial distribution is sufficiently low such that a major factor in its measurement is the inherent drift and inaccuracy of the measuring method. Dome pressure was also a significant contributor to the variability of test article distribution, indicating that this operating parameter, if it cannot be stabilized, must be statistically controlled by a covariant experimental design.

The ability to measure small percentage variations in chamber concentration depends critically on the overall stability of the analytical system used. Analysis of the hydrocarbon analyzer voltages using a single calibration curve suggests that the measured dome propane distribution is low in one region of the dome, possibly as the result of a leak. This region of low concentration involves two of the six hydrocarbon analyzers. However, when the analyzer calibrations are done daily and the measured propane concentrations are computed using these calibrations, no persistent patterns of low or high concentration are found.

These statements apply to operation of the Thomas Domes with gaseous test articles capable of rapid diffusion across airflow paths. Future measurements will indicate the effect of materials of low diffusion capacity, such as heavy vapors and aerosol particles, on dome distribution and operation. These measurement and analytical techniques should be generally applicable to most inhalation exposure chambers. It is hoped that their application to such chambers will eventually provide a quantitative basis for designing inhalation equipment.

ACKNOWLEDGMENT

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QUESTION AND ANSWER SESSION

DR. MacFARLAND (CONSULTANT IN TOXICOLOGY): What's the volume of the chamber?

DR. CARPENTER: The chamber is 702 cubic feet.

DR. YANG (NIEHS): Bob, I would just like to make a clarification. You made a statement that the National Toxicology Program (NTP) recently required animal chamber distribution data. I just want to make a clarification that as long as I have been with NTP we have required data. I think what you are referring to is our recent requirement for developmental chemistry work which includes the animal chamber distribution data to be submitted prior to the initiation of the animal study.

DR. CARPENTER: We'll strike the word recent.

DR. NEWELL (EPRI): I noted the measurements that you have with the access coming from the floor below and up into the chamber. When that's in operation, does that affect the distribution of your gases? In this case, does it change the pressures and, if so, how long before it gets back to equilibrium?

DR. CARPENTER: I haven't had a chance to make those measurements, but I would be very very surprised if it doesn't. Hopefully next year I will be able to tell you.

DR. NEWELL: That would be a useful parameter to have included.

DR. CARPENTER: We'll certainly measure that.

DR. MOSS (BATTELLE PACIFIC NORTHWEST LABORATORIES): What were the air changes per hour in your chamber?

DR. CARPENTER: We ran anywhere from 25 to 95 cubic feet per minute, so you've got to take that and divide by 702 cubic feet. It works out to be from 10 down to roughly one-fourth of that. So, anywhere from 2 to 10. You're not operating at a high rate of exchange relative to what we're used to with the 2000 Chambers.

GAS, VAPOR, AND AEROSOL GENERATION SYSTEMS

Michael E. Hiteshew

Northrop Services, Inc. – Environmental Sciences, P.O. Box 12313, Research Triangle Park, North Carolina 27709

SUMMARY

The generation methodology used to provide test atmospheres for inhalation toxicology research depends upon a variety of factors including physical characteristics and physicochemical properties of the compound, chamber size, and process airflow rates. Proper planning prior to the initiation of any test protocol can help maximize the probability of success of the study.

A simple feedback servo system is presented that is currently used by Northrop for generation and control of gases. This system can be adapted for both vapor and aerosol generation systems. All feedback systems require an on-line monitoring system.

An overview of generation methodologies is presented with considerations given to the proper mixing of compounds and process air streams, compound vulnerability during generation, radiolabeled test atmospheres, and an automated aerosol generator.

INTRODUCTION

Much has been written and documented on the importance of proper generation techniques of gases, vapors, and aerosols for use in inhalation toxicology studies (1-5). The presentation of the test atmosphere must be carefully thought out so that the compound of interest can be presented in the manner specified in the experimental protocols. Care must be taken to not alter the compound's physicochemical properties and to not adulter it with foreign substances. Care must also be taken to provide the proper concentration of the test article within the exposure chamber as this is the single most important variable in a toxicological study. The techniques used for generating such atmospheres must be stable and reproducible if reliable and defensible data are to be derived from the studies. This paper, which is intended to be a review of such generation techniques, will present some unique ideas and approaches currently being developed and/or used by personnel at Northrop Services, Inc. – Environmental Sciences in Research Triangle Park, NC.

Proper inhalation toxicology research begins with thorough planning prior to the initiation of any inhalation study. A joint effort by engineers, toxicologists, and statisticians is required to design an effective and reasonable research protocol. The criteria established in the protocol will have a strong effect on the generation methodology selected by the inhalation engineer.

A primary consideration is the form of the compound or the mode in which it is to be evaluated. Will the compound be presented in gaseous or aerosol form? If it is to be presented as an aerosol, will the particle be wet or dry? What are the desired particle size and the geometric standard deviation of the distribution?

Second, the concentration of the compound has a direct influence on the selection of the generation technique. The combination of concentration, chamber flow rates, and chamber size can preclude the use of many generation methodologies. Certain generation systems might require airflows that exceed the capabilities of some smaller chambers, and some systems might not provide the necessary mass output required to reach the desired concentration in larger inhalation chambers.

Often, the reactivity of the compound is a key consideration. During the generation phase, the test agent can be subjected to extremes in environmental conditions. These include elevated temperatures (vapor generation), surface disruptions (bubblers and nebulizers), and pressure changes (air jet nebulizers). With the compound in such a disturbed state, reactions can occur with any contaminant (e.g. water, oil, particulate matter) in carrier gas streams. The stability of the compound is also a mitigating variable. Will the compound remain stable with respect to its physicochemical properties throughout the course of an exposure, or from exposure to exposure? Reactions can occur with ammonia, water vapor, food dust, animal dander, and chamber surfaces.

Finally, safety is one of the most important factors to take into account when selecting the generation technique. Certain methods might require handling of large amounts of extremely toxic and/or corrosive compounds, which increases the probability of accidentally exposing operating personnel to the compound.

The inhalation engineer must take all of these considerations into account when selecting the generation methodology. Cooperation from other members of the research team is necessary to maximize the probability of success.

Gases and Vapors

For purposes of clarity, gases and vapors can be considered identical, in the respect that they are represented as singular molecules within the test atmosphere. Gases are commonly thought of as those compounds that exist either in a gaseous state or as a liquid with appreciable vapor pressure at standard temperature and pressure (STP). Vapors are normally derived from compounds that are either liquid or solid at STP; regardless of form at STP, both are identical when they are represented in the exposure chamber atmosphere in free molecular form.

Generally, the easiest atmospheres to generate are those in which the compound of interest is prepared in a compressed gas source, as either a pure or mixed gas. Normally, such compounds can simply be metered into the inlet of the exposure chamber by means of a rotameter, capillary, or mass flow controller. When presented to the chamber process (dilution) air in the proper amounts, the gas is diluted to the proper study concentration. Care must be taken to ensure that the contaminant and process airstreams are thoroughly mixed to ensure uniform dosing of the test population and to ensure that samples of the atmosphere used for quantitation are representative of the entire chamber atmosphere. To ensure proper mixing of the contaminant and chamber process airstreams, it is occasionally necessary to introduce a mixing chamber -- a reservoir containing mixing baffles, a metering orifice, or a length of piping with sufficiently turbulent flow (generally, length = 50 pipe diameters with Re >4000). Systems in which the contaminant is diluted directly into the chamber inlet airstream are known as single-dilution systems.

When a researcher is faced with the problem of generating extremely low concentrations of gases while avoiding extremely high dilution ratios, or metering extremely small amounts of the item of interest into the chamber airstream, it might be advantageous to use a double-dilution system. The agent of interest is diluted by a clean airstream, the majority of which is vented as excess and the remainder metered into the chamber airstream where it is subsequently diluted by the chamber process airstream. Whereas this series of dilutions can be repeated as many times as necessary to achieve the desired dilution, in actuality it becomes extremely difficult to maintain a constant and reproducible contaminant stream into the chamber after more than two dilutions (6).

When using devices such as mass flow controllers to control the amount of test article injected into the chamber, it is a simple matter to automate the system via a single feedback control loop, provided that real-time analysis of the chamber atmosphere is available (Figure 1).Northrop currently uses this type of circuitry to control its acute O_3 and NO_2 gaseous exposures and as a backup for its computer-controlled chronic gaseous work. It could easily be adapted for a range of organic compounds, provided that an instrument such as a continuous hydrocarbon analyzer is available.

The relationship between the amount of test gas supplied to the chamber via a mass flow controller and the desired level of the test gas in the chamber is governed by the servo control system. The circuit requires that two voltages be supplied: a voltage from the analyzer, which is indicative of the concentration, and a command voltage, which is indicative of the desired chamber concentration. The command voltage can be supplied by a manually adjusted potentiometer or a computer. The two voltages are supplied to a difference amplifier, which subtracts one from the other. This voltage difference is directed to an integrating amplifier circuit through a resistor, causing the amplifier circuit to either charge or discharge the integrating capacitor. The output of

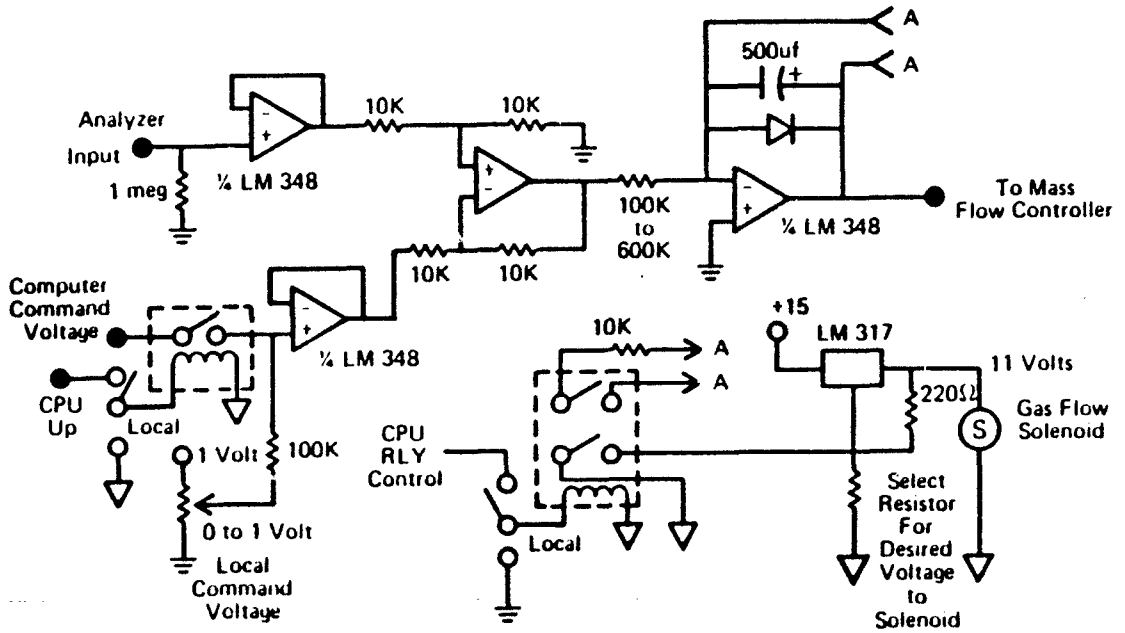


Figure 1. Sample feedback loop control schematic.

this capacitor is used as the control voltage of the mass flow controller. When equilibrium is reached, that is, when the command voltage equals the desired concentration voltage, the capacitor remains in a steady state and holds the mass flow controller open at that particular level. The gain of the system (the rate at which the capacitor charges or discharges) is governed by the resistor value of the integrator. Figure 2 depicts an ideal profile that has been used at Northrop for exposing animals to both O_3 and NO_2, and Figure 3 illustrates the control system capability.

In support of pharmacokinetic and deposition studies, Northrop is performing exposures using radiolabeled gases generated in house, such as ^{14}C phosgene, and stable isotopic gases such as $^{18}O_3$. Radiolabeled gases are prepared by cryogenically transferring small aliquots of the pure labeled compound to a stainless steel sample cylinder and subsequently pressurizing the cylinder with either N_2 or unlabeled mixtures of the same compound. This allows for formulation of a cylinder of labeled gas that is the proper concentration and activity.

For $^{18}O_3$ exposures, two methods have been used to generate the test atmosphere. ^{18}O is a stable isotope of ^{16}O and is commercially available. $^{18}O_2$ is continuously irradiated by a silent arc discharge at liquid nitrogen temperatures and eventually condenses out in the form of liquid O_3. After all the O_2 has been converted to O_3, it is gently warmed and transferred to a stainless steel

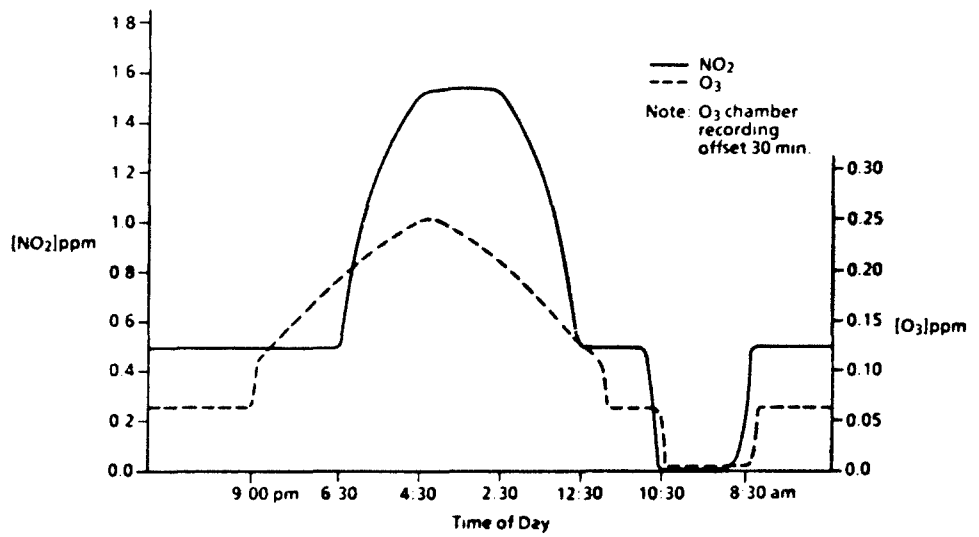


Figure 2. Desired O_3/NO_2 concentration profile.

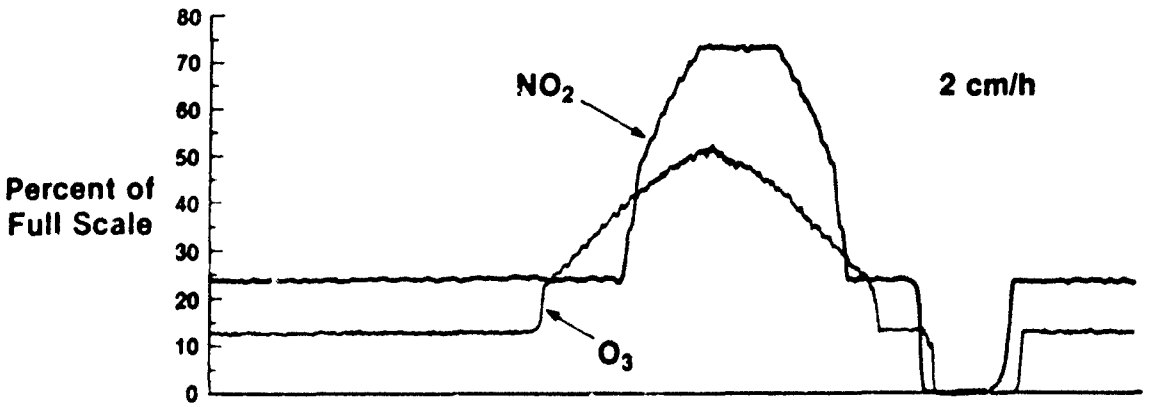


Figure 3. Actual O_3/NO_2 concentration profile.

sample cylinder, after which it can then be metered into an exposure chamber and diluted to the proper concentration. Tissue analysis is performed by isotope ratio mass spectroscopy.

$^{18}O_2$ can also be diluted into a sample cylinder with an inert gas (helium) and irradiated by a silent arc discharge generator to form $^{18}O_3$, which can be metered into an exposure chambe · and diluted to the target concentration.

Methods employed in the generation of vapor atmospheres are governed by the chemical and physical properties of the compound of interest. Vapor pressure, boiling point, and melting point are key factors. Other considerations include the required chamber concentration, given chamber volume and process airflow rates, thermal stability of the compound, and, certainly, safety.

Two of the simplest methods to generate reproducible vapor atmospheres employ permeation tubes (7) and diffusion tubes (8). Both types of devices are commercially available for a wide range of compounds and provide constant outputs of pure vapor. Output capabilities range from the nanogram- to microgram-per-minute range for permeation tubes, and the microgram- to milligram-per-minute range for diffusion tubes.

In the permeation device, a pure aliquot of material is sealed inside a permeable polymeric membrane. At constant temperature and pressure, the compound is held in liquid/vapor equilibrium. Molecular permeation through the membrane provides a uniform and stable flow of the compound to a clean carrier gas stream. Adjustments to the flow rate of the carrier gas stream allow for the production of a range of mixture concentrations. The output of the permeation tube is dependent on the surface area of the permeable membrane (usually dictated by tube length), the membrane thickness, the permeability of the membrane, and the temperature at which the tube is maintained (Figure 4).

Permeation tubes are useful for reactive and corrosive gases, and they require very little maintenance compared to gas cylinder and regulator upkeep; in addition, chemical stability tends to be less of a problem than with reactive gases housed in cylinders. Additionally, they are relatively safe to use because of the small amount of the material present in the tubes.

Adversely, the output ranges of permeation devices limits them to use in inhalation studies. They must be handled carefully because oil from fingerprints or moisture in the carrier gas streams can reduce the output of the tubes. Additionally, the temperature of the tube environment should be maintained within 0.1°C, which requires a somewhat sophisticated temperature control system.

Diffusion tubes (Figure 5) are basically reservoirs of a compound in a liquid state and a capillary tube above the liquid over which flows a clean carrier gas stream. At a fixed temperature, the compound exhibits a constant vapor pressure, allowing the vapor to diffuse through the

Permeation Device Basics

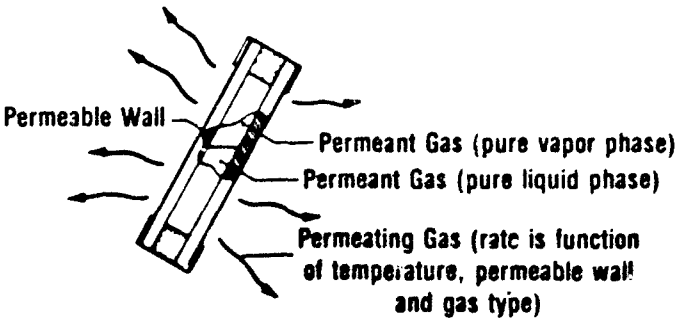


Figure 1: Standard and/or High Emission Tubular Device

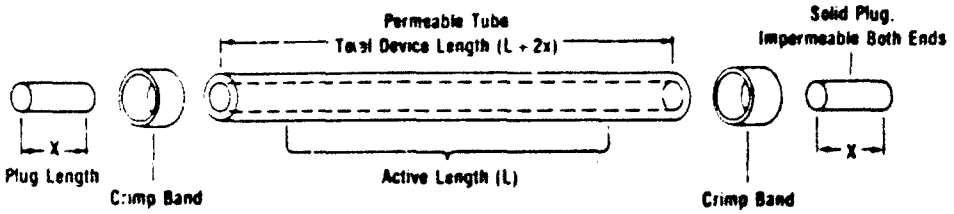


Figure 2: Low Emission (LE) Tubular Device

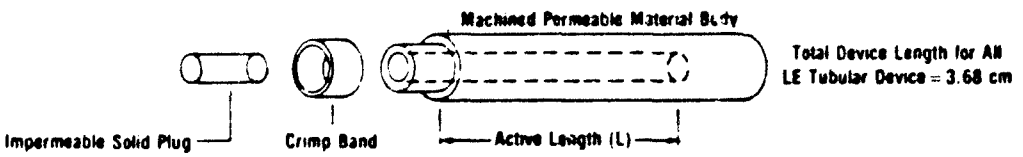


Figure 3: Wafer Device

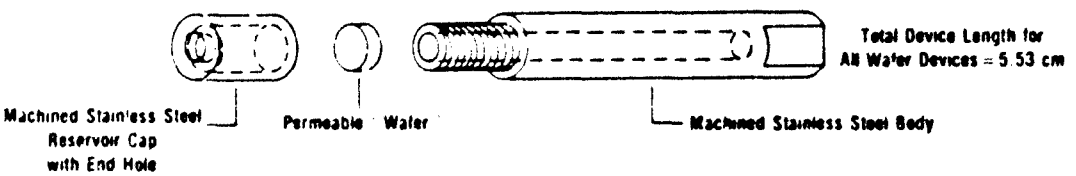
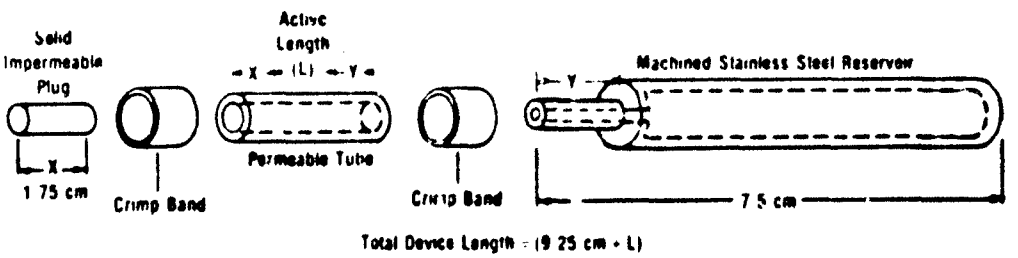


Figure 4: XLT (extended life) Tubular Device



Note: X Dimension in Drawings = 1.75 cm

Figure 4. Permeation tube configurations (courtesy of VICI Metronics).

capillary. The output is dependent on the length and bore of the capillary. As with permeation tubes, diffusion devices require a constant temperature control system because a diffusion tube operates according to a pressure drop across a capillary; back pressures on the capillary can cause fluctuations in the output.

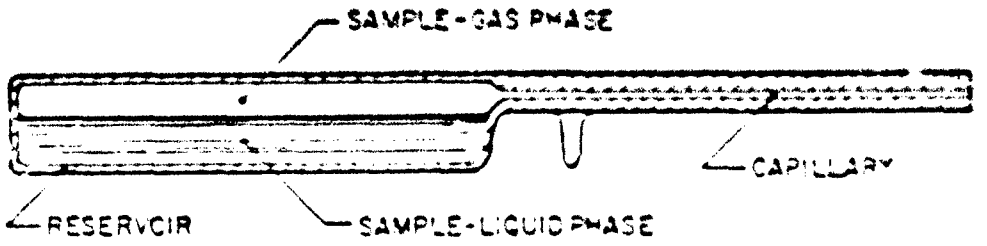


Figure 5. Diffusion tube schematic (courtesy of VICI Metronics)

Several common evaporative methods can be used to provide constant vapor outputs for presentation into an inhalation chamber. The first of these is a simple evaporation system that relies on the inherent vapor pressure of the compound of interest. The compound is allowed to evaporate in a confined and constant temperature environment. The vapor pressure created is used in conjunction with metering valves to deliver the vapor to a dilution system, the output of which is directed into the exposure chamber. This technique is appropriate when the desired concentrations can be achieved with the limited flows of the generator and vapor pressure of the compound.

One can improve this system by heating the reservoir of the compound to a predetermined temperature above ambient, depending on the thermal stability of the compound and the output required, and either bubbling the carrier gas through or passing it over the liquid. The carrier gas then passes through a condenser maintained at a temperature 20°C below ambient, thus ensuring that the carrier gas is saturated with the vapor at that temperature. Immediately upon exiting the condenser, the carrier gas should be mixed with dilution air to prevent condensation in the carrier lines and to prevent possible aerosol formation. The reservoir should be constructed so as to provide a constant surface area as the liquid evaporates. The use of round-bottom flasks is therefore not recommended.

Another system used to generate constant output vapor streams is commonly referred to as countercurrent volatilization. The liquid of interest is metered to the top of a heated reservoir and allowed to cascade down the reservoir over some support medium, either glass wool or glass beads. The support media provides a larger surface area for the liquid to interact with the carrier gas stream. The carrier gas is introduced at the bottom of the reservoir, thus sweeping the vapor into the inhalation chamber. Normally, it is desirable to evaporate all of the liquid and leave no

condensation at the bottom of the column. By metering exact amounts of the liquid onto the column, it is possible to both calculate and control the amount of vapor produced. This type of system eliminates the need for exact temperature control because the amount of liquid added to the column is the limiting factor.

Typically, syringe pumps are used to meter the liquid in this system. They are commercially available in different models with a wide variety of pumping rates. However, the pump drive system often causes the syringe pumps to pulsate, which creates a cyclic output. One solution to this problem is to provide a larger-than-normal mixing chamber prior to adding the vapor stream to the dilution airstream supplying the chamber. This will dampen the syringe pump cycling by providing a longer residence time in the mixing chamber. This problem can be completely avoided by using an HPLC pump/pressure capillary combination. The output of the HPLC pump is coupled to a stainless steel capillary, which provides sufficient back pressure for the pump to operate without pulsation, and also allows for feedback control capability because most HPLC pumps have interface capability.

Aerosol Generation

Numerous systems have been used for generating aerosols of respirable sizes (9-13). This abbreviated discussion will be concerned only with polydisperse aerosol generation techniques. A polydisperse aerosol is one that contains a range of particle sizes as opposed to a monodisperse aerosol, in which all particles are of the same size. Most aerosol distributions found in nature are polydisperse, with the exception of some pollens and dusts. As a general rule, polydisperse aerosols are used in inhalation research. Monodisperse aerosols are generally used for instrument calibration procedures, for studies involving regional pulmonary deposition, or in aerosol physics research.

The sheer number of techniques used to generate polydisperse aerosols prohibits a complete discussion of all types of generation systems. As a result, only a few of the more commonly used techniques will be reviewed: the Wright dust feed (14), the fluidized bed elutriator (15-17), air impaction nebulizers, evaporation/condensation generation systems, and a brief overview of current work being developed at Northrop for an automated, variable output liquid nebulizer.

A variety of generation techniques can be used to disperse dry powders. Perhaps the most widely used device is the Wright dust feed, a generator that uses a variable gear drive to mechanically scrape a prepacked pellet of a compound into a high-velocity airstream. A baffle plate in the airstream serves to break up any agglomerated particles that were removed by the scraping action of the blade. The output of the generator is dependent on the density of the packed pellet and the rate at which the scraper blade moves against the pellet. Generally, the particle size output resembles the particle size characteristics of the bulk powder used to make the pellet. The size distribution may deviate somewhat from the bulk material due to the presence of agglomerated

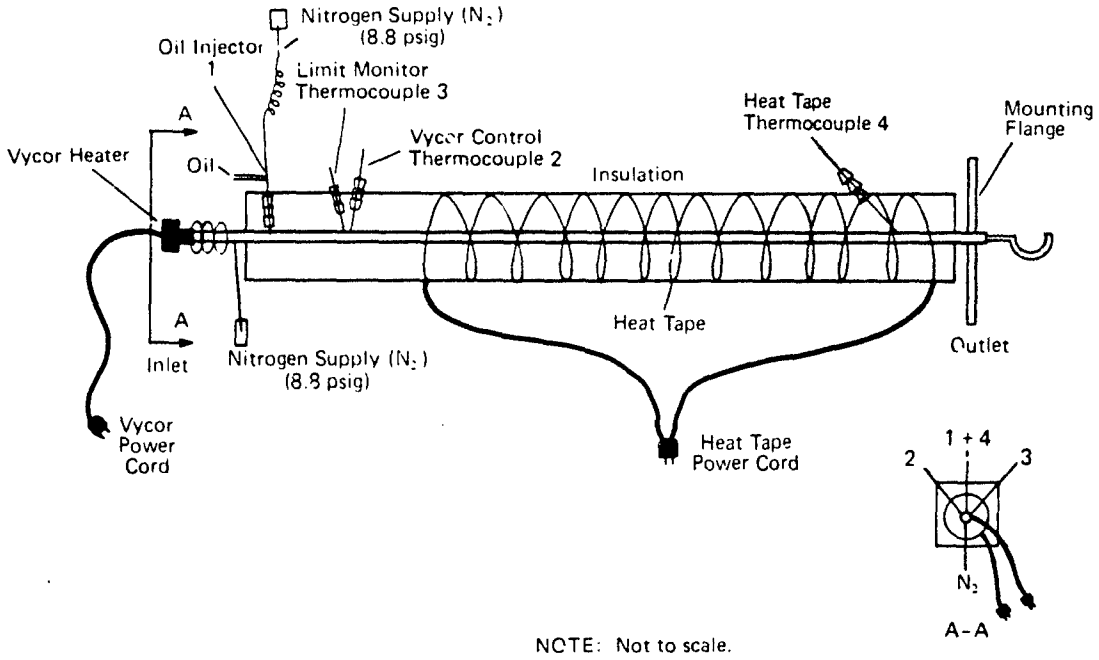
particles or to the breakup of the bulk particles. Some pregrinding or size fractionation may be necessary to provide an aerosol of respirable size if the bulk material contains a high percentage of large particles.

Another method frequently used to disperse dry powders is the fluidized bed eductor. A fluidized bed is produced when air is forced upward through a layer of particles contained in a vertical tube. If there is a mixture of particle sizes in the powder to be dispersed, the fine-mode fraction can be generated by limiting the flow rate of the air moving through the bed. The upward air velocity can be limited so as to not carry the large particles or agglomerates out of the generator. Because only the fine fraction of the particles is being dispersed, it becomes necessary to continuously feed new material into the generator. This can be accomplished by gravity or by chain-drive feeds. The action of dispersing dusts in this manner creates significant electrical charges on the particles, so use of a Kr 85 (beta particle-emitting) source to create an ion field to reduce the charges to a normal Boltzman equilibrium is recommended.

Another type of aerosol generation technique is referred to as evaporation/condensation formation. This type of system has been adapted by Northrop to provide oil aerosols (18, Figure 6). A light oil is metered onto a hot vycor heater contained in an insulated stainless steel tube in an inert atmosphere. The oil is vaporized and swept by a carrier gas to the chamber supply air where it rapidly cools and condenses to form a uniform aerosol.

Perhaps the most commonly used method for producing aerosols for inhalation studies employs the compressed air nebulizer. An example of this type of nebulizer is the Model 3075 constant output nebulizer manufactured by TSI (St. Paul, MN, Figure 7). These generator types force compressed air through an orifice to create a jet stream, which breaks up a liquid stream, creating droplets. The low-pressure region on the immediate downstream side of the orifice continuously aspirates the liquid from a reservoir. The larger droplets are impacted on an adjacent surface and drain back into the reservoir while the smaller particles are carried through the exit of the nebulizer. If the liquid being nebulized is an aqueous solution of a salt, the reservoir contents will gradually concentrate over a period of time as the solvent evaporates. This concentrating of the solution can be minimized by using a larger reservoir or by continuously feeding a fresh solution to the nebulizer. With aqueous solutions, the mass output of the nebulizer is directly related to the concentration of the solution.

Northrop has recently developed a modification of this nebulizer for use in chronic aerosol inhalation studies. The liquid feed line of the nebulizer has been coupled to the common port of a three-way vacuum solenoid valve. The normally closed port of the solenoid is connected to a low-concentration solution of the compound of interest, and the normally open port of the solenoid is



NOTE: Not to scale.

Figure 6. Evaporation/condensation generator.

connected to a high concentration of the same solution. An electronic switching network has been designed to allow the solenoid valve to actuate one per second; the time that the port is left open can be adjusted, from 0.1 to 0.9 seconds (i.e., if the normally opened port is actuated for 0.25 seconds, the normally closed port will be actuated for 0.75 seconds). The cycle rate is dictated by a manually controlled potentiometer. Depending on the set point of the potentiometer, the concentration of the solution supplied to the nebulizer is a mixture of the two stock solutions. Thus, the mass output of the nebulizer can be controlled by adjusting the pulsation of the solenoid valve to provide the nebulizer with the proper concentration of the solution and to provide the desired chamber concentration. By coupling this system with a reliable aerosol mass monitor, it is possible to provide real-time control of aerosol exposures, or to generate varying profiles of aerosol concentrations.

The system does have its disadvantages, in that as it is currently configured, it wastes large amounts of the solution to be nebulized. This could be extremely important if the solute is expensive or very toxic. Additionally, as the concentration increases, the particle size increases. This could be undesirable if particle size considerations are extremely critical, or if the monitor used to quantitate the atmosphere is sensitive to particle size changes.

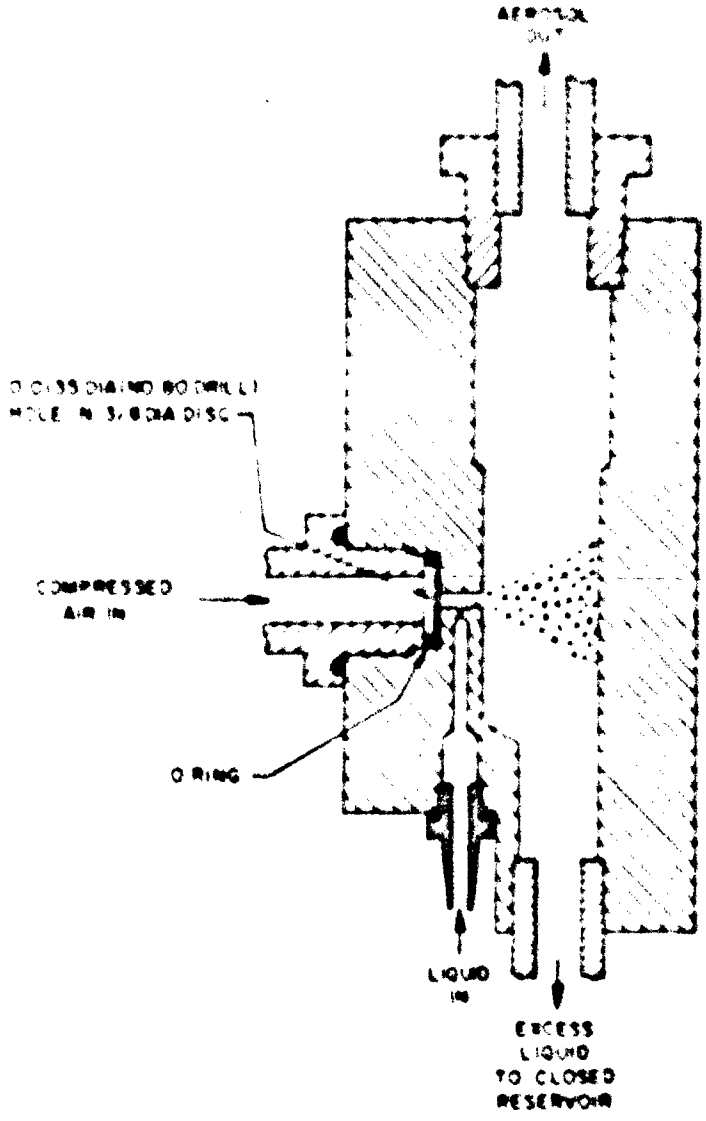


Figure 7. Schematic diagram of TSI Model 3075/3076 constant output atomizer assembly (courtesy of TSI, Inc.)

CONCLUSION

As was stated in the introduction, the amount of literature describing various generation systems for use in inhalation toxicology research is tremendous. The references cited in this paper are a good starting point for information concerning generation and delivery systems.

Automation of various systems can be accomplished by using the simplified servo-feedback control loop described in this paper, provided that reliable real-time monitoring is available.

Proper planning prior to the initiation of any study is necessary to maximize the probability of the study's success. The involvement of the inhalation engineer in this process is crucial, as the generation methodology will be chosen according to the exposure regimen and the physicochemical properties of the compound selected. The generation technique chosen should accommodate the safety of operating personnel and the study.

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MANAGING DATA QUALITY THROUGH AUTOMATION

Robert W. O'Connor and Andrew K. Miller

Northrop Services, Inc. – Environmental Sciences, P.O. Box 12313, Research Triangle Park, North Carolina 27709

SUMMARY

Traditional definitions of data quality deal primarily with individual data sets and the data collection process. Today's standards for ensuring data quality have not changed with respect to the desired results, but have simply been expanded to take advantage of modern technology. Computers are used to acquire, review, store, analyze, and report data. Because each of these steps can be automated, the need for human intervention and manual review is minimized. As a result, the potential for invalid data to reach the data analysis stage has increased significantly. To reduce this potential, efforts must be devoted to developing automated procedures that cover every conceivable validation possibility. Relationships between data and data sets must be well defined (1), and data base support that facilitates ready access to the data for the purpose of analysis must be provided. For small data sets, automation may therefore be impractical; but for large, interrelated data sets, automation is highly desirable. Computer automation has therefore expanded the traditional concept of ensuring data quality to include a complex array of interrelated tasks that must be properly managed to achieve the desired results.

INTRODUCTION

Computer automation and major advances in analytical technology are being applied in an increasing number of scientific and engineering disciplines. Toxicology is certainly no exception. Data are being acquired at faster rates and with greater sensitivity, accuracy, and precision than ever before. Data sets are rapidly growing larger and are becoming more difficult to manage as they grow. Manual methods of data management, although effective in the past, are now becoming too slow, too expensive and, in light of other options available today, ineffective. Efforts must therefore be taken to maintain high standards of data quality and, ultimately, the defensibility of experimental results.

This paper discusses issues that must be addressed in the four major areas of the data management process. The first area covers the research protocol and the requirements for developing a data management plan. The data collection process and its effect on data validity follow. In the third area a comparison is made between manual data validation methods and the

issues that must be resolved in order to automate these methods. The final section deals with automated data base management.

PLANNING FOR DATA MANAGEMENT

Traditionally, research is planned by the principal investigator alone or in conjunction with a committee of concerned scientists (2). Biological end points are identified, a toxicant and its appropriate exposure levels are defined, and other relevant scientific issues are resolved. But is the protocol complete? Have all required data elements (3) as well as the frequency and methods of collection been documented? Have data dependencies and the statistics required to evaluate such dependencies been defined? Have progress reports and subsequent data reviews been scheduled? Have appropriate staff, including statisticians, engineers, and data management and quality assurance (QA) specialists been consulted and, if possible, included on the protocol development committee? These questions are too often left unanswered, primarily because data management has not yet been universally accepted as a key part of the research protocol. Good Laboratory Practice (GLP) regulations and QA activities do, to some extent address these issues, but progress has been slow. Each investigator should periodically review his or her own work as though it were the work of a competing researcher. Attempts should be made to dispute findings, discredit methods, or find weaknesses in the experimental design. Researchers should identify and deal with problem areas early. Practicing good data management techniques does not guarantee defensibility, but it is a major part of a sound approach to producing high-quality research.

Data management, even in the broadest sense, means different things to different people. To some, it is synonymous with data base management. To others, it also includes data validation. In fact, it includes all activities that deal with the data. It is a process that often gets little attention until experimental results are unexpectedly challenged, at which point an anxious review of all data ensues to substantiate or explain the questioned results. Such a review should not be necessary. The investigator should anticipate and plan to discuss controversial results long before the data are made public. Data management, either manual or automated, is a tool that, if properly used by the investigator, can eliminate data collection artifacts that might otherwise bias experimental results. If properly organized, data management can also aid investigators in their study of observed toxicological phenomena.

AUTOMATING THE DATA COLLECTION PROCESS

Industry has taken the first step in automating the data management process. They have, in some cases, integrated microcomputers into their analytical instrumentation (4), thus automating many of the functions that previously required a technician's skills. In our laboratory a variety of

autosampling microprocessor-based analytical instruments are used, some of which include gas and liquid chromatographs and infrared spectrophotometers. Skilled technicians are required to develop methods and establish operating criteria for these instruments. But, once programmed, no further technical support is typically required to sample, analyze, or report data. The analysis process is therefore exact, repeatable, and free of artifacts common to conventional manual methods.

In other cases, stand-alone data management systems have been developed to interface with older analytical instruments to provide essentially the same process control and automated data management functions (5,6,7). Nelson Analytical (Paramus, NJ) has been one of the leaders in providing add-on data acquisition and management support for chromatographic-type instruments. Although we do not use them, other Northrop laboratories have made extensive use of Nelson products in their routine daily operation. This addresses the analytical aspects of an experiment, but what about the collection of other end points that have toxicological interest, such as clinical and necropsy observations (8)? This type of data, and any other data that are subject to human interpretation cannot automatically be acquired without some type of manual intervention. In the worst case, a data clerk is given the job of key punching handwritten descriptions. A more efficient method would be to limit the number of available observations and encode them. The data clerk would then only have to match the selected option from the standardized form, with a similar screen display. An even more efficient approach would be to have data collection forms directly readable by the computer. The use of such data entry forms is likely to come as close as anyone can to dealing automatically with data of this type. Automated data management is a tool, an attempt to standardize the data collection process, eliminate the random human error factor, and make the next step in the data management sequence of events, data validation, a less complicated task.

DATA VALIDATION

The data validation process has traditionally been a manual review of acquired data, the intent of which was to detect and isolate data that, for a variety of reasons, did not belong to the data set. This review process assessed all of the data in small and uncomplicated data sets. As the data sets grew in both size and complexity, a comprehensive review was no longer practical. As a result, random checking was adopted. This, of course, typically detected only gross systematic errors: Sporadic errors usually passed undetected. This is certainly a less-than-ideal approach to validating data. A new, automatic technique was needed for reviewing all data, including relationships between data sets. This technique had to operate quickly, with minimal human intervention, and it had to create sufficient documentation to satisfy both GLP and QA auditing requirements (9,10). The only feasible approach was computer automation. We have developed automated validation techniques that look at time-related data sets objectively. The software, written in Fortran 77, tags

each stored data element with a unique identifying code, which fully describes the validation status of each data element. Both the data and associated status code are stored in the data base.

If the following guidelines are followed, a computer can perform data validation as well as, if not better than, its human counterpart.

1. All data within a data set are presumed to be valid unless it can be shown that specific points within that set are unrelated. For example, outliers are valid data points if they represent a condition that actually existed.

2. No data are ever discarded. Invalid data should be retained and appropriately labeled to avoid inclusion in the data set being processed. A raw data set and a validated data set are therefore one in the same. The difference is merely in the annotation used to identify individual data points.

3. There must exist a set of evaluation criteria that uniquely identifies conditions under which data points can be invalidated. The condition selected must also be recorded and referenced to the data point(s) to which it applies.

Automated data validation is an excellent technique for validating recurring data sets. Unfortunately, the software required to perform this task is not commercially available; it must be developed as needed and tailored to the requirements of the data sets to which it will apply. Once implemented, however, automated data validation is an excellent technique for satisfying QA requirements for methods standardization. It is totally objective in its evaluation process, and it provides consistent results.

After having been validated, the data are then usable but not necessarily accessible for analytical purposes. The data must be integrated into a structured format commonly called a data base.

DATA BASE MANAGEMENT

The task of managing data quality does not end with data validation, but continues into the process of information storage and retrieval, data analysis, and reporting. Information storage and retrieval systems are, in the most basic sense, a repository for information (data base) from which all or part of the information contained within may be easily retrieved. As a manual operation, this might be represented by a single laboratory notebook or a large collection of documents organized and stored in a filing cabinet. As an automated operation, this could be a single computer file, or a collection of files, all of which are interrelated but stored on a single computer system or in some cases different computer systems that are networked together. Both of these concepts are included in our computer system design, conceived to support multiple but independent research activities in

an inhalaton toxicology laboratory. The laboratory is divided into three exposure rooms. Each of two of the rooms have their own Digital Equipment Corporation (DEC) MicroVAX II computer that uses the VMS operating system. The third room has a DEC PDP-11/83 computer that uses the RSX 11M-PLUS operating system. These computers are wired, both together and to a DEC VAX 11/730, using Ethernet. Communication between the computers is handled with DECNet. Data, at the local level, are maintained for a 24-h period in separate independent files. Once daily, the data are sent to the 11/730 for validation and incorporation into the main data base. Should the network be inoperative, however, data can be stored at the local level for several days. Data base support is provided with DEC RDB relational data base software, and some additional support is provided using RS/1 software (Research System 1; Bolt, Beranik, Newman, Inc., Cambridge, MA).

Regardless of whether a manual or automatic method is selected for storing research data, there are two basic requirements that must be met. First, all data, regardless of the potential use in evaluating experimental results, must be adequately identified (8). Second, for all identified data, dependencies, if they exist, must be defined. These criteria must be satisfied to determine an optimal structure for the design of the data base. A superficial effort, even though unintentional, could prove costly, particularly if experimental results are misinterpreted because of missing or inaccessible data base information (11,12).

Having satisfied the two requirements discussed above, the researcher must then develop a method of organizing the data into a logical, easily accessible format. Before the organization of the data is defined, however, some type of unique identifier must be assigned to the data so that it can eventually be archived and, when needed, retrieved. An acceptable method might be to create study and experiment identifiers. A study, or a collection of like experiments, would be given a unique code. The study number identifies the individual study with the year, the day of the year, and where the study was conducted (Figure 1). Data that span the entire study and are common to all aspects of the study are collected under the study identifier (13).

Within the study, experiments or treatments are identified. All data relevant to a specific treatment are therefore accessed by locating the study number and then the individual experiment number. In our data base, data such as animal weights and necropsy data are collected under the experiment number. Combining the study number with the experiment number produces a study/experiment identifier. Given the study/experiment identifier, all data for a group of animals, for example, would be easy to locate.

This organization will work whether one is keeping data in a filing cabinet or in a computer data base. When establishing a computer data base, the user should be familiar with two data models (14). The first is the hierarchical data model, which looks like an inverted tree. The base of

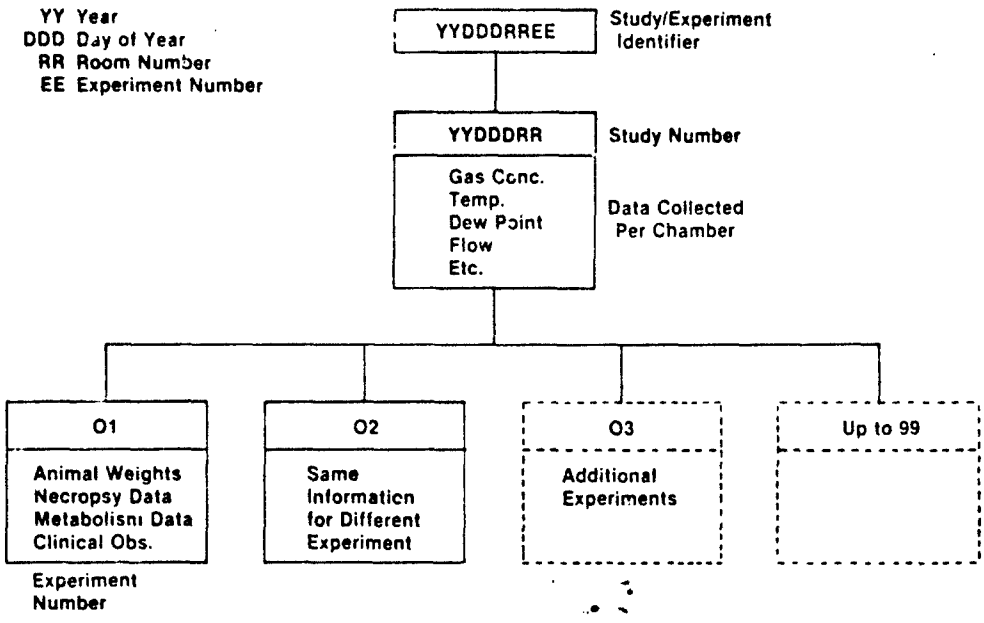


Figure 1. Research data should be divided into logical segments. In the authors' data base each exposure is divided into several experiments. For each experiment common data are stored together. Data common to all experiments are stored separately. Each of these data sets is a unique relational data base linked together in a hierarchical format.

the tree contains the most general information, and the branches of the tree point to more specific information (Figure 1). The other data model to be concerned with is the relational model (15), which treats data as a table. Within the table, no two rows may be identical, but there is some combination of columns, called a key, whose values are used to identify each row uniquely. In our data base, the overall structure of the data is a hierarchical model. The data contained at each level within the hierarchy is stored in a relational format.

At the study level, there is a relational data base for chamber gas concentration data and environmental data. Such data are common to all experiments within the study. At the experiment level, there are multiple relational data bases that contain various animal information (Figure 1). This information is accessed by a common key, the animal number (Figure 2). If the duration of the experiment is different from the duration of the entire study, a separate file containing the dates of the experiment is required. This allows software, used to report study-related data for an individual experiment, to select a finite segment of a much larger data set. This approach helps both the data base management and data validation processes by eliminating redundant data sets.

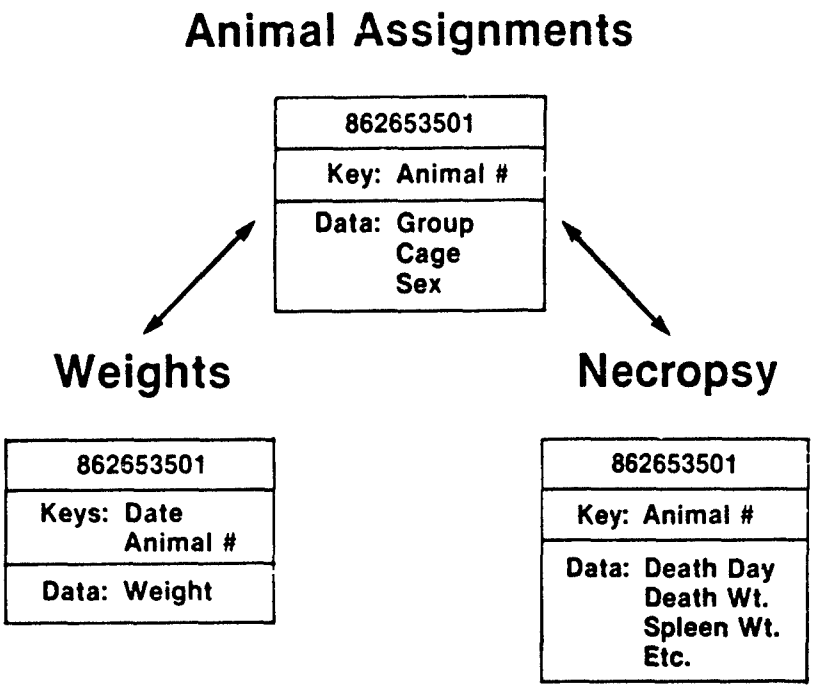


Figure 2. The relational data base format used at the experimental level (see Figure 1) is shown for animal data.

When considering commercial data base management packages, careful attention must be paid not only to their implementation but also to data analysis and reporting capabilities supported within the package. If none exist, or if the ones that do exist are inadequate, then special consideration must be given to communication (data transfer) between the data base and other software packages (e.g., RS/1) designed to handle these functions (16). We made use of the RS/1 research system for this data base application. Within RS/1, data are stored in a hierarchical model, which lends itself very well to separating the different studies and experiments. It also has many graphic and statistical capabilities. RS/1 does not, however, have relational data base features, but it stores its data in a tabular format (Figure 3). In addition to its data analysis capabilities, the package contains a programming language that can be used to extend the basic functions of the package. Because the data were stored in tabular form, the RS/1 language facility was used to implement a simple relational data base within the RS/1 package. The point of this example is to show that the organization of data and the selection of a data base package has a direct impact upon the amount of work required to achieve a functional data base.

2500 PPB
Exposure Dates
Arsine, Study # 8626535

0 Date	1 Daily CxT	2 Length of Exposure	3 Mean Conc. (PPM)	4 No. Obs. (N)	5 Std. Error
1 22-SEP-86	14.952988	6.059444	2.501615	52	0.015592
2 23-SEP-86	14.867825	6.094722	2.478700	50	0.008067
3 24-SEP-86	14.716895	6.088333	2.455231	52	0.009816
4 25-SEP-86	15.239265	6.107778	2.535904	52	0.013227
5 26-SEP-86	14.097750	6.152500	2.343104	48	0.050352
6 29-SEP-86	15.217643	6.076389	2.547135	52	0.013685
7 30-SEP-86	14.955986	6.055278	2.507353	51	0.012453
8 01-OCT-86	15.446678	6.056389	2.583980	50	0.008188
9 02-OCT-86	15.471326	6.125833	2.565880	50	0.020810
10 03-OCT-86	15.161790	6.132500	2.514184	49	0.023696
11 06-OCT-86	15.258127	6.205556	2.545646	48	0.021282
12 07-OCT-86	14.994331	6.175000	2.503811	53	0.009498
13 08-OCT-86	14.795588	6.172222	2.469680	50	0.007502
14 09-OCT-86	14.848971	6.135278	2.460137	51	0.022234
15 10-OCT-86	14.813007	5.929167	2.517860	50	0.008504

0 Date	6 Highest Conc.	7 Lowest Conc.
1 22-SEP-86	2.796	2.125
2 23-SEP-86	2.584	2.241
3 24-SEP-86	2.617	2.322
4 25-SEP-86	2.741	2.136
5 26-SEP-86	2.648	1.356
6 29-SEP-86	2.825	2.369
7 30-SEP-86	2.652	2.172
8 01-OCT-86	2.696	2.438
9 02-OCT-86	2.710	1.626
10 03-OCT-86	2.838	1.570
11 06-OCT-86	3.015	2.338
12 07-OCT-86	2.578	2.125
13 08-OCT-86	2.554	2.245
14 09-OCT-86	2.577	1.472
15 10-OCT-86	2.645	2.272

Figure 3. RS/1 is a versatile tool for storing, analyzing, and reporting data. A sample data table is shown here.

SUMMARY

Data management, either automated or manual, involves a complex set of activities that are often underestimated. It is a key element in experimental design, requiring careful planning and organization. The quality of the research is always interpreted by the quality of the results. It is therefore essential that staff experienced in these activities play an integral role not only in the analysis and presentation of data, but also in the design of the research.

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QUESTION AND ANSWER SESSION

MR. DECKER (BATTELLE PACIFIC NORTHWEST LABORATORIES): You've mentioned quite a bit about validating the data, essentially the raw data as you are taking it. You haven't mentioned anything about validating the processing of that data. For example, the software, statistical packages, and what not. Would you care to comment on that?

MR. O'CONNOR: The subject you've asked about is commonly called software validation, which, at the very least, is a very extensive topic worthy of a presentation all on its own. Briefly, however, it's a process of defining the ability of a software package to perform the function for which it was designed. This is accomplished by providing a range of all possible inputs to the computer and comparing the output to a standard. The standard is often hand-computed results. The measure of acceptability is gauged both by the ability of the software to handle expected inputs and the ability of the software to gracefully handle unexpected data; that is, data which are missing or of some improper format. Software validation is a quality control function and should be performed at the time of acceptance testing, operating system changes, and periodically during the life of the software package.

DR. MOSS (BATTELLE PACIFIC NORTHWEST LABORATORY): My question was what are data in a toxicology experiment? We gave these elaborate systems for collecting data but when are data data? For instance, temperature monitoring in the chamber, that's data. When is the animal data? You put the animal on the scale, and the technicians are watching the animal, and he is jumping around. Maybe they push the button and it's not right – they saw him jump as they pushed. When is that declared data? Then toxicology is an observational science. Most of us involved in the engineering side of toxicology don't want to admit that, but all of our experiments to date normally have to go through the eyes of a Board-certified veterinary pathologist. When are his observations called data?

MR. O'CONNOR: By my interpretation any recorded observation is data. If the trouble was taken to record an observation then it should be maintained as part of the experimental record. It is a simple process to invalidate the data if the observation is not correct. It is not, however, possible to retrieve discarded data which at first might seem incorrect but might have proved at a later date to have a bearing on some facet of the research. With regard to the Board-certified veterinary pathologist, I understand that the rules with regard to recorded observations are different than they are for the toxicologist. I recognize that this disparity exists, but speaking strictly for myself, if I were required to defend my data I would feel much more comfortable knowing my data set was complete.

DR. MOSS: We normally run into pathologists who are trained to enter data directly from their microscopes into the computer, and they will not do that if the data they enter is considered final data – they want to go get a cup of coffee and think about it for a couple of hours, maybe a week, and then come back, look at it again, change it, and then sign off on it. I think that has been

accepted. When a pathologist enters data into a keyboard, it is not data until he does something else to it, which may be a month later.

MR. O'CONNOR: I recognize that this occurs but I don't necessarily agree with it unless the tissues the pathologist is reading are saved for future reference. Most toxicological data are very short-lived, however, and therefore first impressions become much more important. I suspect that is why pathologists are allowed the luxury of modifying their observations without the requirements for an audit trail.

DR. MOSS: I was hoping we could get a discussion going, but you are going to be leaving. I think you will find that pathologists will not work under that condition. There is a tox base data system which has been designed to accommodate the particular personalities of our friends the pathologists. They say it is not data until they sign off on it, so the system has to be able to allow for that. That's generally the accepted rule, that the one exception to the rule is a pathologist. They enter data into a computer system, and they have to do one additional thing, but that may be sometime down the road before it is declared data – they are allowed that. Otherwise they will not use the system.

MR. O'CONNOR: That's a good observation. The only point that I'm trying to make out of this whole discussion on data is that the principle investigator is the one who is ultimately responsible for defending his data. If he doesn't have all of the data available to him to make the decision, then you are putting him in a position in which he can't adequately defend that data.

DR. NEWTON (BIO/DYNAMICS, INC.): In answering his question about validation of software, you said something that the user should be doing. The U.S. Environmental Protection Agency (EPA) and U.S. Food and Drug Administration (FDA) are now developing criteria and are going to look at how you are doing that. I have been told by them that they don't feel that there is any laboratory in the country that could pass the criteria that they are going to be developing. But they want the documentation of the programs, they want the listings of the programs. If you have a commercial supplier of some piece of equipment with a microprocessor, he doesn't want to give you the source. So how are you going to work that problem? Then you have to verify it at some interval that they are going to prescribe. So I think we are just starting to see problems in this area.

MR. O'CONNOR: You have brought up two issues. The first involves the availability of source code for commercial products. The second involves the validation of software. Both EPA and FDA may be working on guidelines or even regulations concerning source code availability and documentation for software but I suspect this will apply only to software developed specifically for the government. If this is not the case I suspect some history-making legal battles are in the works. With respect to software validation it would be very difficult to comment on government requirements until I know what those requirements are.

INHALATION EXPOSURE TECHNOLOGY USED FOR VARYING EXPOSURE MODES AND PROFILES IN TOXICOLOGY STUDIES

Bernard Adkins, Jr., Ph.D

Northrop Services, Inc. – Environmental Sciences, P.O. Box 12313, Research Triangle Park, North Carolina 27709

SUMMARY

Current technology used in inhalation toxicology studies employs various exposure modes and concentration profiles. Inhalation exposure modes typically utilize whole-body techniques, whereas other exposure modes include nose- and head-only exposure systems and, in some cases, whole- or partial-lung exposure systems. The latter two conditions are utilized when safety considerations are warranted by the hazardous nature of the chemical or agent being tested, the test substance may be dermally adsorbed, and/or the costs of the chemical used are of concern. Inhalation exposure studies may span several minutes to 24 h of continuous exposure and from one day to the full life span of the animal being tested. Time-varying profile exposures, on the other hand, are typically used to mimic human exposures to chemical agents and, in some cases, to more accurately extrapolate animal toxicity data in assessing human risk. Automation of inhalation exposure systems has expedited the timely operation of both accurate and repeatable profiles, and it has allowed for reexamination of classical time-weighted average concentration information relating to human health concerns. The toxicological assessment of potential health effects resulting from exposure to airborne substances typically involves thorough characterizing of the test agent via acute, subchronic, and chronic toxicity testing.

INTRODUCTION

In the field of toxicology, the technology involved in conducting inhalation toxicology research and testing is very complex. This process has developed as researchers have continually strived to optimize the test subject's environment while minimizing stress and ultimately acquiring toxicological information that can be used to assess human health risks. The complexities of the discipline have also influenced the utility of the methodology in terms of cost and ease of operation, especially in regard to the technological differences associated with gas/vapor phase exposures compared to test systems involving aerosol/particulate materials.

Inhalation exposure systems are designed to maintain the test atmosphere in an impervious environment, which allows for the controlled dosing of test subjects via the respiratory system. The components in the system that are considered absolutely essential include a means of introducing and quantifying the test material in the enclosed environment, the containment of the test subject

during dosing, and the removal of the test agent via an exhaust/scrubbing system. The technology of the dosing process employed should minimize extraneous variables such as reactivity of the test agent with system components, channeling of the test agent within the exposure environment, stress to the test subject, and, where possible, extra-respiratory tract dosing of test agent.

This paper presents an overview – not a review – of the technology used in current inhalation toxicology testing and research applications. Discussions cover the various modes of exposure, ranging from whole-body systems to head- and nose-only applications and to partial-lung exposure systems. Also presented is a discussion of the use of time-varying exposure profiles with regard to toxicological concerns between treatment parameters, including concentration and exposure duration or time. Information is also provided on the experimental designs currently used in inhalation toxicology research and testing, including acute, subchronic, and chronic regimens. Guidelines for the proper conduct of inhalation toxicology studies are provided in terms of engineering technology discussions and experimental design considerations. More extensive discussions of the methodologies involved in conducting inhalation toxicology research and testing can be found in additional publications (1,2,3).

EXPOSURE MODES

An examination of the toxicology literature reveals numerous publications regarding various applications of exposure modes in toxicity testing and research efforts. Inhalation studies are conducted in systems ranging from very simple and inexpensive aquaria to expensive stainless steel and glass enclosures with sophisticated environmental control systems. Exposure operation may involve either static or, the more accepted and used, dynamic flow-through systems. Test subjects, which predominantly include a variety of laboratory animals, may be housed within the exposure chambers for the duration of the treatment period with external housing during non-exposure periods or, in some cases, housed in the chambers for the duration of their lifetimes. Treatment periods may span a few minutes or days or, under chronic exposures, periods up to the life span of the test subject. Ideal experimental conditions also minimize stress to the test subject so as to maximize the reliability of the toxicity data obtained.

Exposing laboratory subjects to airborne substances may involve the use of five possible treatment systems, including whole-body (immersion), head- and nose-only, and lung-only or partial-lung techniques. The whole-body or immersion exposure system, the most popular technique, is perhaps most frequently used because it presents fewer technical demands in setup time, is less expensive, and is less stressful to laboratory subjects during dosing. The advantages and disadvantages of whole-body exposure techniques have been reviewed in detail (4,5). Using this exposure mode, the investigator can work with large numbers and, in some cases, varieties of

laboratory test subjects; conduct acute, subchronic, and chronic toxicity studies with varying treatment duration and dosing levels (5); be efficient in regard to animal husbandry requirements and technical complexity of exposure operation; and be flexible in regard to the diverse experimental design requirements. Disadvantages of whole-body or immersion exposure systems may include whole-body contamination and dosing of test agent on test subjects; voluminous amounts of test agent required, especially for lifetime studies; elaborate air handling systems for conditioning input process air; expensive exposure chambers and associated supporting equipment; and treatment of effluent air so as to scrub all test agents prior to exhausting the contents of the exposure system into the atmosphere.

Figure 1 presents an idealized whole-body or immersion-type inhalation exposure profile. Exposure duration may span some time, t, from Time Zero to Time t_b, after which the exposure period is terminated in conjunction with the shut-off of input of test agent. Exposure duration may, as in the case with governmental inhalation toxicology studies, span the time between t_a and t_b. In this scheme, t_a may be defined as the T90 for the chamber, that time from when the test agent is "turned on" until the concentration reaches 90% of the nominal level in the exposure chamber atmosphere. It is noteworthy that t_a may also be defined as T99, the time when 99% of the nominal level of the chamber atmosphere is reached. The T99 definition of t_a is perhaps the most commonly used starting point in modern inhalation toxicology studies.

Two types of whole-body or immersion inhalation exposure systems are routinely described in the literature. Figures 2 and 3 present generalized flow diagrams for dynamic flow-through and static exposure systems, respectively. In Figure 2, the elaborate heating, ventilation, and air conditioning (HVAC) system required for maintaining the flow-through quality of the process air is shown. In this system, air is pulled into the process air taken in from the environment, scrubbed through a roughing filter to remove large particulate materials, chilled to reduce the dew point temperature prior to further filtration scrubbing through a high efficiency particulate absolute (HEPA) filter, scrubbed of organic contaminants via a charcoal filter, and rehumidified and reheated to slightly less than ambient laboratory temperatures. The test agent is usually introduced into the process air stream at this point and then mixed via some mechanical mixing process prior to introduction into the inhalation exposure chamber. Test subjects are normally arranged in open-mesh stainless steel exposure cages, with or without excreta collecting pans, and arranged in rows, columns, and/or tiers within the chambers. The test agent-laden process air is then exhausted from the exposure chamber through a HEPA filter, charcoal filter, or suitable scrubbing device, and diluted with laboratory and/or building exhaust air prior to exhausting into the environment. Various parameters are routinely monitored during this process, including an operational check on the transmural pressure across the HEPA filter, and dew point temperature of the conditioned and

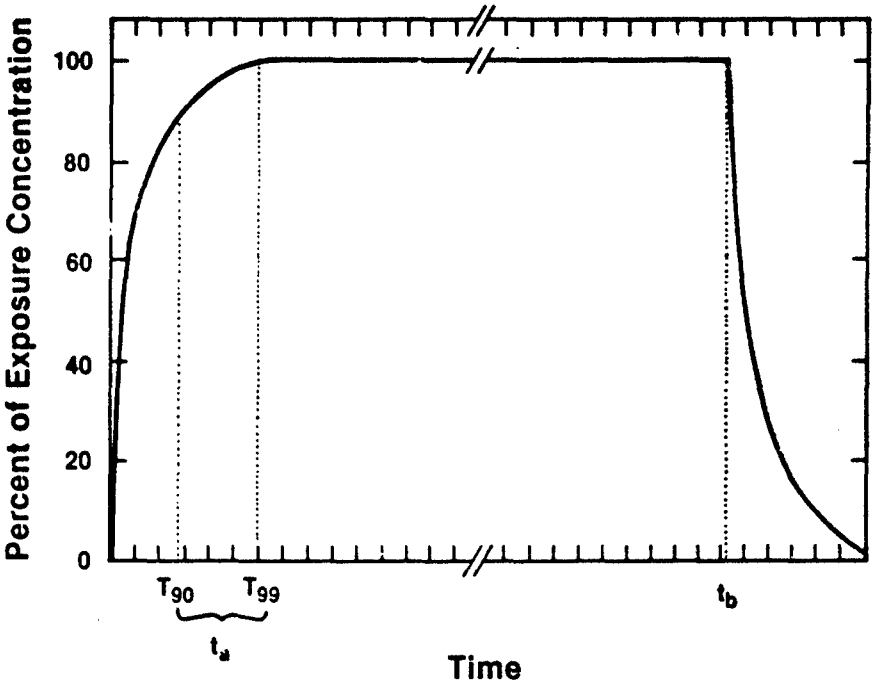


Figure 1. Idealized whole-body or immersion-type inhalation exposure profile. In this representation, exposures may span minutes to hours, t_a to t_b, starting at time zero, or in many cases, at T90, or in few cases, at T99.

barometric pressure. Some systems may scrub the process air of interfering chemical contaminants that would affect the quality of the test agent quantification process.

Most critical to the conduct of any whole-body or immersion-type inhalation exposure study is the monitoring of parameters in the breathing zone of the test subjects. In Figure 2, the dew point and ambient temperature, chamber static or differential pressure, chamber airflow, and chemical concentration, or mass in the case of particulate exposures, are routinely measured with microprocessor-based instrumentation. Many of these systems have communication interfaces for external data acquisitions. In the chamber exhaust air, a check on the efficiency of the HEPA filter operation is monitored via differential pressure across the filter. Also, scrubber efficiency can be determined by monitoring the effluent air downstream of the charcoal and/or scrubbing device.

Figure 3 presents a flow diagram of a typical static or closed inhalation exposure system used for whole-body or immersion toxicity studies. This system is most popular in pharmacokinetic and/or toxicokinetic study applications (6,7) and is also used in experimental designs to test expensive and/or hazardous test agents. In the static system, process air is routinely supplied in bottled form under high pressure and, in most cases, is dried (via a water scrubber) and scrubbed of CO_2 prior to mixing with the test agent. An important and unique aspect of the static or closed exposure system is the requirement for O_2 input, which is naturally depleted during normal respiration of the test

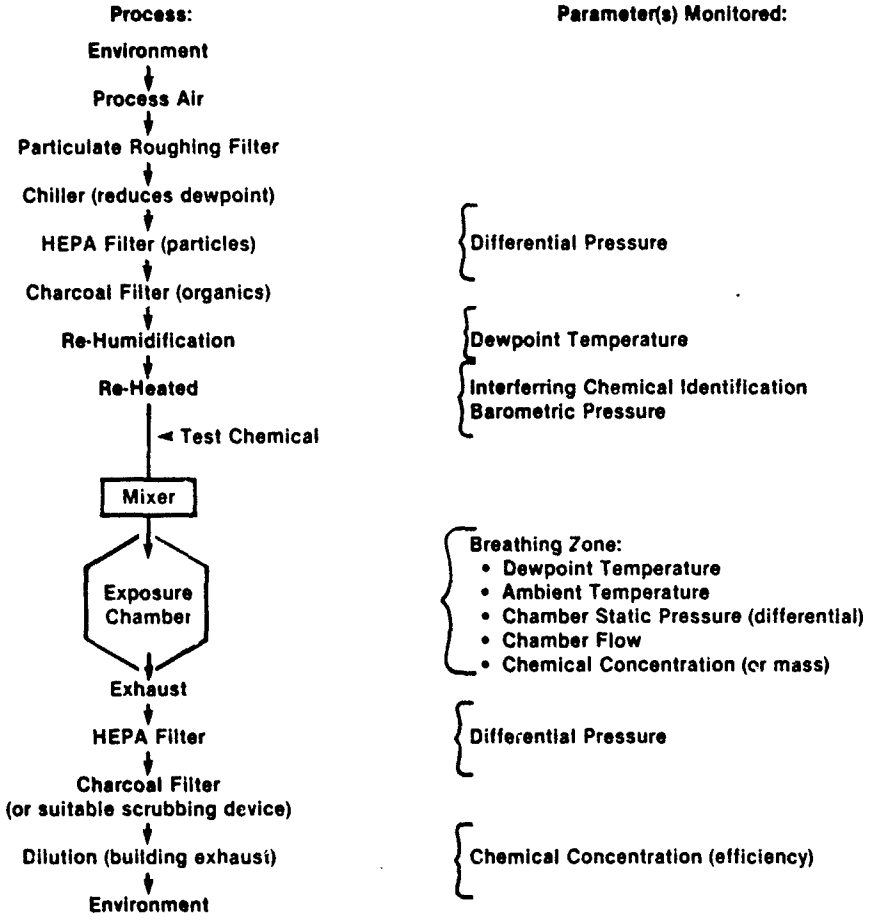


Figure 2. Typical dynamic flow-through inhalation exposure system. The flow of process air through the system is shown in the left panel, while the parameters typically monitored during this process are shown in the right panel.

subjects. In the exposure chamber, which may consist of simple vessels such as aquaria or pickle jars, test subjects may or may not be restrained or housed in the wire-mesh exposure containers used in dynamic exposure systems. Process air laden with test substance is normally recirculated in the static or closed system as shown by the recirculation arrows. At the termination of the exposure, the entire content of the closed system is exhausted through HEPA and charcoal filters and diluted with laboratory or building process air prior to exhausting into the environment. Parameters routinely monitored in the static or closed inhalation exposure system include O_2 concentration via a feedback control system, differential pressure for ensuring the nonpressurization of the closed exposure apparatus, ambient and dew point temperature, and chemical concentration via a suitable monitor for quanitifying the test agent. HEPA and charcoal filter operating efficiencies can be similarly monitored in the dynamic exposure system via a differential pressure measurement across the filter and via a chemical concentration determination downstream of the filters/scrubbers, respectively. It

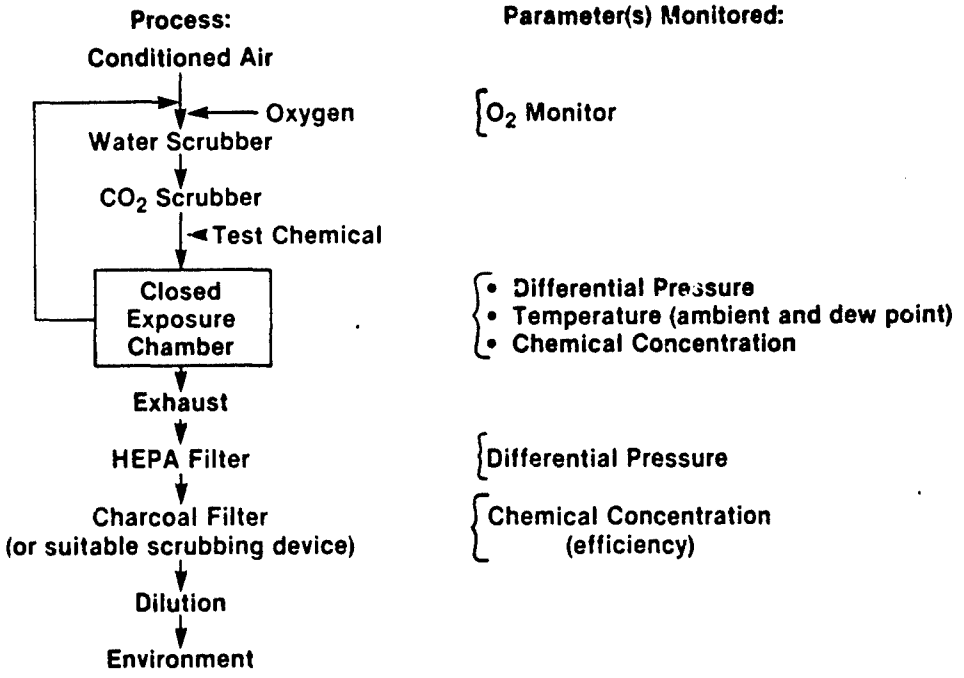


Figure 3. Typical static or closed inhalation exposure system. The flow of process air is also given in the left panel with corresponding parameters monitored in the right panel.

is noteworthy that static or closed inhalation exposure systems are not practical for use in particulate exposures or, in many cases, routine testing applications.

Examples of both dynamic and static whole-body (immersion) inhalation exposure systems are thoroughly discussed in the literature (2,5,8-11). Figure 4 depicts an example of a whole-body or immersion-type inhalation exposure system.

The second and third types of exposure modes for the dosing of laboratory subjects to airborne test agents are head- and nose-only exposure systems. Because these two systems are so similar in design and, in many cases, application, both will be discussed together in this effort. Both systems have applications that make them ideal for use with hazardous and/or radioactive test agents, and, in some cases, for routine testing of substances that do not have these unique characteristics. Whole-body contamination by the test substance, a major problem with whole-body exposure modes, can be collectively reduced or eliminated by using head- or nose-only exposure systems. These systems tend to operate much more efficiently than whole-body systems; they may utilize fewer animals and require less test agent for equivalent dosing because the internal volume of the exposure system is reduced. It is noteworthy that partial oral dosing of test subjects can occur with head-only systems, which is not the case with properly designed nose-only systems.

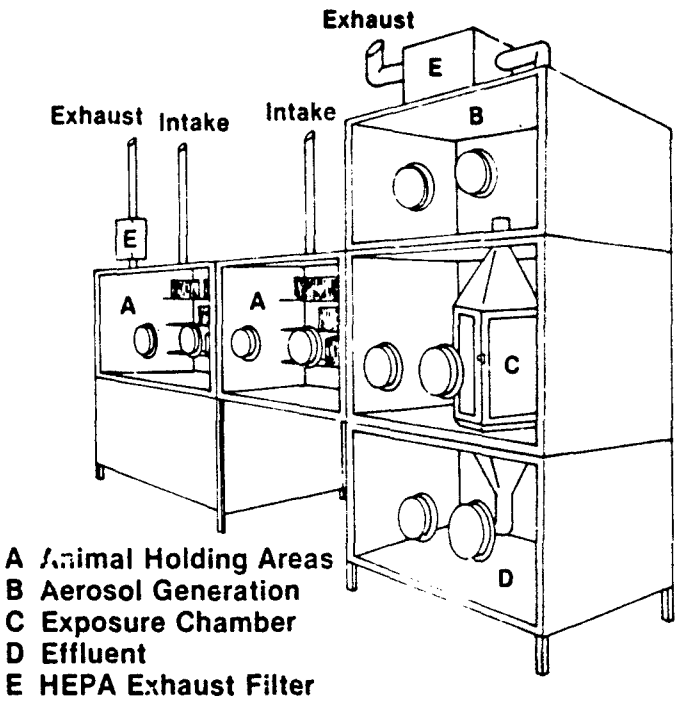


Figure 4. Representative whole-body or immersion-type inhalation exposure system used for hazardous test substances. The legend provided describes the various components of this exposure and containment system.

The disadvantage of head- and nose-only exposure systems is their technical complexity. Obviously, these specialized exposure systems tend to be more labor-intensive and require a much more technically complex design than commercially available whole-body systems. From a physiological standpoint, the use of these systems appears to cause stress in the laboratory subjects, which must be considered in regard to the effect on experimental design and on the parameters under investigation. One report (12), however, based upon an extensive evaluation of clinical/physiological parameters in both restrained and unrestrained animals, indicates that the nose-only exposure system does not cause stress in the animals. More extensive descriptions of head-only exposure systems (3-5,13,14) and nose-only exposure systems (3-5,15-18) have been described in the literature.

The last exposure modes to be discussed in this overview are the very specialized systems used for lung-only and partial-lung exposures. These systems, which have been reviewed (3,4), will also be discussed together because of their similarities. Neither system lends itself to large-scale testing or screening of routine substances, but both are used almost exclusively in research applications (e.g., metabolism studies) in which specific dosing of components of the respiratory tract by airborne

chemicals is required. These systems are very sophisticated and technically complex. These systems are also similar to head- and nose-only exposure systems when hazardous and/or radioactive substances are used. Lung-only and partial-lung exposure systems are also, from an engineering standpoint, the most efficiently operated inhalation exposure systems, and they allow for fractional dosing of components of the respiratory tract by the test substance. With partial-lung exposure systems, an additional advantage, which may not be possible with any of the other systems described, is the ability to precisely dose specific areas of the respiratory system. Also, control tissue can be obtained from the same test subject, thus eliminating any experimental variability between treatment and control comparisons when different test subjects are used.

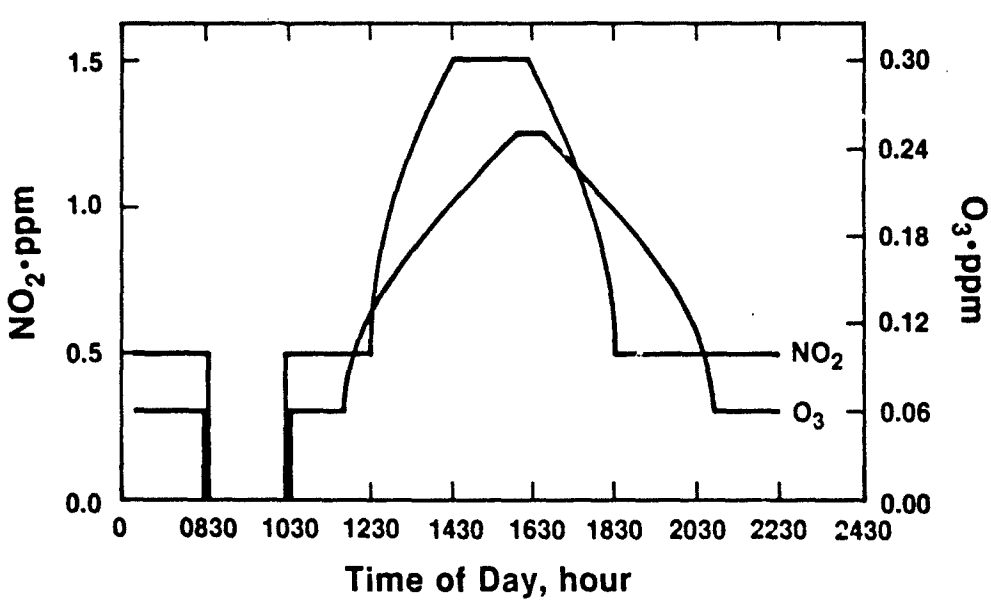
The disadvantages of these specialized exposure modes are more numerous than those for whole-body and head- and nose-only systems and must be considered in context to their research applications. Both lung-only and partial-lung exposure systems require the use of appropriate anesthetic and surgical procedures in test subjects for proper application of cannulation, intubation, and/or dosing devices. Both systems are extremely complicated to operate because, in many cases, physiological monitoring of test subjects must be performed in conjunction with dosing of the test agent. Stress exerted on test subjects must also be considered when interpreting test data. Another fact often overlooked with the interpretation of lung-only exposure data, especially in the case of regional deposition of test agents, is that the normal scrubbing of larger airborne particles and aerosols in the upper respiratory tract is eliminated with this type of exposure. When the data are being used in regulatory decision-making, it is particularly important to consider this latter factor.

EXPOSURE PROFILES

Time-varying exposure profiles represent a special case of inhalation toxicology study designs. Their applications involve experimental designs that allow for more appropriate simulation of environmental and/or occupational exposure conditions, since many real world exposures to airborne substances rarely mimic steady-state conditions. Exposure profiles typically include low-level treatment of test agents superimposed by spikes or excursions at higher treatment levels (19,20). The product of concentration times time, or CT, can be held constant by varying the various components in the exposure regimen without superimposing spikes or excursions on a basal treatment level (21,22). Exposure profiles allow for better examination of the relationship between CT and toxicity of test agents. In pharmacological applications, time-varying profile treatment with chemotherapeutic substances, even with the same CT, can give profound differences in response. These latter examples of time-varying exposure profiles can also be categorized as studies designed to test differences between intermittent and continuous exposures. Several reports have made these comparisons with environmental chemicals (23-25). Time-varying exposure profiles also

provide more data for risk assessment of test agents in relation to potential health risks of airborne substances. Finally, microprocessors and/or less sophisticated machine-controlled systems interfaced with inhalation exposure systems have provided a more reproducible profile for chronic time-varying profile exposures.

Current studies with regulatory implications, such as that designed to investigate chronic real-world exposure regimens of O_3 and NO_2 toxicity in laboratory rats (26), mimic so-called worst case environmental conditions for the chemical agents being tested. This study is designed to investigate the time course of development of chronic respiratory disease associated with these test agents. Figure 5 summarizes the experimental exposure profile design used in a study conducted for the U.S. Environmental Protection Agency, in which the NO_2 background exposure is maintained at 0.5 ppm for 16 h with a 6-h progressive spike to 1.5 ppm during the afternoon of each normal weekday. Weekend exposures in this effort are designed for continuous exposure to 0.5 ppm NO_2. The O_3 exposures in Figure 5 consist of 0.06 ppm for 13 h and a progressive excursion to 0.25 ppm over a 9-h period. In both curves, the 2-h valley in the curves shown between 0830 and 1030 h represents the daily animal care and chamber maintenance periods.



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Figure 5. Exposure profile used for chronic O_3 and NO_2 inhalation toxicity exposures. Both NO_2, left ordinate, and O_3, right ordinate, are graphically presented versus time for a 24-h period.

EXPERIMENTAL DESIGNS FOR INHALATION TOXICOLOGY STUDIES

Current inhalation toxicology studies, whether conducted for research or testing applications, or for academic or governmental regulatory reasons, typically include acute, subchronic, and chronic exposure regimens. These studies have over the last few decades evolved with similar experimental designs, regardless of their origin (i.e., academia, industry, or government). Because the experimental designs of government studies tend to set the stage for all studies that follow, a discussion of the salient features of these studies is presented below. More detailed discussions may also be found elsewhere (27,28).

Acute Inhalation Toxicity Studies

Table 1 presents an overview of the major considerations involved in typical acute inhalation toxicity studies. These studies are designed to provide critical information on the acute toxicity of the test agents to be tested, the maximum tolerated concentration (MTC), and the LC_{50} of the agents used, and to define dose ranges for additional toxicity studies that may be conducted, including both subchronic and chronic studies. Variations in the use of two sexes and species of laboratory subjects, as well as the number of dose levels investigated, may be found in experimental designs. The number and variety of animals used in control groups is usually dictated by the nature of the test agent used. Some consideration must be given to whether the study is conducted with the test subjects housed individually or in groups; the former condition being preferred. The treatment period usually spans a single treatment interval, indicated as starting from Time Zero, or T90, and is, in most cases, followed by a 14-day observation period. Other specialized acute inhalation toxicity studies may, however, be conducted with treatment periods that last a few minutes or that continue nonstop for the entire 14-day observation period. Normal animal husbandry procedures are followed during nonexposure periods with food and water provided *ad libitum*. Complete necropsies and histopathological examinations of tissues are typically performed during the study on all interim animals that do not survive and on all remaining animals after the 14-day observation period.

Table 2 presents an overview of a second type of acute inhalation study used in modern toxicity testing and research operations. Repeated-dose toxicity studies, including those studies conducted for less than one month, are extremely important to dose-range determinations for subchronic and chronic toxicity studies. Repeated-dose studies are usually performed with two sexes and species of test subjects and five treatment levels of test agents. Corresponding control animals are incorporated into the experimental design, including both chamber or sham control animals as well as untreated control animals. Treatment periods typically span 6 h/day (from T90) for 10 working days with 2 weekends as nonexposure periods interspersed in the study. Or they may

TABLE 1

ACUTE INHALATION TOXICITY STUDIES

Experimental design
- 5 animals/2 sexes/2 species/5 dose levels
- Various control animal groups
- Individual versus group housing
- Inhalation period: 4-6 h (+ T_{90})
- Animal observation periods: 14 days posttreatment (food/water – *ad libitum*)
- Complete necropsy/histopathology

Dose selection for prechronic/chronic toxicity studies

Other considerations
- Duration: minutes → hours → days
- Chemical dose
  - ☐ Lethality range finding (MTC)
  - ☐ LC_{50} determination

TABLE 2

REPEATED-DOSE ACUTE INHALATION TOXICITY STUDIES

Repeated-dose experimental design
- 5-20 animals/2 sexes/2 species/5 treatment levels
- Control animals
- Treatment period: 6 h/day (+ T_{90}); 10 days + 2 weekends; 14 days; 20 days + 4 weekends
- Individual versus group housing
- Animal observation period: 2 x daily for 14-day treatment period (food/water: *ad libitum* during nonexposure period)
- Complete or partial (target organ) necropsy and corresponding histopathology
- Dose selection for prechronic (90-day) and/or chronic toxicity studies

span 14 days of continuous treatment and, in some cases, last for 20 days, with 4 nonexposure weekend periods interspersed throughout the study. Again, the issue of individual versus group housing of animals during treatment is of concern, with the former condition being preferred. Animal observations must be performed twice daily, with food and water provided *ad libitum* during nonexposure periods. Both partial and complete necropsies of test subjects with corresponding histopathological examinations of tissues vary depending upon whether target organ determinations have been made from other acute toxicity studies and whether additional subchronic or chronic studies are to be conducted with the test agent.

Subchronic Inhalation Toxicity Studies

Inhalation toxicity studies conducted for treatment periods ranging from 1 to 12 months in duration are normally referred to as subchronic toxicity studies. As is summarized in Table 3, the most popular experimental design used currently is the 13-week or 90-day study, in which two sexes and species of test subjects as well as corresponding control animals are treated with up to five different doses of the test agent. Sentinel animals, the animals used to monitor the health status of the entire colony, are incorporated into the study and serve as additional controls. The treatment period most often used is 6 h/day (from T90) for 5 days/week for the duration of the 13-week period. Animals are also observed twice daily, with food and water provided *ad libitum* during nonexposure periods. In studies utilizing the Hazleton 2000 style chamber (Lab Products Company, Aberdeen, MD), originally developed and described by O.R. Moss (29), food and water may be provided continuously because the test subjects are housed in these chambers during exposure and nonexposure periods. Complete necropsies of all test and control subjects and corresponding histopathological examinations of tissues are the standard of performance for subchronic studies.

TABLE 3

SUBCHRONIC TOXICITY STUDIES

13-week (90 day) experimental design

- 10-20 animals/2 sexes/2 species/5 dose levels
- Control animals (sentinel animals)
- Individual versus group housing
- Treatment period: 6 h/day (+ T_{90}) – 5 days/week
- Animal observation period: 2 x daily (food/water: *ad libitum* during nonexposure period)
- Complete necropsy/histopathology
- Dose selection for chronic toxicity studies

Chronic Carcinogenicity Studies

Toxicity studies conducted for periods longer than one year and for periods approaching the life span of the test subject are typically referred to as chronic toxicity studies. As summarized in Table 4, these studies can accomplish several goals; namely, the determination of chronic toxicity and carcinogenic potential of the test agent. Two governmental guidelines (27, 28) recommend the use of two sexes/species of test subjects and three treatment levels with corresponding control animals. Sentinel animals are extremely important in chronic toxicity studies because some subclinical diseases in laboratory species can go undetected (e.g., Sendai virus) (30), thus influencing the histopathological assessment of test agent treatment. The preferred experimental design uses individual housing of test subjects, rather than multiple or group housing as was used in earlier

chronic toxicity and carcinogenicity studies. Treatment periods vary considerably, from the routine 6 h/day to 23 h/day (using the Hazleton 2000 style chamber) and may span either 5 or 7 days of treatment and be conducted for 52 to 104 weeks. Animal observations are made twice daily, with food and water provided *ad libitum* during exposure and nonexposure periods. Complete necropsy of test subjects and histopathological examination of tissues are also extremely important for assessing both the chronic toxicity and carcinogenic potential of test agents.

TABLE 4

CHRONIC CARCINOGENICITY STUDIES

Experimental design

- Determines chronic toxicity and carcinogenicity potential of test agent
- 20-60 animals/2 sexes/2 species/3 dose levels
- Control animals
- Sentinel animals
- Individual versus group housing
- Treatment period: 6-23 h/day (+ T_{90}); 5-7 day/week; 52-104 weeks
- Animal observation period: 2 x daily (food/water: *ad libitum* during exposure and nonexposure periods)
- Complete necropsy/histopathology

DISCUSSION

The precise treatment of test subjects with airborne substances is a complex undertaking. Inhalation exposure chambers may span design configurations that are very simple (17) to very complex (29), with or without elaborate support equipment, to accomplish whole-body to partial-lung dosing of test substances. Various dosing regimens, referred to as time-varying exposure profiles, similarly span intermittent/continuous exposure considerations to complex profiles designed to mimic actual human exposures to environmental or occupational substances. All of these facets of the discipline of inhalation toxicology fit together in a variety of toxicity evaluations, ranging from acute to subchronic and chronic treatments, directed toward making assessments important to human health.

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QUESTION AND ANSWER SESSION

DR. GARDNER (NORTHROP SERVICES, INC.): It might be useful, Dr. Adkins, if you would give us some idea of what a complete carcinogenicity test would cost.

DR. ADKINS (NORTHROP SERVICES, INC.): That's a difficult question to answer. Studies conducted in government facilities would typically cost $100,000 to $200,000 a year. To support such a study in a nongovernmental facility the cost may escalate to as high as one-half million dollars a year. Again, a lot of the cost factors are the sophistication of the analytical chemistry requirements for the test agent that you are working with, any special redundancies in the system that you need to have to comply with governmental regulations, and things of that nature.

DR. CARPENTER (NORTHROP SERVICES, INC.): You should also point out that the total cost of a study like that, no matter where you do it, is highly dependent on the type of end points you are looking at. If you go beyond the basic histopathology into more sophisticated functional testing the costs rapidly go up.

DR. FURST (UNIVERSITY OF SAN FRANCISCO): How do you deal with the fact that when you put the animals in a restrainer they weigh less than the cage controls? Is that because they eat less and because they eat at a different time? Furthermore, they usually live longer than the cage controls. How do you interpret that at the end of your experiment?

DR. ADKINS: We match our sham control animals to the same housing and/or treatment conditions as the treated animals. Factors such as food/water deprivation and stress of containment may contribute to the weight reductions observed. Results from chronic studies that we have conducted indicate that treated animals do, in some cases, live longer than control animals. We feel that this phenomenon may be the result of the stress of the treatment on these animals which is obviously absent in the control animals. Control animals receive essentially no stress even though they may be going in and out of chambers. This is not that same magnitude of stress that you would have in treated animals.

PANEL DISCUSSION IV

Owen Moss, Ph.D. – Rapporteur

Battelle Pacific Northwest Laboratories

DR. MOSS: I would like to thank the speakers this afternoon for their presentations. We heard Dr. Adkins speak to us about the experimental equipment and the experimental procedures for controlling the time of exposure, and the importance of that in the basic thought of inhalation experiments. Mr. Hiteshew talked about the generation equipment for aerosol from gases, the techniques that they are using, and the considerations in producing the atmospheres for the animals. Mr. O'Connor talked about data management and the real challenge represented by the flood of data that comes out of one of these studies. Dr. Carpenter just finished talking about the Thomas Domes and some thoughts on how to calibrate a large chamber.

I have questions for each of the speakers. What I would like to do first is talk to Bernie Adkins. At the end of your presentation, you talk about chronic studies, and one of the questions that came to mind is the length of the study. Now a Fischer 344 rat lives 2 1/2 to 3 years. You hear people proposing that instead of a two-year study for Fischer 344 rats, you would gain an awful lot by exposing them for another six months. In your reading and thinking, what do you really gain by exposing those animals for another six months?

DR. ADKINS (NORTHROP SERVICES, INC.): Let me first of all respond to that by saying that I am not an advocate of lifetime exposure studies. In conversations with pathologists we have found that when you compare a lifetime study, versus a two-year study, the expense of the operation versus the higher frequency of tumors and so forth, you gain very little by doing the lifetime exposure.

DR. YANG (NIEHS): I just want to make a comment about this particular question. I want to bring to the attention of the group that Lovelace had conducted diesel studies, which were really a confirmation of other studies all over the world. At 24 months they saw nothing, and yet at 36 months significant toxicological effects of diesel fuel exhaust became evident.

DR. MOSS: Next we have discussions on aerosol generation and gas generation. I have a question regarding aerosol generation, especially of the nebulizer system described. In terms of the sensitivity of the particle size distribution, you run the risk of changing your particle size distribution with that system while still maintaining the mass, and it may not be very much, but you still run the risk. So there are two points that might influence the quality of your experiment. Bob Carpenter just talked about one in terms of the change of the spatial distribution in the chambers because of different particle sizes. The other has to do with your feedback system for optically monitoring the aerosol. Some of the light-scattering devices have windows where they get a fairly flat plateau for a range of

particle sizes, and then the light scatter or the signal drops off on either end. How do you propose to account for that?

MR. HITESHEW (NORTHROP SERVICES, INC.): You're right, Owen, about the advantages of having an automated generation system in that case. Although real-time measurement is desirable, there has yet to be an instrument developed that will provide all characterizations of different size particles. As far as that particular system, it was put in to allow for changes in nebulizer output without having to change the flow rate. Now I believe that an automatic control system, where one might be running overnight, would be better if you could slightly pulse your generator. This would keep the concentration of the solution constant rather than changing the dynamic mixing characteristics of the chambers. But as far as feedback control, typically we use an optical particle monitor (RAM) for a real-time indicator. We do quantitate by other methods, but we work in a very narrow window as far as concentration ranges. I don't think you are going to see that much of a shift in particle size from what we are talking about.

DR. MOSS: You also talked about reactive aerosols. Bob Carpenter reported on an experiment in which the aerosol reacted with the animals. There was a big change in the distribution in the chamber. That's a subject that is really very old. It goes back to the 1940s when Silver was doing research, at Edgewood Arsenal, in terms of aerosols or materials that react with the surface of animals. We have not solved that problem yet except in individual cases. I call inhalation toxicology high-technology toxicology, yet we are great as long as the chamber is empty. Then we put the animals in, and the test article concentration is a function of how the animal surface area reacts to the compound of interest.

MR. HITESHEW: I want to clarify something. Our distribution work was done with a very reactive gas. Even after the chamber had been freshly cleaned the chamber surfaces had to equilibrate. I'm not sure what the mechanism might be, but rise times and fall times in the chambers increase, even though you are putting in the same amount of gas. I wanted to clarify that it was a gas I was discussing and not an aerosol.

DR. MOSS: Well, a lot of the early research was with gases. In fact, one of the numbers that we often use was based upon a gas experiment. That's the recommended ratio of the volume of the chamber to the volume of animals. Do you know what that percentage is?

DR. CRAIG (BATTELLE COLUMBUS LABORATORIES): Five percent.

DR. MOSS: That experiment was done by Silver in 1947 – he came up with that recommendation. That was based on the reaction of animal fur to a war gas; and the chamber was a very small chamber, a 356-liter chamber. There were 38 air changes per hour, and what he found was that if he had over 5% volume of animals in his chamber, he had a significant drop in the concentration of the

aerosol as it went from the inlet line to the outlet line. So that's where that recommendation came from. It has nothing to do with temperature of animals, it has to do with an experiment done in 1947 trying to solve the problem that we have talked about twice today – the reaction of animal surface area to the compound. It's a function of how reactive the compound is.

DR. CARPENTER: Owen, maybe you are making a good argument for occasionally going back and trying to reinvent the wheel. I think there are a number of cases in which you see conventional wisdom enshrouded in mystery. When you try to find out what the bases for those paradigms are, you in fact discover that it doesn't have anything to do with the work you are doing.

DR. MOSS: We'll never be allowed to repeat that experiment because that was part two of a two-part experiment, and part one was with human subjects. Let me read it. Clothed and naked men were exposed to various established concentrations of a dichloroethyl sulfide vapor in a 20,000-liter chamber operated at an airflow of 3600 liters per minute (about 10 air changes per hour). The entry of the men into the chamber caused an almost immediate drop in concentration, which then remained for the duration of the exposure. At this new lower level the concentration drop was much more pronounced with clothed men than with naked men because of the difference in the nature and quantity of the added surface. Then he presents the data. We will never be allowed to repeat that experiment, and I don't know whether it needs to be or not. It's a classic example.

I had a question for Bob Carpenter. A lot of work goes into calibrating a chamber, and you ask why do we have to spend so much time? Twice during his talk Bob talked about trying to find out the minimum number of sample points. If you are a pure fluid dynamist you would say two is all you need. Let me present the assumptions that people use. Silver based his chamber operation assuming a perfectly mixing box or a stirred, closed-volume reactor. Now there are four assumptions in operating a stirred, closed-volume reactor which we assume but very seldom talk about. First, we assume that there are no leaks in or out of the reactor. Two, we assume there is no reaction of the material that you are putting into the reactor with the walls or the reactor or whatever is inside. We assume that the reactor, to get the equations that we use, is thoroughly mixed (some kind of fan blade or something that is really mixing). You put one atom in and it is thoroughly distributed throughout the reactor, which is impossible, but that is what you assume. Then you assume that the material coming into the reactor is thoroughly mixed. In other words, you don't have a high concentration coming down the tube on the left side, and on the right side, clean air. With those four assumptions then you can develop the equations for that reactor and use them. But if you have a perfectly thorough reactor then you only need to measure the inlet and the outlet. So why don't you shoot down those assumptions? Which ones do you think really failed?

DR. CARPENTER: Well, I don't know that you are going to shoot them down, but you are going to modify them somewhat. First of all, you know quite well that these chambers leak, and if they don't before you run them through the cage washer for six months, they will afterward. So you do have to deal with leaks. You do have to deal with imperfect mixing. Recently I found a paper by Chalette and Cloutier. They provided additional models for mixing vessels; that second plot that I showed was an application of his models. Basically, that straight line plot should go through 1.0 for a perfectly mixed closed reactor or tank. If the intercept moves to the right, you will also find slope changes. And the reciprocal of the slope, or that intercept, gives you the measure of the unmixed volume. This has been called dead volume. In addition to that, you find that if you have a situation in which you have a partially mixed reactor and some plug displacement, the slope will also change. You can find situations in which you are effectively short-circuiting the inlet to the outlet. In other words, imagine that instead of our exposure chambers you had a 55-gallon drum with a mixing paddle in it. You were pouring liquid in the top and taking some out of a hole on the side. If part of the liquid that you poured on the top of the contents of your 55-gallon drum simply ran across the surface and exited, then you would get a nonlinear curve. Applying those concepts to our exposure systems will provide a handy way to make these analyses. We know that the old Rochester-type chambers or Hinners-type chambers with the tangential entry would show a short-circuiting from the inlet to the outlet before the contents mixed.

DR. MOSS: That's leading up to my next question. First, let me comment on the leaks. At Battelle, in developing these chambers and the computer-controlled systems for two-year exposures, we had to address the problem of leaks. One of the most difficult things to do in this high-technology field is to close the door to your chamber every day. Food gets stuck in it as well as in the watering tubes, the gasket doesn't get squished down properly, and you have a leak. Since you run your chamber at a slightly negative pressure, that's clean air coming in, and the animals will find it. They are guaranteed to. So we had to do a flow balance. The air coming in is equal to the air being sucked out, and if it's not, then you have a leak. You alarm automatically and the alarm doesn't go off until it's fixed. Technicians get upset, but they learn how to close the door. When we first initiated that system we had not realized how poor we were at closing doors. That is something that those who have chambers should really think about. Don't assume you know how to close a door. You were talking about streaming through chambers, and you did your experiment with a light gas. The equations that you have talked about really are designed for fluids, liquids. Do you have mixing in the streamlines of the liquid so that it's all uniform and everything is mixed? They don't assume that there is a very high diffusion, but the gas you used, I would guess, would diffuse maybe one centimeter a second, so if you had a gradient, it could, in about a minute and a half, diffuse anywhere in the chamber. Assuming that you have a straight line from where it comes into that

ring, then the farthest distance is about a meter in any direction before it runs into a source – that it has only a meter or so to travel. That's a thousand seconds. How many minutes is that? Sixteen minutes. You had a half-time in your chamber of about 35 minutes. Two air changes per hour. So, if you were streaming and had dead zones in your chamber with the high gas diffusion coefficient, you may not see the dead zones. What I would do is take the heavy aerosol and put it in the chamber and measure the inlet and the exhaust and see if you get the same results with your light gas. If they are the same, you are done. If not, you will be back here for the next four years telling us. What's your reaction to that?

DR. CARPENTER: Yes, you are right. In fact, this study, although I call it a distribution study, is as much a study in analytical method analysis as it is in distribution. We deliberately chose the gas that way. We chose a gas that would give us the best diffusion that we could get. The purpose in doing this was to see how good a set of measurements we could get. This was our baseline. We will go ahead and do it again with a gas that has half the diffusion coefficient, therefore half the mobility, and I suspect that we will begin to see the effects you are talking about. And yes, you are right, we should look at the inlet and outlet – and we will. What I really hope to get out of these two sets of data is some indication of how many points you have to measure if you want to learn about the distribution in that chamber when you start using aerosols. I don't have analyzers that can respond and give me an answer in a minute or minute and a half. So the aerosol studies will have to involve a lot fewer points. These considerations also affect the experimental regimens when it comes to inhalation toxicology. To date I have not been happy with just simply picking a whole set of points and going in and measuring them because I always have this feeling that if I measure twice as many points I would have a different number. I know if I measure half as many points I have a different number. I would like to have some idea of just what I want to do to have some degree of confidence in the results. So hopefully, by taking these Dome data sets and artificially reducing the number of data points, we can begin to get some idea of how many points we have to measure.

DR. MOSS: Well, what I was getting at is that you may have the complete data set or very close to it. You may have to do the aerosol once and then for each compound you would need to verify that – you may be able to get by with just two points. If it's there, you are done, and if not, you go back to work. I would like to now open this up for discussion from the audience.

MR. WEINER (UNIVERSITY OF CINCINNATI): I'd like to comment on the discussion that you were just pursuing, as far as aerosol diffusion in the chambers. It seems that most of your chambers are dependent on turbulent diffusion to move the aerosols around in order to be able to provide them to the animals for innalation. I was wondering if you had ever pursued looking at the scale of turbulence it goes through, because that would also tend to define the scale in which you would

have to sample – what the distance is going to be between different points and your matrix – to be able to determine how well your aerosol is distributed.

DR. CARPENTER: I haven't, but I would like to, and that's a good point about the scale suggesting how frequently you would have to sample.

DR. MOSS: I was speaking in terms of air velocities. In the exposure chambers that we normally have, the highest velocity outside of the inlet line is essentially still-air conditions, under three miles an hour, so you have very laminar flow.

MR. WEINER: If you have laminar flow then how are you getting turbulent diffusion?

DR. MOSS: But you have a concentration gradient. In other words, you are putting in an aerosol or a gas at a high concentration into a bucket which supposedly is perfectly mixed but it isn't, and now you have a gradient that is driving that mixing in one way or the other. That's if you have a gas. If you have an aerosol you want the streamlines to be thoroughly mixed throughout the chamber and you have to do something to do that such as using a fan blade which will help to ensure turbulence.

MR. WEINER: If you want the aerosol to be homogeneously mixed, and generate homogeneous turbulence, then fan blades aren't quite going to do it. You are still going to have a scale.

DR. CRAIG: One of my Bibles is the NTP Statement of Work, and I'm wondering if we are not overemphasizing this question about ensuring homogeneity in the exposure chambers. Other than as a method of detecting whether or not you have leaks, it has been my experience that with any well-designed chamber, by far the most profound influence on the homogeneity measurements is the presence of a leak in the system. But beyond that the NTP Statement of Work requires routine rotation of the animals from one position to another in the chambers; and, providing the nature of the distribution in the chamber is not changing from day to day, I believe that that rotation should take care of a lot of the minor differences in the distribution of the material providing, as I say, you haven't developed any leaks. Now of course there are other ways of testing for leaks, such as pressure drops and so on.

I want to ask Bernie Adkins a question. In his presentation he mentioned something about head-only exposures – that one of the disadvantages of a head-only exposure is that it gives you a partial oral dose. Obviously, it would serve as a whole-body exposure. I have always adopted the view that aerosol exposure provides not only an inhalation exposure but also an oral exposure, because everything that has not been taken up into the bloodstream immediately is cleared by the ciliary epithelium to the mouth and swallowed. So that gives you your oral dose. You can't avoid that, and that may be as much as 75% of the total dose. Have you got any comments on that?

DR. ADKINS: I can't agree with you more. The oral dosing in the context that I presented concerns what the animals pick up in preening. Obviously we know that there is a certain amount of oral dosing associated with the normal flushing of the respiratory system. So regardless of the type of inhalation exposure mode that you use you are going to have that.

DR. MacFARLAND (CONSULTANT IN TOXICOLOGY): You don't have to restrict that to aerosols. If you're breathing a gas it's not only the ciliary activity that eases the lung particles – you must also consider a fluid flow. The gases dissolve, and you swallow.

MR. DECKER (BATTELLE PACIFIC NORTHWEST LABORATORIES): I want to ask Doug how he rotates his animals.

DR. CRAIG: I'm sorry, but I'm not the person who does this routinely. Maybe I should turn it over to somebody who does. It is a regular rotational scheme that requires cages moving from level to level and side to side on a daily basis. That is done in all our exposures.

DR. ADKINS: In our system we assign random number generation tables with the cage numbers. The animals that are housed outside the chambers have two cage cards. One stays with the polycarbonate cage regardless of whether the animal is in that cage or is in the exposure chamber, and then a corresponding card goes with that animal inside the chamber. That number is what is used as the random number in the number generation table to define where that animal goes in the chamber, and we use the computer to generate a randomization scheme. Our statisticians tell us that this achieves what we want to do.

MR. DECKER: I agree that that's a good way. I asked because I know for a fact that we have a problem with our rotation. With the Hazleton chamber, the H chamber, it's not Hazleton anymore I guess, our animals live in the chambers and our rotation scheme takes the cage and moves the cage around within the chamber but does not move the animals around within the cage. So you end up with an animal in the front left corner being in the front left corner for two years in that cage. This eliminates bias from left to right and top to bottom, but not from back to front. So I guess what I'm saying is that I don't think we have answered the question by that means, unless you are doing it in the way you are. You have to have that rotation on a random basis.

DR. YANG: I have a kind of philosophical question, which is actually not mine, that I would like to address to the panel, Dr. Moss, and some of the expert inhalation toxicologists in the audience. This question was raised earlier this month by Dr. Robert Dietrich of NIH in a pharmacokinetic workshop related to risk assessment. He personally asked me this question on another occasion and I really cannot answer him very well. The usual design of exposure scheme is something like six hours per day, sometimes T90 plus six hours, five days a week for rats. He asked why was this schedule or regimen implemented? He, of course, realized that there is a possibility of imitating occupational

exposures and sc ..n and so forth, but I think more importantly his question derived from the fact that rats as animals have much fewer cells than humans. Also, the lifespan of a rat is much shorter than that of a human. Therefore is it reasonable to use approximately the same kind of an exposure scheme to treat animals? I would like your comment.

DR. CARPENTER: You raise a couple of interesting points. In fact, we set out to do a set of experiments in which we wanted to mimic the lung burden of materials that the Consumer Product Safety Commission was concerned about – materials that people normally wouldn't be exposed to until adulthood. So we did an experiment in which we scaled lung burdens based on what we anticipated human exposure to be. We scaled the time course of the experiment on the basis of the relative proportion of age between longevity in humans and Syrian hamsters. When we completed that experiment we used the ICRP (International Committee on Radiological Protection) model to look at lung burden as a function of time. We did a deposition and retention study to get the constants from the model. Several things came out of that that were interesting from an experimental standpoint. In terms of the exposure regimen, we used animals that were as young as possible because, in fact, 18 years or so of age for a human is not very long for a Syrian hamster. A curve of lung burden could then be computed as a function of age for the human. When translated to the Syrian hamster that would mean exposing the animal all day long, every day, for as long as possible, because it is a step function. Then we used the model to tell us on what day to expose the animals, to what concentration, and for what time period in order to keep the animal's lung burden within ± 10% of what it is anticipated to be in humans. We carried out that experiment, and what we found out was not unanticipated. If you do a 30-day study with animals to get time constants that are measured on the order of 30 or 40 months, your concept of what those time constants are isn't quite as good as you would like it to be. So halfway through the experiment there was a "midcourse correction" in which we fiddled with those constants to get things back into line because the lung burdens were higher than we thought they should be. You can conduct a study on that basis, and I wonder if that isn't a rational way of doing some studies. Equally important though, I would like to see some experiments done comparing the effects of doing the exposures at night as opposed to daytime, because I have noticed over and over again as you go in in the evening to shut things down, that just about the time the aerosol generator comes off the rats stretch and begin to wake up and begin to behave like something besides little powder puffs all curled up in the corner. I suspect that we would see a difference in distribution characteristics and a difference of inhaled dose if in fact animals didn't spend the entire time of the exposure curled up in a little ball with their nose tucked up under their belly.

DR. MOSS: Yes, Dr. Adkins mentioned intermittent versus continuous exposure in his talk, and while he was talking I was writing down "what is one day in the life of a rat?" It's equal to about 30 days in

the human. That means every hour is about a day so we really should expose for 20 minutes or 30 minutes, and then stop. Then, considering what Bob just said, I decided wait a minute – they are active at night, they sleep during the day, why bother with trying to duplicate on the microscale? What we really need to do is expose them when they are active instead of when they are not. That would probably be the major improvement.

DR. CARPENTER: I would really like to see experiments carried out that way.

DR. MOSS: We can do that now with the computer-controlled systems that people are getting. We saw a control system that was $10.00 today. At least you could control the feedback system. Of course they didn't say it would take three years labor to attach it to your experiment.

DR. ADKINS: Owen, I would like to respond to Dr. Yang's question and comment. The six-hour period is a treatment time interval that is used ultimately in risk assessment determinations, and regardless of whether you treat that animal for one hour, six hours, or continuously, the information is used to extrapolate effects in humans. Again, I draw on the wealth of knowledge that exists here in the audience, especially from those who have been in this business longer than I have. When you consider that the majority of what we are talking about here are government-funded projects, the reason why we do six-hour exposures is because it fits conveniently in an eight-hour work schedule and still allows our technicians to make observations before and after the exposure. We may or may not have the same group of technicians doing the maintenance activities, and these activities would fit conveniently in an eight-hour work schedule since our customer does not like to pay overtime. Maybe some others can respond to this issue.

DR. PEIRANO (U.S. EPA): About the nighttime exposure. About four or five years ago, before Cincinnati shut down their inhalation facilities, one of the things we were doing in the diesel program was shifting light cycles on the animals. So you don't have to worry about the problem of having the technicians work at night. You just close off the chambers so that the animal's light cycle is switched, so that the animals are active during the technician's active cycle. But I can't remember how they correlated with the light and dark cycle as far as how we had been doing the studies. We were doing that type of work, and I think that's one thing everyone should be considering.

DR. CRAIG: What was the dose comparison?

DR. PEIRANO: The dose comparison as far as inhalation? That was four or five years ago, and I can't remember how in-depth we went into comparisons there. But we were considering that. We did put the animals on shifted light and dark cycles.

DR. MacFARLAND: When I first started doing inhalation studies 45 years ago in 1941, this business of how long you expose animals to simulate an industrial exposure was just beginning to emerge. In

those days you did it for eight hours. It began to be realized after awhile that this forced you to get to work a little early and you were late getting home. There was a period when that eight-hour period was dropped to seven hours, and a rationalization was produced to allow that, and then it switched over to six hours. The rationalization of course is that the worker in the occupational situation takes time off for lunch, and he has a couple of breaks morning and afternoon, and six hours is a pretty fair shot on how long he is actually working in an eight-hour day. That's simply how the thing arose. Rationalization of this in terms of burdens and so on enter into it.

DR. CRAIG: I think maybe we are just wasting our time even worrying about this because ultimately what is important is the dose that the animal gets. And unless the dose distribution changes dramatically from a daytime exposure to a nighttime exposure, for example, because of tidal volume changes, then it really isn't going to make a great deal of difference when the animal acquires that dose. Of more concern is whether or not the two-day break has a dramatic impact on what happens to the toxicity of the material. I have studied at least two different materials where we exposed animals for five days, we broke for two, and then, following the next exposure, all the animals died. There was a sixth exposure after a two-day break, so the implication is that there was some sensitization occurring there. Now, at least in the pharmaceutical industry, the requirement is that we expose for seven days a week and we have routinely done that. The length of time of course depends on what the client wants in terms of length of exposure and so on.

DR. KUTZMAN (NORTHROP SERVICES, INC.): I have to disagree with Dr. Craig a little bit. I think the physiological state of the animal should also be considered because what you are talking about is not a delivered dose having an effect, but dose at the target organ. For example, glutathione levels change in the rat between day and night. You also have to take physiological changes into account.

SESSION V

TOXICITY OF THERMAL DEGRADATION PRODUCTS

INTRODUCTORY REMARKS

LCDR John F. Wyman, MSC, USN

Naval Medical Research Institute/Toxicology Detachment, Wright-Patterson Air Force Base

Good Morning and Welcome to our final session. We are very glad that you are here on this last day of our conference. The session this morning is on the toxicity of thermal degradation products. It is very appropriate that this session follows yesterday afternoon's session on inhalation toxicity because combustion toxicology is an extension of inhalation toxicology in that the primary route of exposure for fire gases and combustion products is by inhalation.

Combustion toxicology is a concern to all communities, but it is of particular concern to the military in that much of the work that we do is carried out in closed atmospheres aboard ship and in submarines. If there is a fire in a submarine, it is not possible to go outside to get fresh air. The design for this session is to be an overview. We want to relate to you how combustion toxicology research is carried out. This will include the type of chambers being used, the analytical methodologies, the choice of animal models, and, in terms of interpretation of the toxicity data that are collected, which potential toxicant is causing this toxicity. And finally, after you have collected all of the data, how will these data be utilized by regulatory agencies? The Air Force laboratory at Wright-Patterson and the Navy laboratories are developing programs in combustion toxicology. The Air Force has had a program for some time. The Navy's program is new this year. Something that I have come to appreciate while working in the field of combustion toxicology is how technically difficult this field is. Yesterday's session on inhalation toxicology illustrated very well the technical challenges involved. In addition to these challenges in combustion toxicology, you must also consider the fact that you may start off with a simple compound or group of compounds, but when you burn them you have created a very complex mixture that is analytically very difficult to characterize.

We are very fortunate this morning in that today's overview will be provided by an august group of specialists in the field of combustion toxicology. They are all recognized experts in the field of combustion toxicology, and we are very glad to welcome them.

COMPARISON OF TEST SYSTEMS (NATIONAL BUREAU OF STANDARDS, PITTSBURGH, AND DIN) FOR ASSESSING FIRE TOXICITY

Ghonda Sangha

Manuscript Not Submitted

ANALYTICAL METHODS FOR CHARACTERIZATION OF COMBUSTION ATMOSPHERES

Arthur F. Grand, Ph.D.

Southwest Research Institute, San Antonio, Texas 78284

DEFINITIONS AND REFERENCE DOCUMENTS

It is important to review certain definitions prior to discussing the methods appropriate for analyzing combustion atmospheres. The following definitions were taken from the referenced ASTM standards.

Smoke - the airborne solid and liquid particulates and gases evolved when a material undergoes pyrolysis or combustion (ASTM E176)

Fire gases - the airborne products emitted by a material undergoing pyrolysis or combustion, which at the relevant temperature exist in the gas phase (ASTM E176)

Combustion products - airborne effluents from a material undergoing combustion; these may also include pyrolysates (ASTM E800)

From these definitions, one should note that smoke is not simply the visible solids and liquids to which we often refer, but encompasses everything airborne from either pyrolysis or combustion of a material. "Fire gases" refer specifically to the gaseous component of smoke, and these are usually the species that undergo chemical analysis. The term "combustion products" is used widely and was therefore cited in ASTM E800 (which will be described below). However, combustion does not technically include pyrolysis (thermal decomposition in the absence of oxygen); therefore, the term "smoke" is preferred. Note that combustion does not necessarily imply the presence of flame; there can be nonflaming combustion and smoldering as well as flaming combustion.

Two important documents have been developed in the standards organizations ASTM (which used to stand for American Society of Testing and Materials, but the name has been dropped in favor of the acronym only) and ISO (International Organization for Standardization). All of the important methods for characterizing fire gases are described in these documents. ASTM E800, *Standard Guide for Measurement of Gases Present or Generated During Fires,* is a survey of available methods for analysis of the most important gases present in fires. The latest version available is dated 1981; however, it is currently undergoing revision which should be complete early in 1987. It is intended to be a guide book and so does not provide details of any techniques. The details may be found in the references within that document. The advantages and disadvantages of the analytical techniques are summarized, but one particular technique is not recommended over another.

The other document, entitled *Methods for the Analysis of Gases and Vapors in Fire Effluents,* is a draft proposal being developed under the auspices of ISO, Technical Committee 92, Sub-committee 3, Working Group 2. This document will be part of a comprehensive technical report by the working groups of Subcommittee 3 regarding toxic hazards of fire. This manuscript contains more detail on analytical methods than presented in ASTM E800; however, fewer procedures are described. Recommended analytical techniques are indicated for all the fire gases surveyed. In both of these reports, the emphasis is on the major toxic products contained in smoke.

REASONS FOR ANALYSIS OF FIRE GASES

There are four primary issues surrounding the analysis of the components of smoke: toxicity considerations, fire research, detection of smoke, and air pollution. They are summarized below.

Toxicity Considerations

The toxic products of smoke, in addition to oxygen and carbon dioxide, are analyzed in an effort to help determine why people die in fires. Generally, these products are present in relatively high concentrations (hundreds or thousands of parts per million) and are toxic even under short exposure conditions, up to 30 min. Certain studies, such as those that address fires in submarines or space vehicles, are concerned with lower concentrations of toxic fire gases over longer exposure periods. The main purpose of analysis of gases for toxicity considerations is to identify the toxic species and determine their concentration in smokes evolved from various materials under typical fire scenarios. When, occasionally, an unknown toxicant is responsible for causing severe toxic effects, an analysis of the atmosphere is necessary to identify the causative species.

Fire Research

Identifying the components of smoke is an important aspect of the research to define mechanisms of combustion and identify the contribution and mode of action of fire retardant additives. Also, analytical techniques have been important in determining mechanisms of formation and rates of evolution of soot from polymeric materials. Measuring low concentrations of short-lived species in flames has been essential to understanding the chemistry of flame reactions.

Detection of Smoke

In order to develop smoke detectors, which in practice are often detectors for only the gases in smoke, knowledge of the components of various smokes is essential. Smoldering fires and flaming fires produce vastly different compounds; this fact is particularly significant in development of appropriate smoke detectors. In certain applications, such as smoke detectors in air ducts and for the

future space station, detectors must be sensitive to much lower concentrations than normally required for either residential or commercial business areas.

Air Pollution

The stack gases from incinerators or industrial combustors may contain compounds that are either directly toxic or otherwise injurious to the environment. For these reasons, stack gases are often monitored to determine their composition. Techniques for sampling and analyzing stack gases are similar to those used for measuring smoke in full-scale laboratory combustion experiments.

There are obviously enough subjects under these four categories for an entire book and they cannot all be covered in this brief treatise. Therefore, the topics to be covered will be those with toxicological significance. In particular, the emphasis will be on those species that are predicted to have acute toxic effects and are generally present in relatively high concentrations in fire atmospheres; however, some discussion of measurement of lower concentration species will be presented. The gases to be discussed will be long-lived species (i.e., no free-radicals or other transient compounds).

SAMPLING

Many researchers consider sampling of fire gases the most critical part of the analytical process. It is stated in ASTM E800 that "More errors in analysis result from poor and incorrect sampling than from any other part of the measurement process." The reader is encouraged to consult the two standard documents described earlier for more detailed discussion and references to equipment for both sampling and analysis.

If sampling is done improperly, critical species may be missed without the operator's knowledge. Incorrect sample treatment may also lead to high values from interferences. One must have the proper analyzer, also, but concentrating on that aspect exclusively can lead to deceptive analytical results.

Before setting up sampling lines, one must determine the significant data to be obtained from the experiment. When looking for toxic products, the researcher must be aware that many of them are at relatively high concentrations (i.e., measurable in ppm rather than ppb). This permits some leeway in the sampling parameters and influences the decision to be made on allowable accuracy. For example, generally one does not need to use a GC/MS (gas chromatograph/mass spectrometer) to analyze for common products. There are continuous, on-line instruments available for many common gases, and there are batch sampling techniques with analysis after the experiment. Each method has its own advantages and disadvantages.

Sampling locations are a serious consideration when analyzing areas where fires have occurred. In laboratory experiments, sampling locations may be influenced by the presence of animals, by any dilution or surface loss of products, and by the presence of other sampling probes. The presence of soot, liquid pyrolyzate, and corrosive gases needs to be taken into consideration. In large-scale fire experiments, high temperatures and smoke stratification occur, requiring that the location of sampling probes be carefully planned.

There are specific sampling problems that need to be considered for each of the gases to be discussed below. There is not much problem in transporting noncondensable, nonreactive gases such as carbon monoxide (CO), carbon dioxide (CO_2), or oxygen (O_2). Carbon monoxide, however, could be lost in certain reactive filters. Heated sampling lines need to be used for condensable gases and to avoid condensation of water. Soot filters are often required in order to use a particular instrument; however, they can absorb reactive components. The filter should be heated and, if necessary, the filter could be analyzed for specific components and a correction factor applied to the analytical results.

Whenever possible, short sampling lines should be used. Sometimes no sampling line is required, as in the case of solid absorption tubes. For continuous analyzers, the sampling lines generally cause a time delay in addition to that for the analyzer response. Intermittent (batch) sampling often has no time delay. This type of sampling may result in an instantaneous or a time-integrated result. The physical characteristics of the sampling probe need to be considered when sampling high-temperature atmospheres and/or when sampling is conducted in very corrosive environments. Finally, since the sampling line may have an effect on the concentration of the gases, a check of the calibration gases should be made through the actual sampling system.

GASES AND ANALYZERS

Introduction

In this section, both common and less common (often more expensive) techniques will be covered, including continuous and batch methods. The reader is referred to the standard documents already described for a more detailed discussion and for reference to equipment and procedures. This treatise will not deal with commercially available detector tubes. These devices have some utility for quick and easy indication of the presence of a gas of interest, but have no real place in so-called analytical procedures. There are numerous interferences in smoke, and the accuracy of some tubes is only ± 20% under ideal conditions.

Carbon Monoxide (CO) and Carbon Dioxide (CO_2)

Carbon monoxide and CO_2 are the most common gases present in fire atmospheres; therefore, they are also the most likely to be measured. The toxicity of CO is of major importance in determining the overall toxic effects of smoke. The presence of a high concentration of CO_2 may be important in toxicity considerations because of its effect on respiration rate. These two species are produced, in varying ratios, during the combustion of all carbon-containing polymers.

Carbon monoxide and CO_2 are noncondensable gases, which can be analyzed directly from a combustion atmosphere or collected in syringes or plastic bags for subsequent analysis. CO_2 can be lost on certain absorbents (e.g., soda lime), which may be present in the sampling line in order to protect the analyzer from acid gases.

The commonly used analytical techniques for measuring CO and CO_2 are described below.

1) NDIR (nondispersive infrared) is one of the more commonly used techniques for continuous analysis of CO and CO_2. Analyzers are employed that are specific for the gas of interest and have relatively few interferences. A soot filter is required in the sampling line, and removal of moisture is recommended.

2) Gas chromatography is suited only for batch analysis; however, it is a commonly used procedure. Column selection is critical because CO_2 is irreversibly absorbed onto certain columns. Both CO and CO_2 can be analyzed on a porous polymer column with TC (thermal conductivity) detector. Lower concentration analyses can be achieved by hydrogenation and analysis by FID (flame ionization detector).

3) Instruments based on electrochemical measurement of CO were developed primarily for long-term (ambient air) monitoring. The response of these types of instruments is often too slow for combustion atmosphere monitoring.

Oxygen (O_2)

It is important to analyze for O_2 in toxicity studies to ensure that there is sufficient O_2 to prevent oxygen-depletion problems with the animals.

1) Gas chromatography, as a batch analysis technique, can be performed with O_2 using the same columns as those used for separation of CO, but not those used for CO_2.

2) A paramagnetic O_2 analyzer is an instrument specifically intended for analyzing oxygen, based on the unique paramagnetic properties of O_2. Such instruments have had widespread use in the continuous analysis of O_2 in fire gases.

3) Another continuous measurement device for O_2 is the polarographic analyzer. It generally is not as precise or rapid as the paramagnetic analyzer; however, it has had considerable application in analysis of O_2 in fire gases.

Hydrogen Cyanide (HCN)

HCN is often considered the second most important acutely toxic species in fire atmospheres. It can be formed during the combustion of many nitrogen-containing polymers such as urethane foams, ABS, and wool.

1) Analysis of HCN often entails collecting the species in aqueous solution over some time period (time-integrated batch sampling), with subsequent analysis by any of several techniques: ion-selective electrode (ISE), ion chromatography (IC), colorimetry, or titration. Another sampling technique utilizes soda lime tubes (SLTs).

2) There are several procedures for analyzing HCN by gas chromatography. Direct injection of the combustion gas is possible; samples are taken in gas-tight syringes for instantaneous, intermittent sampling of the atmosphere. Hydrogen cyanide in solution can also be analyzed by GC. Alkali-flame or nitrogen-phosphorus detectors are recommended over the flame ionization detector, which is less specific for HCN.

3) Fourier transform infrared (FTIR) instruments and nondispersive infrared instruments are available for analysis of gaseous HCN. Long path-length instruments are often necessary for the low concentrations that exist in fire atmospheres.

4) There are two continuous analyzers for HCN, in addition to the infrared instruments. One is an apparatus based on continuous chemical reaction of HCN with sodium picrate and colorimetric analysis of the complex formed. The other is a commercial instrument that analyzes HCN from an electrochemical response with silver/silver chloride electrodes. Use of this latter instrument is limited to lower concentrations of HCN (less than 150 ppm) than are sometimes found in acutely toxic HCN-containing smoke.

Hydrogen Chloride (HCl) and Hydrogen Bromide (HBr)

The toxicity of halogen acid gases has recently received increased attention. Halogen acid gases are produced during combustion of halogen-containing polymers (e.g., PVC and FR polymers containing Br). They are the most difficult of the major gases to sample because of their reactivity with moisture in the atmosphere and corrosiveness to sampling lines. Also, gaseous calibration standards are often unreliable.

1) Absorption into aqueous solution is the traditional analytical sampling technique. This is still used; however, it is plagued by experimental difficulties in preparation of bubblers, length of

sampling lines, efficiency of absorption, and plugging of the air inlet tube by smoke particulates. Subsequent analysis is performed by titration, ISE, pH measurement, conductance, and IC.

2) Absorption tubes have supplanted aqueous impingers for many applications. Dry SLTs have been used successfully in many studies at Southwest Research Institute (SwRI). Absorption tubes containing caustic solution on glass beads have been used by researchers at the Federal Aviation Administration. The extract from either of these methods can be analyzed by titration or ion chromatography.

3) Two methods can be used for continuous analysis of HCl. One method employs a commercial gas filter correlation technique based on infrared analysis. The other is based on a French standard (described in the ISO document), and is currently in use at SwRI. This method uses a commercially available stat titrator system to titrate chloride continuously by silver in a sample stream. In the SwRI setup, the sample flow rate is controlled by a mass flowmeter. The output of the mass flowmeter and the rate of flow of silver nitrate, the only two variables, are fed into a dedicated microprocessor, which continuously displays and records the concentration of HCl.

Compounds Containing Fluorine

Some of the most perplexing questions in combustion toxicity research today concern the highly toxic species produced during the combustion of PTFE (polytetrafluoroethylene). Analyses have been made of many of the components, but the results do not add up to the observed toxic effects. Other fluorine-containing compounds produce HF as the primary toxic species.

1) Analysis of HF may be performed by some of the same techniques used for analysis of HCl and HBr (above). However, reactivity of the HF, and a reduced tendency for dissociation of HF, make the techniques less reliable. Adsorption tubes containing activated charcoal have been used, followed by desorption and analysis by fluoride ISE. Analysis of fluoride by IC can also be performed if sampling is done in aqueous solution.

2) A continuous method using a fluoride ISE has been reported by the French (described in the ISO document).

3) Analyses of other toxic products from fluorine-containing polymers have been reported, including carbonyl fluoride and perfluoroisobutylene (PFIB). Gas chromatographic and GC/MS techniques are required.

Nitrogen Oxides

Nitric oxide (NO) and nitrogen dioxide (NO_2) are likely components of fires involving nitrogen-containing polymers; however, there have not been definitive studies to show that toxicologically significant concentrations of these species exist in typical smoke atmospheres. Analytical methods

for measuring these species are based on techniques for atmospheric analysis. Often, the analytical result is expressed as NO_X for the sum of the NO and NO_2 components. The more likely compound in fire is NO; NO_2 generally is a secondary combustion product.

1) Chemiluminescence is the usual means for analyzing the nitrogen oxides. It is a continuous analysis procedure, producing results for NO and NO_X (NO_2 is calculated by difference). The analytical range of available instruments is sometimes a concern because they were developed for ambient air monitoring.

2) A colorimetric method for analyzing the nitrogen oxides involves collecting the species onto a solid sorbent, with subsequent dissolution and analysis.

Sulfur Compounds

Sulfur oxides (SO_2 and SO_3) may be present in combustion atmospheres when a material containing sulfur is involved. Many of the comments given above for nitrogen oxides apply to the sulfur oxides: they have not been demonstrated to be in toxicologically significant quantities in most smoke atmospheres, and available instruments were developed primarily for ambient air monitoring.

1) NDIR instruments are available for continuous analysis of sulfur oxides. There are specific detectors available for GC analysis of sulfur oxides, and colorimetric procedures have also been reported.

2) Carbonyl sulfide (COS) is a highly toxic product that has been identified in certain combustion atmospheres. Analysis of this species is by GC/MS.

Acrolein, Other Aldehydes

Aldehydes, especially acrolein, have been identified in the combustion products of wood and polypropylene, and are probably present in the smokes of numerous other linear and oxygen-containing polymers. They have been associated with postexposure lethalities of rodents in smoke toxicity tests. Identification of specific aldehydes is difficult because many of them are chemically similar to one another. By the same token, the toxicological effects of many of the aldehydes may be similar.

1) Absorption into solution with subsequent wet-chemical (colorimetric) procedures is the most common technique for identifying aldehydes. Sampling may need to be performed for long periods (e.g., 15 to 30 min) to obtain a sufficient concentration for analysis. In another procedure, a reaction product of the aldehydes is measured by high-performance liquid chromatography (HPLC). Direct analysis of combustion atmospheres may be accomplished using gas chromatography (with a flame

ionization detector). If the combustion atmosphere is very complex, identification of individual atmospheres is more difficult.

Isocyanates and Nitriles

Toluene diisocyanate (TDI) and numerous other isocyanates and nitriles may be produced by the combustion of urethanes. Although there have not been definitive studies relating these specific products to the toxicity of urethanes, the toxicity to rodents of the combustion atmospheres of flexible and rigid polyurethane foams may not be accounted for simply by CO and HCN.

Analysis of specific isocyanates and nitriles is generally performed by gas chromatography or GC/MS.

Other Organics

There are many possible organic species in smoke. Studies have reported attempts toward elucidating them. Generally, the ones noted above are those most often cited in studies relating to toxicology. Analyses are usually by GC/MS.

Total Hydrocarbons

The generally misleading term "total hydrocarbons" refers to any and all hydrocarbons that respond to a specific detector, usually an FID. Thus, all unburned hydrocarbons will respond in some manner and are reported as parts per million of the calibration gas (usually methane). The analytical result, which at best must be treated as semiquantitative, probably does not have a great deal of relevance to toxicological studies.

Some commercial detectors for total hydrocarbons are simply an FID alone, which will respond to any combustible gas or vapor. Hydrocarbons without oxygen give a greater response in this type of detector than do oxygenated species. In conjunction with a gas chromatograph, some separation of the organic species will be achieved, and the analytical result will depend on the calibration. Hydrocarbon detectors based on infrared will also detect numerous hydrocarbons, depending on the wavelength selected and the calibration standards used.

CONCLUSIONS

The discussion in this paper focused on sampling considerations and analytical methods for characterizing combustion atmospheres (smoke), with an emphasis on toxic products. The science of combustion toxicology relies on analytical support to help elucidate the reasons for toxic effects of combustion atmospheres. In fact, some researchers in the United States and Europe (England, in particular) are seeking ways to predict toxic effects from chemical analysis alone, without the need for animal testing. Unfortunately, we are not as close to this goal as we could be because much of

the laboratory combustion toxicity testing in the United States has not had adequate analytical support. Thus, we have a large quantity of LC_{50} data on smoke from various materials, but we do not have the relevant analytical data to determine why the animals died.

A laboratory does not need extensive (or expensive) equipment to make certain analytical measurements on some of the major toxic products in smoke. For example, analysis of carbon monoxide concentration alone will tell a researcher whether rodent lethality was due primarily to CO. If CO is not the only toxicant present, certain simple analyses of the smoke atmospheres, with consideration for the chemical makeup of the polymer, can be very useful. Animals need not be present for the analytical experiments. If nitrogen is present in the polymer, one might look for the presence of hydrogen cyanide; whereas if halogen atoms are in the material, and postexposure lethalities are observed, one might attempt to quantify the level of halogen acid (e.g., HCl) present in the smoke. More sophisticated analytical procedures might be warranted; however, these should not be the first priority if an analysis of animal exposure effects is the primary purpose of the experiment.

TOXICOLOGICAL INTERACTIONS BETWEEN CARBON MONOXIDE AND CARBON DIOXIDE

Barbara C. Levin, Ph.D., Maya Paabo, M.S., Joshua L. Gurman, Ph.D., Steven E. Harris, and Emil Braun

National Bureau of Standards, Gaithersburg, Maryland 20899

SUMMARY

Fischer 344 male rats were subjected to 30-min individual or combined exposures of carbon monoxide (CO) and carbon dioxide (CO_2). All deaths from CO occurred during the exposures, and the LC_{50} values were 4600 and 5000 ppm, depending on experimental conditions. Animals exposed to CO_2 concentrations ranging from 1.3 to 14.7% for 30 min were neither incapacitated nor fatally injured. The addition of nonlethal concentrations of CO_2 (1.7 to 17.3%) to sublethal concentrations of CO (2500 to 4000 ppm) caused deaths of the exposed rats both during and following (up to 24 h) the 30-min exposures. The most toxic combination of these two gases (2500 ppm CO plus 5% CO_2) increased the rate of carboxyhemoglobin (COHb) formation 1.5 times that found in rats exposed to 2500 ppm of CO alone. The COHb equilibrium levels were the same. Exposure to both CO and CO_2 produced a greater degree of acidosis and a longer recovery time than that observed with either single gas. The results fit a mathematical model indicating a synergistic interaction. Combustion of 11 materials at their LC_{50} values indicated that CO was probably the primary toxicant in one case and that the combined CO plus CO_2 was the cause of the deaths in three other cases. Additional fire gases need to be studied to explain deaths from the other materials.

INTRODUCTION

The physiological, biochemical, and toxicological effects of CO and CO_2 have been intensively studied as separate chemical entities since the early physiological research of Haldane and Smith in 1892 (1). There have been few studies, however, that examine the toxicological effect of combined exposure to CO and CO_2. The lack of such research is surprising since both CO and CO_2 are common products of the combustion of carbon-containing materials. Thus, accidental exposure to high levels of CO will rarely occur without simultaneous exposure to CO_2. Carbon monoxide, however, is estimated to be 25 to 40 times more toxic than CO_2 in humans and has been shown to be about 80 times more toxic in rats (2,3). Therefore, it is understandable why the role played by CO in the deaths and injuries resulting from smoke inhalation has received much more attention than that of CO_2.

CO exerts its toxicity by binding to the hemoglobin of a victim's blood, forming the easily measured and stable compound, COHb. CO combines with hemoglobin at a rate of about 1/10 that of oxygen (O_2) and dissociates from hemoglobin at a rate of about 1/2400 that of O_2. Therefore the affinity of hemoglobin for CO is approximately 240 times greater than for O_2 (4). The presence of COHb in the blood prevents the binding of O_2 to hemoglobin and the formation of oxyhemoglobin

(O_2Hb); death thus occurs from the lack of transport of O_2 from the lungs to the tissues (tissue hyposia).

CO_2 is a normal byproduct of cellular metabolism and is extremely important in the regulation of the vital functions of the body. Changes in the CO_2 concentration will produce marked effects on respiration, circulation, and the central nervous system (5,6). For burning combustibles without O_2 in their molecular structure, the maximum obtainable concentration of CO_2 is 21%, which would be reached only if all the O_2 were converted to CO_2, a highly unlikely event under actual fire conditions. In the experiments to be reported here, 17.7% was the highest concentration of CO_2 tested, well below the 40% reported to be lethal to rats exposed for 30 min (3).

The few published studies on exposures to both CO and CO_2 have produced conflicting results. In two studies, a possible antagonistic or reduced additive effect was noted (3,7). In two other laboratories, no significant differences were noted when rats were exposed to the combined gases as compared to the separate gases (8,9). On the other hand, some data have been published that indicate a positive interaction between the combined gases (10,11).

In view of these controversies, the present studies were performed to evaluate the interrelationships between CO and CO_2. The results provide evidence that exposure to combined sublethal concentrations of the individual gases will produce death in rats. The data were compared to the LC_{50} values for 11 solid materials thermally decomposed in the same apparatus to determine whether the concentrations of CO and/or CO_2 could account for the deaths. This information is important in the evaluation of the toxicity of combustion gases, the determination of concentrations of such gases that would be compatible with life in areas away from the room of fire origin, and the development of a fire hazard computer model.

METHODS

Materials

The CO evaluated in this study was commercially supplied by either Roberts Oxygen Company, Inc. (Rockville, MD), or Matheson Gas Products (Dorsey, MD), in concentrations of 1.2% in air, 3.2% in nitrogen, and 100% CO. (Impurities were checked in the cylinder of 100% CO and found to consist of 0.001% total hydrocarbons, 0.035% argon and O_2, 0.09% nitrogen, and 0.018% CO_2.) The CO_2 was supplied by Matheson Gas Products in concentrations of 10 and 20.2% in air, 50.5% in 20.9% O_2 and 28.6% nitrogen, and 100% CO_2.

The solid materials used in this study, namely, an acrylonitrile-butadiene-styrene (ABS), a Douglas fir, a flexible polyurethane, a modacrylic, a polyphenylsulfone, a polystyrene, a polyvinyl

chloride (PVC), a PVC with zinc ferrocyanide, red oak, a rigid polyurethane, and a wool, are the same as those studied previously in this laboratory (12).

Animals

Male Fischer 344 rats weighing between 200 and 300 g were obtained from the Harlan Sprague-Dawley Co. (Walkersville, MD) or Taconic Farms (Germantown, NY). The animals were allowed to acclimate to our laboratory conditions for at least 10 days prior to experimentation. Animal care and maintenance were performed in accordance with the procedures outlined in the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals." Each rat was housed individually in suspended stainless steel cages and provided with food and water *ad libitum*. Twelve hours of fluorescent lighting per day was provided using an automatic timer.

Animal Exposure System and Chemical Analysis

The 200-l rectangular animal exposure chamber designed for the National Bureau of Standards (NBS) Toxicity Test Method was used in both the gas and material experiments (Figure 1) (13). In all experiments, the CO, CO_2 (both measured by nondispersive infrared [NDIR] spectroscopy), and O_2 concentrations (measured by either a galvanic cell or by a paramagnetic analyzer) were monitored continuously throughout the exposures and recorded by an on-line computer every 15 s. Any gases removed for analysis were returned. In the gas experiments, the animals received either square-wave or gradual exposures. In the square-wave exposures, CO and/or CO_2 were mixed with nitrogen and/or air in the animal exposure chamber, and a fan in the chamber provided adequate mixing of the gases. The gas concentrations in the chamber were monitored by NDIR spectroscopy; when the desired concentrations were reached, the animals were inserted into the chambers. In the gradual exposures, the animals were first inserted into the chamber and the gases were then brought linearly to the desired concentrations over a 5-min period.

In the experiments examining combustible solids, the materials were decomposed in a cup furnace located directly below the animal exposure chamber such that all the combustion products from the test materials were evolved directly into the chamber and remained in the chamber for the duration of the exposure. The materials were examined under separate nonflaming and flaming conditions, which were achieved by setting the furnace 25° below and above each material's predetermined autoignition temperature (the temperature at which the material will spontaneously ignite into flames). The animals were inserted into the chamber before the material was placed into the furnace so that the animals experienced the initial generation of the smoke. In most cases, the CO (an indicator of the completeness of combustion) reached a steady state in 5 to 10 min (13). Douglas fir and red oak continued to produce CO throughout the 30-min exposures.

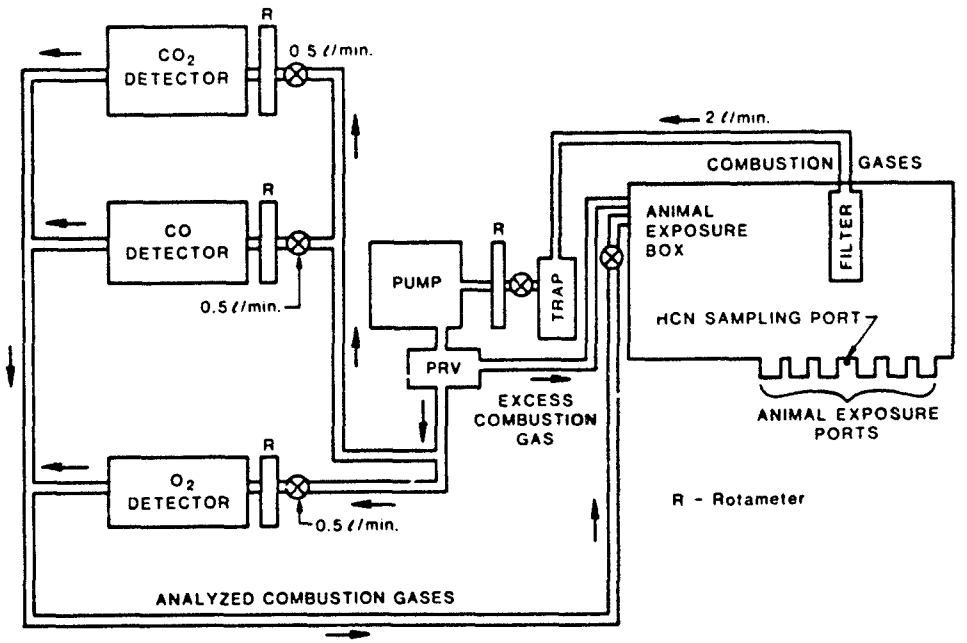


Figure 1. Schematic of the National Bureau of Standards Toxicity Test Method.

Blood Analysis

Animals designated for blood analysis underwent cannulation 24 h before exposure. A cannula was surgically inserted into the animal's femoral artery, thereby allowing blood samples to be taken during and following the exposures from unanesthetized rats. Total hemoglobin (THb), oxyhemoglobin (O_2Hb), COHb, methemoglobin (metHb), and volume percent oxygen (vol. % O_2) were measured with a Co-Oximeter (Model 282, Instrumentation Laboratory, Inc., Lexington, MA). The partial pressures of CO_2 (pCO_2) and O_2 (pO_2), bicarbonate (HCO_3^-), base excess (BE), total CO_2 (TCO_2), and pH were measured with the Corning 168 pH/Blood Gas System (Corning Medical, Medfield, MA).

Test Procedure

Six rats were exposed in each experiment. Animals were placed in restrainers which were then inserted into six portholes, located along the front of the exposure chamber, such that only the rats' heads were exposed. To achieve square-wave exposures, the portholes were fitted with rubber stoppers during the time the gas concentrations were reaching equilibrium. Insertion of the restrained rats caused the stoppers to fall into the chamber and simultaneously exposed the rats to the chamber atmosphere. In the gradual gas and material experiments, the rats were inserted in the

portholes first so that they were exposed to the initial (usually 5-min) generation of the test atmosphere as well as the later steady-state conditions. All gas concentrations are average exposure values, calculated by integrating the area under the instrument response curve and dividing by the exposure time.

Exposures were 30 min (unless otherwise noted), during which time blood (0.5 ml) was taken from cannulated animals (one or two animals per experiment) at zero time, about halfway through the exposure, and just before the end of the experiment. All animals (including controls but excluding cannulated animals) were weighed daily from the day of arrival until the end of the postexposure observation period; cannulated animals were sacrificed following exposure.

The toxicological end point was death. The number of animals that died at each gas concentration was plotted to produce a concentration-response curve from which an LC_{50} (30 min and 14 days) was calculated. The LC_{50} in this case is defined as the concentration of test gas in the chamber (parts per million or percent, where 1% = 10,000 ppm) or amount of material placed in the furnace divided by the exposure chamber volume (mg/l) that caused 50% of the animals to die during the exposure plus the 14-day postexposure observation period. The LC_{50} values and their 95% confidence limits were calculated via the statistical method of Litchfield and Wilcoxon (14).

Kinetic Blood Uptake Study of CO with and without CO_2

Experiments were designed to determine whether an increased rate of formation of COHb due to the respiratory stimulation of CO_2 was sufficient to explain the increased toxicity observed at sublethal levels of CO and CO_2. In these experiments, all six rats were cannulated and exposed to pre-set concentrations of the gases. Blood (0.5 ml) was sampled at approximately 1-min intervals sequentially from different rats such that an excessive amount of blood was not taken from any one rat. The exposures lasted 30 min, but blood sampling was continued an additional 60 to 90 min to observe postexposure recovery.

Statistical Modeling

Two regression models were fitted to the data using the Generalized Linear Interactive Modeling (GLIM) statistical software package distributed by the Royal Statistical Society. GLIM reports the goodness of fit of a model in terms of a scaled deviance. The scaled deviance has an approximate chi-squared distribution, with degrees of freedom equal to the sample size minus the number of parameters in the model. For a standard regression model, the deviance reduces to the residual sum of squares.

RESULTS

Carbon Monoxide

The animals received square-wave exposures to CO concentrations ranging from 1470 to 6000 ppm and gradual exposures to average 30-min concentrations ranging from 3280 to 5700 ppm. As these were all closed-system exposures, average 30-min CO_2 concentrations produced by the respiration of the rats ranged from 0.13 to 0.24%. The average O_2 concentrations never fell below 16.5% (Table 1).

TABLE 1

ANIMAL MORTALITY FROM CARBON MONOXIDE EXPOSURES

CO[a] (ppm)	CO_2[a,b] (ppm)	O_2[a] (%)	COHb[c] (%)	Within Exposure Deaths[d]
5,990	1,270	16.5	–	6/6
5,740[e]	1,390	16.9	89.9	5/6
5,030	2,170	20.4	85.1	4/6
4,960[e]	1,620	–	87.6	3/6
4,860	1,500	17.3	–	5/6
4,610	2,180	20.5	83.9	3/6
4,570	2,100	20.5	–	5/6
4,330	2,230	20.3	83.8	1/6
4,080[e]	1,520	17.8	84.6	0/6
3,720[e]	1,500	–	82.7	0/6
3,620[e]	1,860	–	83.4	1/6
3,280[e]	1,900	16.8	–	0/6
2,520	1,900	20.8	78.0	0/5
1,470	2,430	20.6	67.3	0/6

All exposures were square wave except where noted.

[a] Average gas concentration over 30-min exposure.

[b] CO_2 concentration from animal respiration.

[c] Value prior to end of 30-min exposure.

[d] No postexposure deaths were ever observed from 30-min CO exposures.

[e] Gradual exposure (i.e., gases introduced into chamber over 5-min period).

Regardless of whether the rats received gradual or square-wave exposures to CO in air, all deaths except one occurred during the 30-min exposures; the exception was a death noted at 40 s postexposure. The LC_{50} value was 5000 ppm with 95% confidence limits of 4400 to 5600 ppm for the gradual exposures, and 4600 ppm with 95% confidence limits of 4400 to 4800 ppm for the square-wave exposures (Figure 2).

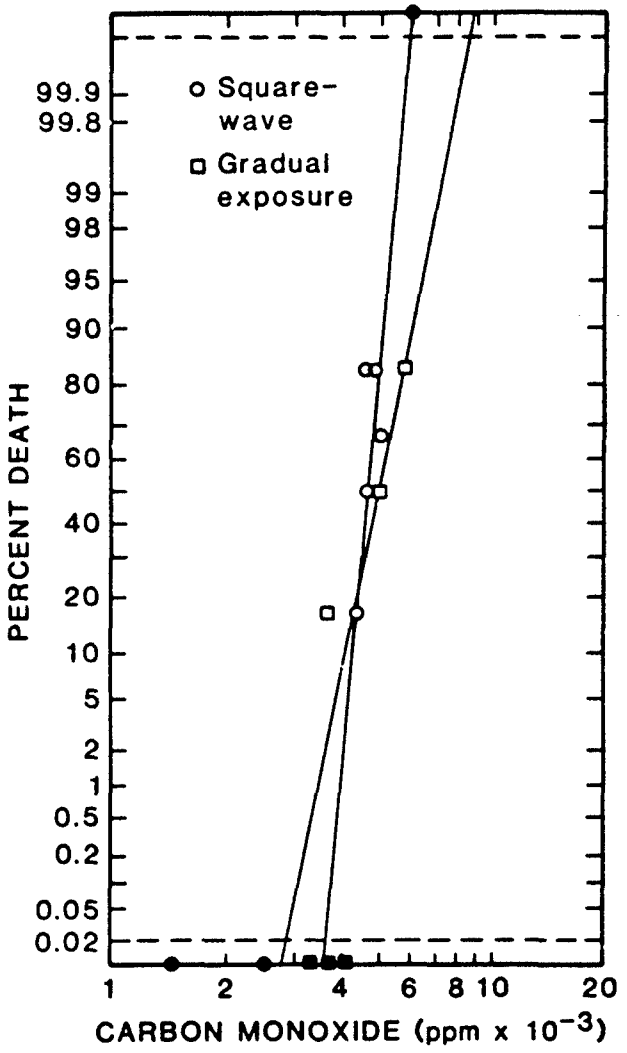


Figure 2. Concentration-response curves for 30-min gradual and square-wave exposures to CO. Filled symbols above top dashed line and below bottom dashed line indicate 100% and 0% deaths, respectively.

In most experiments, arterial blood was sampled through surgically implanted cannulae in one or two of the six exposed unanesthetized rats before (control values), during, and following the 30-min exposures. Table 2 shows the mean control values of eleven blood parameters and their standard deviations that were calculated for the animals used in this experiment and arterial blood control values previously obtained from our laboratory, from the University of Utah (15), from SRI International (16), and from published human values (17). All data are in good agreement except the pO_2 values from the University of Utah; their low value is attributable to their location in Salt Lake City, which is at an altitude of 4500 ft and has an atmospheric pressure of 640 mmHg (17). At this altitude, the pO_2 should be and is approximately 70 mmHg (18).

Figures 3A-H show the measured blood values in animals exposed to different average atmospheric concentrations. Blood samples were taken from different animals during the 30-min exposures at times ranging from 19 to 30 min. Carboxyhemoglobin and pO_2 increased as the concentration of CO increased, while O_2Hb; vol. % O_2; pH; pCO_2; HCO_3^-; BE; and TCO_2 decreased.

The time to reach the COHb equilibrium levels depended in part upon the atmospheric CO concentrations (Figure 4). At concentrations close to the 30-min LC_{50} values, the COHb levels reached equilibrium approximately 10 min after the start of the test, regardless of whether the animals received square-wave or gradual exposures. In all of the CO exposure experiments in which one or more deaths occurred, the COHb levels were greater than 83% (Table 1).

Carbon Dioxide

Animals exposed for 30 min to square-wave concentrations of CO_2 in air ranging from 1.3 to 14.7% showed neither mortality nor incapacitation (as indicated by lack of righting reflex) during or following any of these exposures; even rats exposed to the highest CO_2 (14.7%) had normal righting reflexes, posture, and exploratory behavior when tested immediately following the exposures. In all these experiments, the O_2 levels were greater than 17% and the background CO was negligible (ranging from 25 to 50 ppm; that is, within instrumental noise).

At three CO_2 concentrations (5.25, 5.72, and 14.7%), either one or two of the six exposed animals were cannulated and had blood drawn at time 0 (baseline) and at 15 and at 30 min into the tests. The results shown in Table 3 indicate that the percent O_2Hb decreased (lowest value was 83.4%) as did the pH (7.00) and the base excess (-7.1 mM). On the other hand, pCO_2 rose (highest value was 126.8 mmHg), as did the pO_2 (122.6 mmHg), HCO_3^- (30.5 mM), and total CO_2 (34.3 mM). All of the extreme values occurred at the highest concentration of CO_2, 14.7%. With the exception of pO_2, all of the changes that were greater than two standard deviations from the baseline values occurred as early as 15 min into the exposures.

TABLE 2

CONTROL BLOOD VALUES FROM DIFFERENT LABORATORIES

Arterial Blood Parameters	NBS[a]	NBS[b]	UTAH[c]	SRI[d]	Human Values[e]
Total hemoglobin (g/100 ml)	14.8 ± 1.2 (180)[f]	14.4 ± 1.2 (39)	15.4 ± 1.6 (146)	NR	15
Oxyhemoglobin (%)	94.1 ± 1.8 (180)	93.5 ± 1.0 (39)	90.4 ± 6.4 (285)	NR	97.1
Carboxyhemoglobin (%)	1.7 ± 0.8 (180)	2.4 ± 0.7 (39)	1.6 ± 1.4 (286)	NR	NR
Methemoglobin (%)	0.7 ± 0.3 (180)	0.3 ± 0.3 (39)	NR	NR	NR
Volume percent oxygen (ml O_2/100 ml blood)	19.3 ± 1.7 (180)	18.7 ± 1.5 (39)	NR	NR	20.3
pH	7.44 ± 0.06 (173)	7.42 ± 0.07 (30)	7.4 ± 0.04 (289)	7.42 ± 0.07 (10)	7.40
Partial pressure CO_2 (mmHg)	35.0 ± 4.1 (151)	30.6 ± 3.1 (30)	27.0 ± 4.9 (283)	35.0 ± 2.5 (10)	40
Partial pressure O_2 (mmHg)	95.6 ± 8.1 (138)	97.2 ± 11.4 (30)	70.2 ± 8.8 (289)	101.5 ± 16.2 (10)	100
Bicarbonate (mM)	20.5 ± 2.5 (141)	20.1 ± 4.1 (30)	17.0 ± 3.0 (283)	24.9 ± 4.8 (10)	25[g]
Base excess (mM)	-1.7 ± 2.6 (141)	-2.7 ± 4.6 (30)	-5.2 ± 2.8 (283)	NR	NR
Total CO_2 (mM)	21.4 ± 2.6 (141)	20.9 ± 3.9 (30)	NR	NR	21.9

[a] National Bureau of Standards control data from Fischer 344 male rats from experiments not reported here.

[b] National Bureau of Standards control data from Fischer 344 male rats used in current experiments.

[c] University of Utah data from Long Evans male rats (15).

[d] SRI International control data from Long Evans male rats (16).

[e] Data from healthy resting young men (17)

[f] Mean values ± standard deviations (number of animals)

[g] Data from Davenport (27).

NR = Not reported.

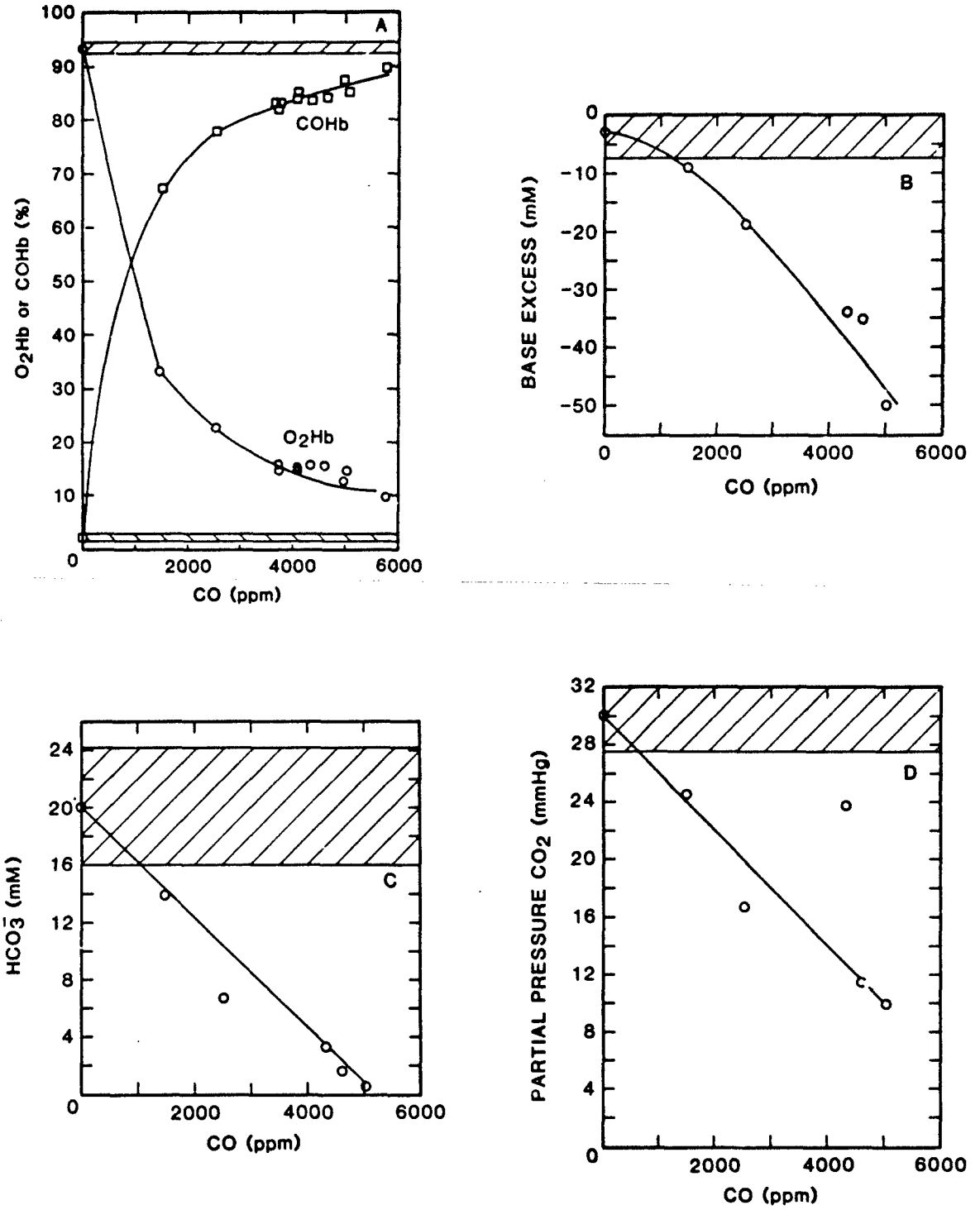
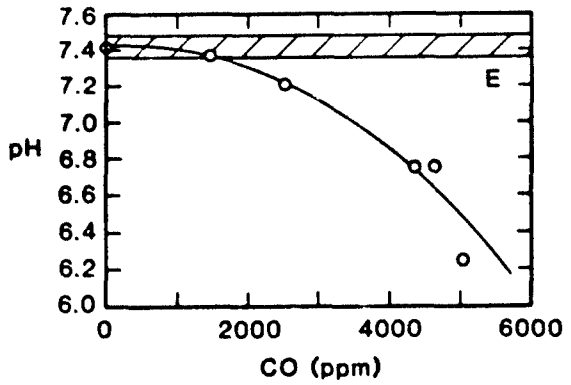
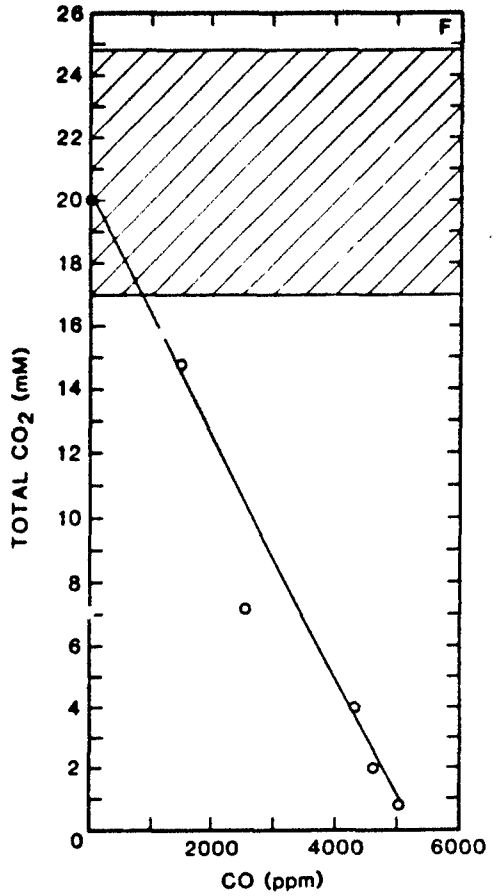
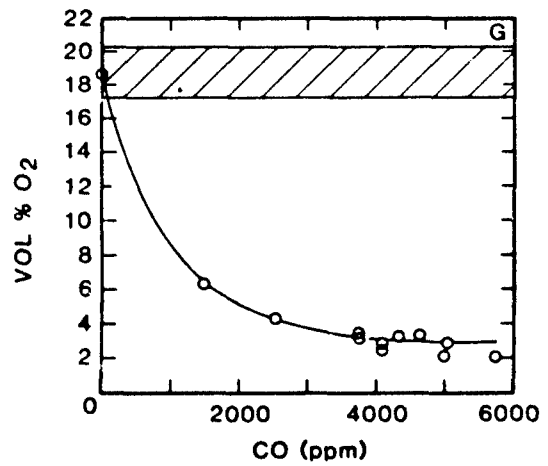


Figure 3. Equilibrium levels of various blood parameters from 30-min exposures to CO. Crosshatched areas indicate one standard deviation around the mean of the controls. (A) COHb and O_2Hb; (B) Base excess; (C) HCO_3^-; (D) pCO_2.







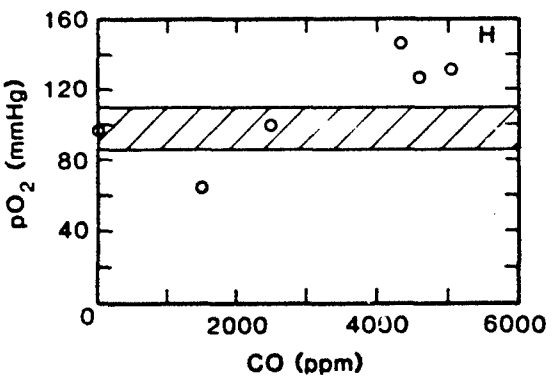


Figure 3 (continued). Equilibrium levels of various blood parameters from 30-min exposures to CO. Crosshatched areas indicate one standard deviation around the mean of the controls. (E) pH; (F) Total CO_2; (G) Vol. % O_2; (H) pO_2.

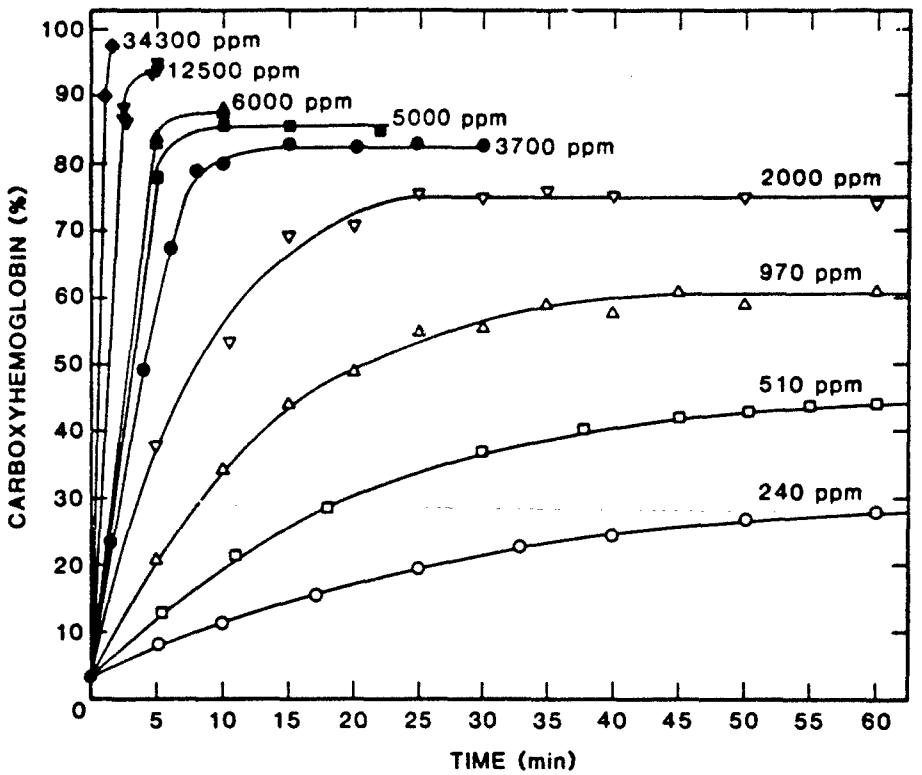


Figure 4. Rate of formation of COHb from exposures to various atmospheric concentrations of CO (ppm refers to CO concentrations).

Combined Carbon Monoxide and Carbon Dioxide Experiments

When nonlethal levels of CO_2 (1.7 to 17.3%) were added to sublethal concentrations of CO (2500 to 4000 ppm), some animals died either during or following the 30-min exposures (Figure 5 and Table 4). (Although 17.3% CO_2 was not tested by itself, it is considered nonlethal because 17.7% CO_2 in the presence of 3190 ppm CO did not cause deaths [Table 4].) As the CO_2 concentrations increased to 5%, the deaths occurred at increasingly lower levels of CO (down to approximately 2500 ppm). Above 5% CO_2, the curve shifted back to the right such that at the highest CO_2 concentration tested (17.7%), deaths were not observed in the presence of 3200 ppm CO. All postexposure deaths occurred within 24 h. No deaths were observed below 2500 ppm CO regardless of the CO_2 concentration. Carboxyhemoglobin levels observed in the combined experiments during which animals died ranged from 76 to 84% (Table 4). These values overlapped those in which only postexposure deaths were observed. There were other combined CO and CO_2 experiments, however, in which the COHb levels ranged from 76 to 81% and no deaths occurred (Table 4).

TABLE 3

BLOOD VALUES FOLLOWING CO_2 EXPOSURES

Lab	Time Exposed (min)	Gas Concentration		Blood Values								
		CO_2 (%)	O_2 (%)	O_2Hb (%)	COHb (%)	O_2 (%)	pH	pCO_2 (mmHg)	pO_2 (mmHg)	HCO_3^- (mM)	BE (mM)	TCO_2 (mM)
NBS	0	0.03	20.9	93.1[a]	3.2[a]	18.7[a]	7.47[a]	32.5[a]	91.9[a]	23.6[a]	1.6[a]	24.7[a]
				1.0[b]	0.7[b]	1.4[b]	0.05[b]	3.5[b]	9.2[b]	1.8[b]	2.3[b]	1.8[b]
	15	5.25	18.8	92.7	2.0	18.4	7.30*	62.8*	120.1*	30.6*	2.5	32.6*
		5.72	19.5	93.4	3.0	17.8	7.31*	49.9*	109.2	25.0	-1.6	26 6
				92.6	3.4	18.2	7.32*	50.0*	101.4	25.8	-0.6	27.4
		14.7	17.2	83.6*	0.4	16.3	7.00*	121.6*	108.5	29.6*	-6.4*	33.4*
				83.4*	0.6	16.4	7.03*	116.2*	104.9	30.4*	-5.0*	34.0*
	30	5.25	18.8	89.3*	2.1	15.0*	–	–	–	–	–	–
		5.72	19.5	93.7	2.9	16.8	7.31*	52.0*	108.8	26.0	-1.0	27.6
				93.2	3.4	18.5	7.33*	56.9*	111.1*	29.7*	2.6	31 5*
		14.7	17.2	86.6*	0.4	17.3	7.00*	126.8*	122.6*	29.5*	-7.1*	33.4*
				84.2*	0.6	17.3	7.00*	122.6*	114.3*	30.5*	-5.5*	34.3*
SRI[c]	0	–	–	–	–	–	7.42[a]	35.0[a]	101.5[a]	24.9[a]	–	–
							0.07[d]	2.5[d]	16.2[d]	4.8[d]	–	–
	30	8	–	–	–	–	7.28[a]	64.9[a]	113.9[a]	30.8[a]	–	–
							0.07[d]	8.8[d]	2.9[d]	1.9[d]	–	–
	30	16	–	–	–	–	7.10[a]	105.1[a]	109.4[a]	32.8[a]	–	–
							0.05[d]	13.6[d]	6.0[d]	2.0[d]	–	–
	30	18.8	–	–	–	–	7.08	103.5	108.0	31.2	–	–

[a] Mean.

[b] Standard deviation of controls (n = 5).

[c] Data from Rebert et al. (16).

[d] Standard deviation (n = 10).

* More than two standard deviations from controls (0 time values)

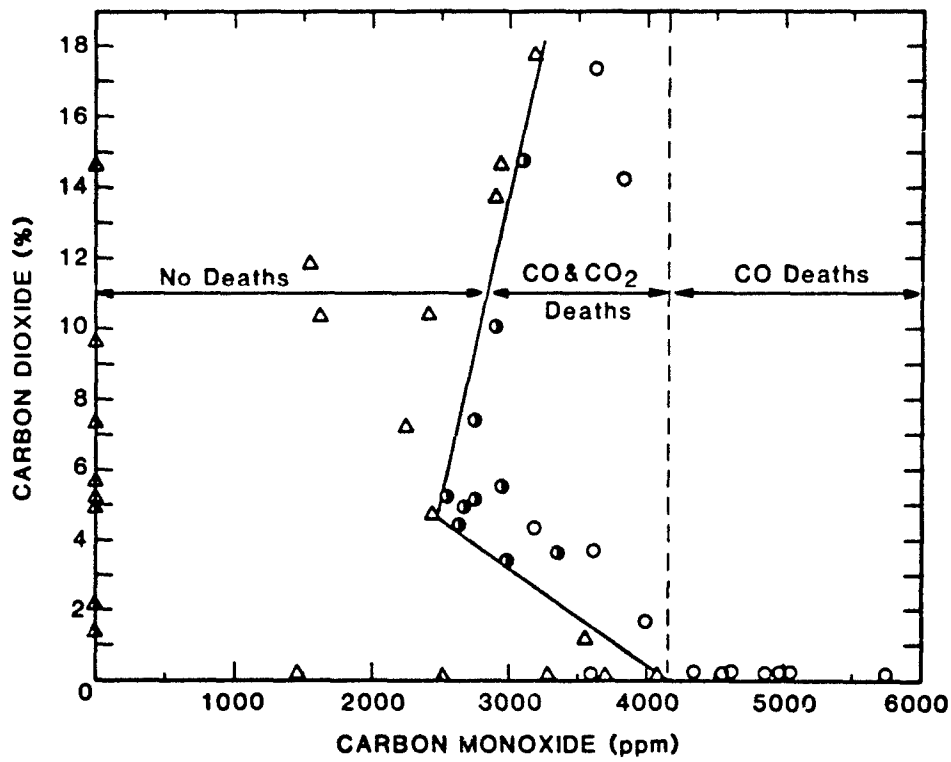


Figure 5. Deaths resulting from CO or CO_2 alone and from combined exposures to CO plus CO_2. No deaths (Δ); Deaths within-exposure (O); Deaths within- and postexposure (O). The solid line separates the experiments in which no deaths occurred from those in which one or more animals died.

It is well known that the presence of CO_2 will increase the animal's respiratory minute volume. This, in turn, will affect the rate of uptake of CO and consequently, the rate of formation of COHb. As shown in Figure 5, in the presence of 5% CO_2, the lowest lethal level of CO was 2500 ppm; therefore, the rate of formation and ultimate equilibrium levels of COHb were examined with this concentration of CO with and without 5.25% CO_2 (Figure 6).

Calculation of the slopes of the first seven values in each of the curves shown in Figure 6 indicates that the initial rate of formation of COHb was 1.5 times greater from 2500 ppm of CO in the presence of 5.25% CO_2 than in the absence of CO_2. The COHb equilibrium level was the same (78%), but was reached in approximately 10 min when CO_2 was present and in about 20 min without CO_2. Although roughly the same amount of blood was taken from each animal in both experiments, no animals died during or following the 30-min CO exposure (up to 90 min postexposure); whereas, in the CO plus CO_2 experiment, two animals died during the 30-min exposure and two died during the

TABLE 4

ANIMAL MORTALITY FROM CO AND CO_2 EXPOSURES

CO[a] (ppm)	CO_2[a] (ppm)	O_2[a] (%)	COHb[b] (%)	Deaths			Total Deaths No. Died/ No. Tested[d]
				Within Exp.	Postexp.	SPE[c]	
4,000[e]	16,900	17.5	84.0	4/6	0/6	0/6	4/6
3,820	142,400	17.5	83.1	6/6	0/6	0/6	6/6
3,620	173,200	20.7	82.0	1/6	0/6	2/6	1/4
3,610	37,500	19.5	–	4/5	1/5	0/5	5/5
3,560[e]	10,800	17.7	81.1	0/6	0/6	2/6	0/4
3,360	36,600	19.6	80.6	2/6	1/6	1/6	3/5
3,190	43,400	19.4	–	3/6	0/6	0/6	3/6
3,190	177,100	20.7	81.0	0/6	0/6	2/6	0/4
3,100	147,600	17.5	80.9	0/6	1/6	1/6	1/5
2,990	34,300	19.5	79.2	0/6	1/6	1/6	1/5
2,960	55,600	19.7	77.3	0/6	1/6	2/6	1/4
2,940	146,900	17.5	80.0	0/6	0/6	2/6	0/4
2,900	137,600	17.5	80.3	0/5	0/5	2/5	0/3
2,900	100,300	18.3	77.5	3/6	1/6	0/6	4/6
2,760	74,300	19.0	80.6	1/6	1/6	1/6	2/5
2,750	51,100	18.5	76.8	1/6	2/6	1/6	3/5
2,680	49,700	19.3	78.2	0/6	1/6	2/6	1/4
2,640	44,800	–	76.0	2/6	1/6	0/6	3/6
2,540	52,500	19.7	77.3	2/5	2/5	1/5	4/4[f]
2,450	47,500	19.5	68.1	0/6	0/6	2/6	0/4
2,410	104,700	18.1	77.8	0/6	0/6	2/6	0/4
2,240	73,000	19.1	75.9	0/6	0/6	1/6	0/5
1,620	103,600	18.2	70.2	0/6	0/6	2/6	0/4
1,530	118,800	17.7	69.2	0/6	0/6	2/6	0/4

All exposures are square wave except where noted.

[a] Average gas concentration over 30-min exposure.

[b] Value prior to end of 30-min exposure.

[c] Sacrificed postexposure because animals were cannulated.

[d] Number tested = number exposed minus number sacrificed for blood.

[e] Gradual exposure (i.e., gases introduced into chamber over 5-min period).

[f] All animals were cannulated.

postexposure period, at 7 and 31 min. In a 60-min exposure in which fewer animals were cannulated and less blood was sampled, animals exposed to 2660 ppm of CO reached 76.5 and 77.8% COHb by 15 and 30 min, respectively, and did not die either during or following the 60-min exposure. In other words, 78% COHb was not lethel, even for exposure times greater than 30 min. In a comparable experiment, exposure to 2640 ppm CO plus 4.4% CO_2 for 30 min caused the deaths of three of the six tested animals, one within the 30-min exposure and the other two at 30 s and 2 h postexposure; in this experiment, COHb levels were 76%.

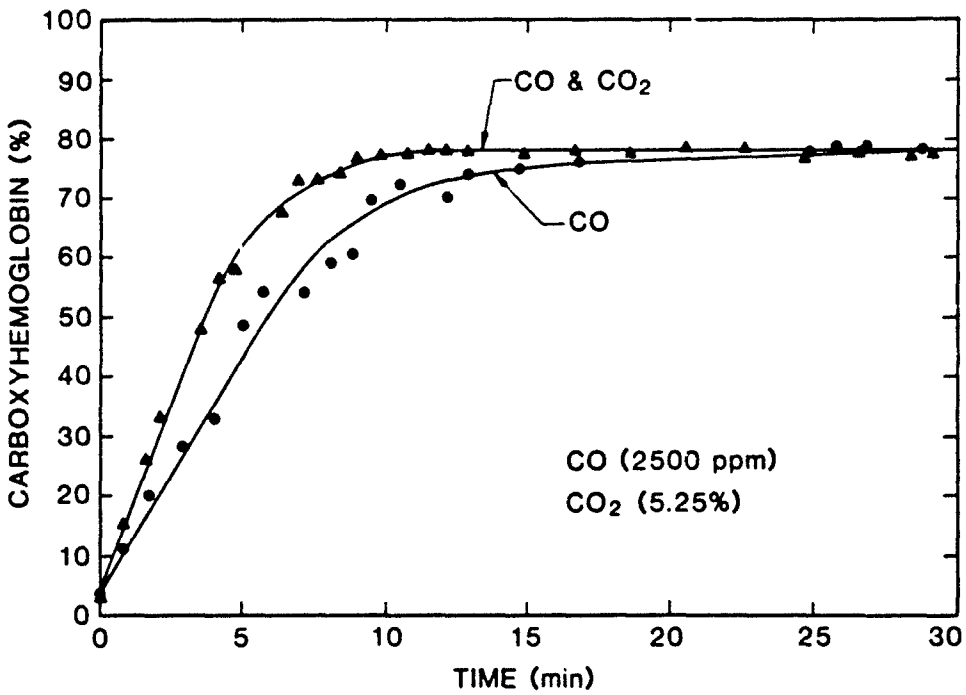


Figure 6. Rate of COHb formation during exposure to 2500 ppm CO alone (O) or with 5.25% CO_2 (Δ).

The O_2Hb levels, as expected, showed the inverse relationship to that observed for COHb; namely, levels decreased to approximately 23%, with and without 5% CO_2, and the rate of decrease was slightly faster with the CO_2 present. Recovery rates and O_2Hb concentrations appeared about equal in the two cases (Figure 7A). Volume % O_2 also showed results similar to that observed with O_2Hb (Figure 7B). The equilibrium levels seen with COHb, O_2Hb, and vol. % O_2 all appeared directly related to the CO concentration. Five percent CO_2 alone did not produce a significant change in these blood parameters (Table 3).

The pH (mean control value was 7.42 ± 0.07) decreases when the animals are exposed to CO alone (2500 ppm CO produces a pH of 7.2 in about 30 min), or CO_2 alone (5.7% CO_2 produces a pH of

7.3 in about 30 min), but the combination (2500 ppm CO and 5.25% CO_2) produces both a more rapid rate of decrease and a lower 30-min pH level (pH 6.8) (Figure 7C). The recovery rate was also quite different in that the pH started to rise about 5 min following the end of the CO exposure and reached normal levels by 30 min postexposure; whereas, in the case of CO plus CO_2, the pH continued to fall for at least an additional 30 min, reaching a final low of pH 6.6. These pH levels did not return to normal until 60 to 90 min following the end of the exposure.

The TCO_2 and HCO_3^- levels showed very similar profiles; that is, they initially rose slightly higher in the presence of CO and CO_2 than only CO, but in both cases, the levels started to decrease about 4 min into the exposure (Figures 7D and 7E). The rate of decrease and the levels were approximately the same throughout the 30-min exposures. During the recovery from the CO exposure, however, the TCO_2 and the HCO_3^- immediately started to rise; whereas, in the combined CO and CO_2 case, the levels remained low for at least another 30 min.

The base excess decreased in both cases, but the rate of decrease was faster and reached a lower level (-29.3 mM) at the end of the 30-min exposure to the combined CO_2 and CO than to either CO alone (-18.7 mM) or CO_2 alone (-1.0 to 2.6 mM) (Figure 7F and Table 3). Within 10 min following the CO exposure, the base excess started to rise; whereas, in the case of the CO plus CO_2, the base excess continued to drop, ultimately reaching levels as low as -36.8 mM by 30 min post-exposure. Sixty minutes after the end of the combined CO and CO_2 exposure, recovery was apparent.

The pCO_2 decreased throughout the 30-min exposure to CO alone and started to recover approximately 5 min following the exposure (Figure 7G). However, when the animals were exposed to CO plus CO_2, the pCO_2 increased above the control values during the exposure and immediately dropped below the control levels following the exposure. The pCO_2 remained low for at least 20 min before starting to show signs of recovery.

The pO_2 results were scattered but on a whole were above normal both during and following the exposures to CO or CO plus CO_2 (data not shown).

Application of Empirical Mathematical Models

To determine whether an additive or synergistic interaction was occurring between CO and CO_2, two empirical mathematical models were examined and fitted to our data by F. O'Sullivan and R. Wyzga. One model presumed that the response was purely additive and took the following form,

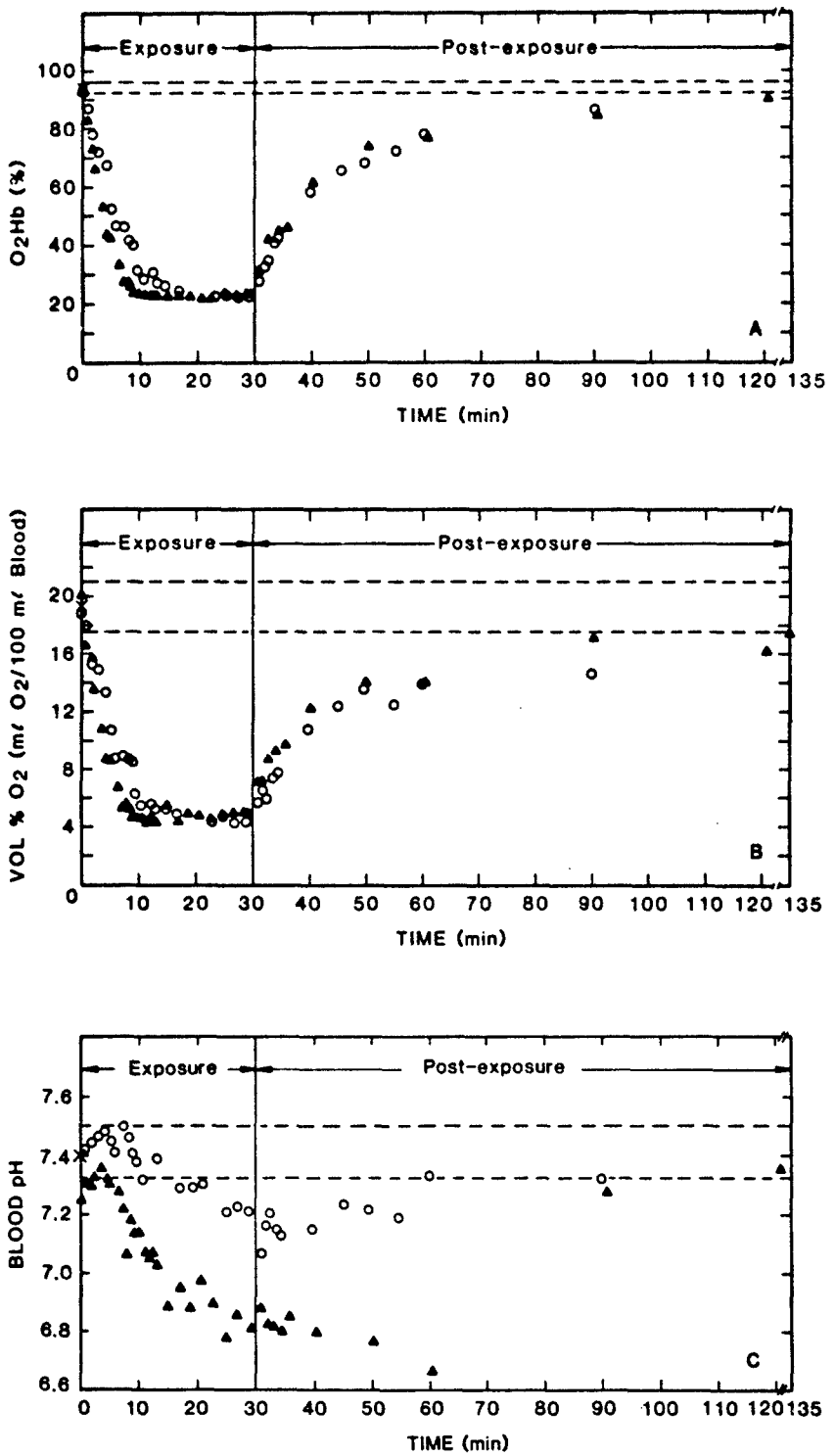


Figure 7. Changes in various arterial blood parameters during and following exposures to 2500 ppm CO alone (O) or with 5.25% CO_2 (Δ). Mean and standard deviation of the controls is indicated by the X and dotted lines. (A) O_2Hb, controls = 180; (B) vol. % O_2, controls = 180; (C) pH, controls = 56.

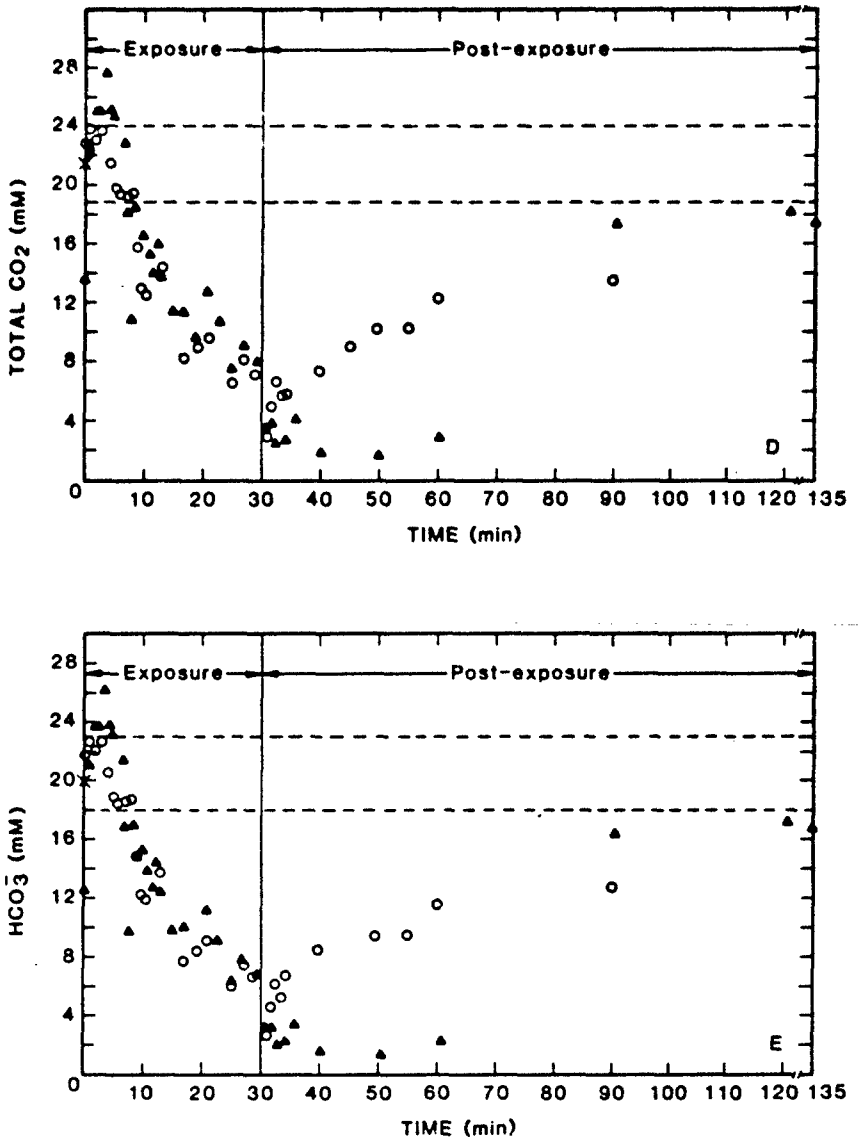


Figure 7 (continued). Changes in various arterial blood parameters during and following exposures to 2500 ppm CO alone (O) or with 5.25% CO_2 (Δ). Mean and standard deviation of the controls is indicated by the X and dotted lines. (D) TCO_2, controls = 141; (E) HCO_3^-, controls = 141.

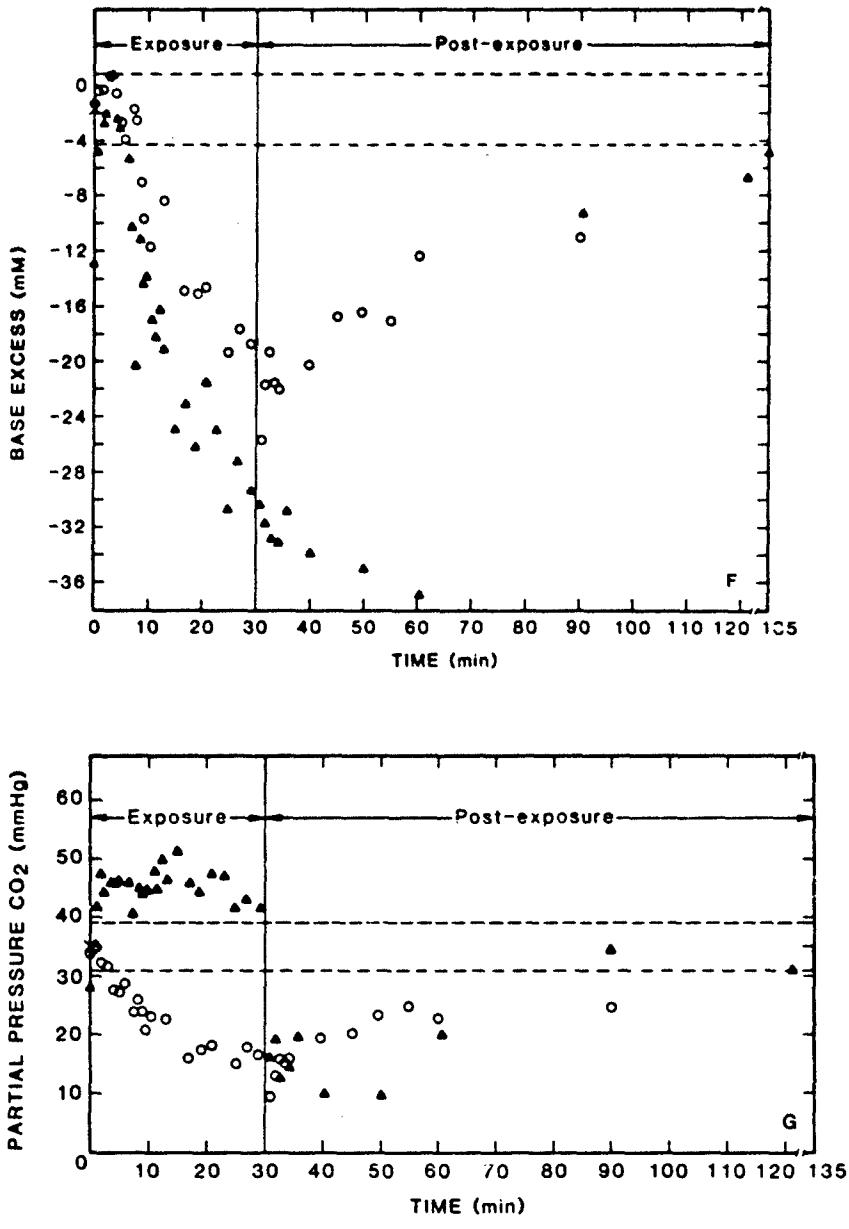


Figure 7 (continued). Changes in various arterial blood parameters during and following exposures to 2500 ppm CO alone (O) or with 5.25% CO_2 (Δ). Mean and standard deviation of the controls is indicated by the X and dotted lines. (F) base excess, controls = 141; (G) pCO_2, controls = 151.

$$\log \frac{P}{1-P} = \alpha + \beta_1[CO] + \beta_2[CO_2]$$

where P represents the probability of death occurring in an experiment and α and β are derived coefficients from the least-squares calculations. Terms in brackets are the average concentrations of CO and CO_2 in an experiment.

The other multivariable regression model presumed the existence of an interaction term (i.e., synergism exists between CO and CO_2). This model was an extension of the previous one and took the following form.

$$\log \frac{P}{1-P} = \alpha + \beta_1[CO] + \beta_2[CO_2] + \beta_3\frac{[CO]}{[CO_2]}$$

The results of this analysis showed that for the additive model, the scaled deviance was 30.27 for 19 degrees of freedom; whereas, for the interaction model, it was 24.17 for 18 degrees of freedom. An analysis of residuals for the interactive model showed that one outlier existed. This outlier existed because all the animals in this experiment were cannulated and more blood was taken from these animals than from animals in other experiments. Using GLIM to refit these data without the outlier showed that the scaled deviance was minimally affected in the linear model (29.90 for 18 degrees of freedom), whereas the interaction model had a scaled deviance of 17.87 for 17 degrees of freedom. The reduction of 6.3 units for 1 degree of freedom in the interaction model is highly significant. Residuals from this latter fit were all within two units of zero.

Carbon Monoxide and Carbon Dioxide Generation from Burning Solids

A series of natural and synthetic materials were thermally decomposed under both flaming and nonflaming conditions. The LC_{50} values for 30-min exposures and 14-day postexposure observation periods obtained from each test material in each combustion mode are presented in Table 5 along with their respective average CO, CO_2, and final COHb concentrations.

Polyphenylsulfone, decomposed in the nonflaming mode, was the only material that produced sufficient CO (4400 ppm) and COHb levels (84%) to implicate CO as the primary toxicant. The concentrations of CO produced from all the other materials decomposed at their LC_{50} values were too low (<4000 ppm) to attribute the resultant deaths to CO alone. Douglas fir, polyphenylsulfone, and red oak all decomposed in the flaming mode, generated lower-than-lethal CO levels, but produced COHb levels that appear to be sufficient (≥82%) to produce the deaths. In these three cases, the combination of CO and CO_2 was sufficient to account for the resulting deaths.

TABLE 5

CONCENTRATIONS OF CARBON MONOXIDE, CARBOXYHEMOGLOBIN, AND CARBON DIOXIDE FROM MATERIALS EXAMINED AT THEIR LC_{50} VALUES

Material	Mode	LC_{50}[a] (mg/l)	CO[b] (ppm)	COHb[c] (%)	CO_2[b] (%)	Deaths
ABS	F	19.3	1500	42	1.07	W
	NF	30.9	670	27	0.53	P
Douglas fir	F	39.8	3400	83	3.71	W
	NF	22.8	2700	81	0.69	W,P
Flexible polyurethane	F	>40	960[d]	65[d]	4.79[d]	W
	NF	22.6	820	54	0.32	P
Modacrylic	F	4.4	400	22	0.39	W,P
	NF	5.3	430	16	0.53	W
Polyphenylsulfone	F	19.8	3500	82	2.27	W,P[e]
	NF	9.5	4400	84	0.51	W
Polystyrene	F	38.9	1300	78	1.95	W
	NF	>40	72[d]	6[d]	0.21[d]	*
Poly (vinyl chloride)	F	17.3	1100	49	0.55	P
	NF	20.0	590	27	0.46	P
PVC + zinc ferrocyanide	F	15.0	2200	68	0.97	W,P
	NF	11.3	1200	36	0.53	P
Red oak	F	56.8	2800	83	4.06	W
	NF	30.3	2400	80	0.72	P
Rigid polyurethane	F	13.3	1700	61	1.20	W
	NF	>40	1700[d]	47[d]	0.94[d]	*
Wool	F	28.2	700	43	ND	W,P
	NF	25.1	920	41	0.71	W,P

[a] Based on deaths during 30-min exposures plus 14-day postexposure periods.

[b] Average 30-min concentration.

[c] Value obtained prior to end of 30-min exposure.

[d] Value obtained when 40 mg/l of material was tested.

[e] Only postexposure death occurred 5 min following exposure.

F = Flaming material decomposition.

NF = Nonflaming material decomposition.

W = Within-exposure deaths.

P = Postexposure deaths.

* = No deaths in range studied.

ND = No data.

DISCUSSION

Most toxicological experiments are designed to examine the effect of a single compound. In real life, however, the body is constantly bombarded by multiple compounds and drugs. It is now apparent that the ability to escape from a fire does not depend solely on the concentration of CO and the exposure time, but rather on the biochemical and physiological effects resulting from the interaction of the fire gases. If two compounds are introduced simultaneously, the response may be equal to the sum of the effects of each chemical by itself (an additive effect), or the combined effect may be greater (i.e., synergistic) or less (i.e., antagonistic) than that predicted by simple additivity (19). Another possible interaction is described as potentiation, the situation in which a compound that is noneffective at the tested concentration enhances the toxicity of another compound (19). In this particular case of CO and CO_2, potentiation was not considered the appropriate descriptive term because CO_2, while not lethal at the concentrations tested, does have profound physiological effects on respiratory rate, tidal volume, and the cardiovascular and central nervous systems (5,16,20).

The experimental results presented here are qualitatively similar to those of Nelson et al. (11), who found that the 30-min lethal concentration of CO in rats was 6000 ppm (when the levels of CO_2, approximately 1600 ppm, were primarily generated by the animals' respiration) and decreased to 2560 ppm CO when 1.44% CO_2 was present. Quantitatively, however, it appears from our experiments that the probability that this particular combination will produce deaths is low (Figure 5). It is more difficult to compare our results to their 2-h studies, which also showed interactive effects, since our experimental design did not include 2-h exposures.

Rodkey and Collison (10) also found decreased mean survival times in rats exposed to 6000 ppm CO plus 4.5% CO_2 (16.8 ± 0.6 min) compared to those exposed to 6000 ppm CO alone (22.4 ± 0.8 min). The results of Crane (9), however, disagree with those of Rodkey and Collison. Crane found no differences in the times-to-incapacitation and times-to-death in experiments in which rats were exposed to CO concentrations ranging from 5000 to 14,000 ppm with and without CO_2 concentrations of 4 to 13%. Both of these studies were performed at CO concentrations in which death of all the animals would be expected during 30-min exposures from CO alone. Although there are no experimental details in the report by Crane (9), it is assumed that the end points of incapacitation and death were monitored, as done previously in his laboratory, by observing when the animals stopped moving within rotating cages. In the study by Rodkey and Collison, the animals were allowed to move freely within the exposure chamber. Our experiments show that at 5000 to 6000 ppm of CO, the rate of uptake of CO is very rapid and the addition of CO_2 makes little difference in the rate of COHb formation. Also, animals being forced to perform work will have higher ventilation rates than those held still in restrainers, as in our current experiments.

The combination of being exposed to high CO levels and being forced to exercise could explain why the animals in Crane's experiments did not show shorter times-to-incapacitation or times-to-death. In other words, they were already loading the CO as fast as possible. Synergism could not be observed because the rats were already experiencing the maximum toxic effects from the CO alone. His protocol, which does not indicate a postexposure observation period, also would not detect the postexposure deaths due to the extensive and prolonged acidosis.

The studies by Edginton and Lynch (8) were similar to those of Crane in that the CO concentrations examined were by themselves high enough to be lethal in 30 min. According to our data, the low levels of CO_2 (0.89 or 1.2%) that they tested would not be expected to produce a significant difference in results when added to 5600 and 6000 ppm of CO.

An antagonistic effect was suggested by Pryor et al. (3) to explain the results observed in their animals exposed to combinations of CO and CO_2. In 4-h experiments on mice, they found the minimal lethal concentration of CO by itself was 1250 ppm (1 out of 10 animals and 7 out of 10 animals died in two experiments). At the minimal lethal concentration of CO_2 (40%), 4 out of 10 of the exposed mice died; the earliest death occurred at 35 min. The combined CO plus CO_2 experiments produced erratic results. The investigators concluded that fewer deaths occurred (e.g., 1250 ppm CO plus 30% CO_2 produced 4 out of 10 deaths) than with CO alone. Considering the variation of their results from 1250 ppm of CO alone, the CO plus CO_2 results could be considered greater than, less than, or the same as the CO alone.

Gaume et al. (7) performed very short experiments (3 min) to determine the time necessary to cause the collapse of mice in an exercise wheel when exposed to CO, CO_2, or a combination. Their results indicated that 13,000 ppm CO produced collapse in 54 ± 6.1 s, and 15 and 17% CO_2 caused collapse in 43 ± 6.1 s and 28 ± 1.2 s, respectively; whereas, 13,000 ppm CO plus 5% CO_2 produced collapse in 62 ± 2.0 s. Whether these results truly represent a significant antagonistic effect is debatable.

CO Experiments

The experimental evidence provided here showed that exposure to CO in air for 30 min causes death of rats at concentrations of 4600 to 5000 ppm and at COHb levels greater than 83%. All deaths from these CO exposures occurred during the exposure or the first minute following exposure. Although the pO_2 in the arterial blood increased, the greater affinity of hemoglobin for CO (240 times that of O_2) prevents the formation of O_2Hb. In addition, the presence of COHb shifts the O_2 dissociation curve to the left, such that the small amount of O_2 bound to the hemoglobin is less likely to be released at the partial pressures present at the tissues (21). The CO deaths are,

therefore, primarily attributable to the high COHb, the low O_2Hb, the greater difficulty in unloading O_2 to the tissues, and the subsequent tissue hypoxia.

In addition, the low blood pH observed in the animals exposed only to CO is indicative of a state of metabolic acidosis which is most likely due to the increase in anaerobic metabolism and the production of lactic acid. The pCO_2 and TCO_2 decrease because of the reduced respiration at the mitochondrial level due to the lack of O_2. The HCO_3^- and BE are consumed trying to compensate for the acidosis; the bicarbonate is not replenished because the CO_2 is not being produced.

Experiments performed with humans, dogs, and rats have shown that no increase in respiratory rate occurs from CO exposures regardless of the concentrations (22,23). Matijak-Schaper and Alarie (24) have shown that exposure to increasing concentrations of CO causes decreasing respiratory rates. As long as the pO_2 remains sufficiently high and the pCO_2 low, increased ventilation should not occur, although some data in the literature indicate that delayed hyperventilation may occur from spinal fluid lactic acidosis (21).

CO_2 Experiments

The CO_2 concentrations (1.3 to 14.7%) tested in this series of experiments were neither incapacitating nor lethal to rats exposed for 30 min. These results agree with those of Herpol et al. (23) who also found that rats exposed to CO_2 concentrations of up to 15% for 30 min never lost consciousness and with those of Pryor et al. (3) who found the minimal lethal CO_2 concentration in mice was 40% for 4 h.

Carbon dioxide does have extensive physiological effects. First of all, at concentrations of up to 10%, it acts as a respiratory stimulant. Above 10%, the minute volume and respiratory rate decrease (20,23). Schaefer et al. (25) exposed 38 human males to 3.3, 5.4, and 7.5% CO_2 for 15 min and noted increases in the respiratory minute volume. At 3 and 5% CO_2, the increase was primarily due to an increase in the tidal volume. At 7% CO_2, the respiratory rate also increased. Pulse rate also showed a 4% increase at 5% CO_2 and a 24% increase at 7% CO_2. Carbon dioxide also acts as a potent vasodilator which may produce a greater cerebral blood flow (18). The vasodilation of the blood vessels in the brain acts to increase the blood flow and to bring more O_2 to the brain tissues.

As expected from increased atmospheric levels of CO_2, we found that the arterial pCO_2 and TCO_2 rose; this, in turn, caused the HCO_3^- to increase. The high pCO_2 causes the arterial pH to decrease and, as a consequence, the base excess also is depleted in an attempt to compensate for the respiratory acidosis. The decreased pH shifts the oxyhemoglobin dissociation curve to the right, which reduces the O_2 saturation of hemoglobin even though the pO_2 has not decreased. This shift resulted in an O_2Hb of 83 to 86%. The pO_2 is higher due to a better ventilation-perfusion relationship created by the increase in tidal volume associated with the increased pCO_2. Table 3 also

shows the blood results from experiments performed at SRI International (Menlo Park, CA) in which rats were exposed for 30 min to concentrations of CO_2 ranging from 8 to 18.8% (16). These investigators also found that the pH decreased while the pCO_2, pO_2, and HCO_3^- increased.

Combined CO and CO_2

When sublethal amounts of CO_2 were added to sublethal concentrations of CO, some of the rats died during either the 30-min exposures or in the following 24 h. The increase in tidal volume and, to some extent, respiratory rates that have been known to occur in the presence of increased partial pressures of CO_2 (20,23) suggest that the deaths may be due in part to an increased rate of formation of COHb. The rate of formation of COHb depends in part on the atmospheric concentration of CO (Figure 4). Other factors that affect the rate of CO absorption include the rate and depth of breathing, the exposure duration, the blood volume, the barometric pressure, and the diffusivity of CO through the lung (26). The COHb equilibrium levels, however, are directly dependent upon the ambient CO.

The results indicated that the rate of formation of COHb from an exposure to approximately 2500 ppm of CO was 1.5 times greater in the presence of 5.25% CO_2 than in its absence. The COHb equilibrium level, although reached faster in the presence of CO_2, was exactly the same, 78%. The increased rate of COHb formation and the maintenance of the equilibrium level at 78% for a greater time period was not sufficient to explain the deaths that occurred with the combined CO and CO_2 for a period of 30 min, because in 60-min experiments, animals with COHb levels of 78% (induced with CO alone) for longer than 30 min did not die. Therefore, the death of the animals from these sublethal concentrations of CO and CO_2 does not appear to be due solely to the slightly increased rate of formation of COHb.

Examination of other blood parameters showed that the final levels of O_2Hb and vol. % O_2 appeared to be primarily dependent upon the CO concentrations. The only effects on O_2Hb and vol. % O_2 noted in the combination experiments were slightly faster rates of decrease. The pH, however, in the presence of the combined gases, decreased faster, reached a lower level during the exposure, continued to fall for at least 30 min following the exposure, recovered at a slower rate than the pH values seen in the CO exposures, and indicated a severe and prolonged acidosis.

The main differences between the CO and CO plus CO_2 experiments with regard to the TCO_2 and HCO_3^- blood values were seen in the recovery period where the values remained low for at least 30 min before showing signs of returning toward normal. Base excess also was affected by the addition of CO_2 to CO; it decreased to a lower level during the exposure and continued to decrease for at least 30 min following the exposure before some recovery was observed. The pCO_2 values were high during the exposure but dropped to quite low levels following the exposures and

remained low for at least 20 min. This effect of low pCO_2 following initial high levels of pCO_2 is termed hypocapnia and has been examined previously (16,25). The drop has been attributed to the increased respiratory activity which continues even after the pCO_2 has decreased. Schaefer et al. found that 5% CO_2 provided the maximum respiratory stimulus for the hypocapnia (25). We have shown here that hypocapnia still occurs even in the presence of CO.

Therefore, it is apparent that a combination of respiratory and metabolic acidosis occurs in the animals when exposed to both CO and CO_2. The blood values indicated that signs of recovery were also delayed for at least 30 min following the end of the combined gas exposures and full recovery did not occur until 60 min postexposure; in contrast, in the CO experiments, recovery occurred within the first 5 min of the postexposure period.

Examination of Solid Materials

The question still remains whether deaths in fires can be attributed to the major toxic gases produced from the thermal decomposition of the involved materials or if the toxicities of minor or more obscure gases need to be examined. Eleven materials decomposed at their LC_{50} values (based on 30-min exposures and 14-day postexposure periods) under both flaming and nonflaming conditions produced CO concentrations that in only one case could explain the lethalities that occurred. That case, polyphenylsulfone in the nonflaming mode, produced an average CO concentration of 4400 ppm and a COHb level of 84%. Also, the deaths only occurred within exposure as expected from CO toxicity.

To determine whether the combination of CO and CO_2 was sufficient to account for the resultant deaths from exposure to the thermal decomposition products from the other materials, an empirical mathematical relationship was derived from the rather sharply bounded death/no death region shown in Figure 5. This relationship is shown below.

$$\frac{m[CO]}{[CO_2]-b} \geq 1$$

$[CO]$ and $[CO_2]$ are the atmospheric concentrations of CO and CO_2 in parts per million, respectively; m and b are the slope and y intercept of the solid line in Figure 5 and equal -28 and 117,000 ppm, respectively, if the atmospheric concentration of CO_2 is $\leq 5\%$, and 150 and -313,000 ppm, respectively, if CO_2 is $>5\%$. If the product of the equation is < 1, the animals will live.

Based on this formula, the resultant deaths from the flaming decomposition of Douglas fir, red oak, and polyphenylsulfone would be predictable based on the concentrations of CO and CO_2. The deaths occurring from the other materials, however, could not be explained based on these two

gases either alone or in combination. Thus, in these other cases, the toxic interactions of additional fire gases are being examined.

In conclusion, the experiments reported here show that above a certain concentration of CO (4100 ppm), some of the animals will die from CO poisoning, and adding CO_2 will not make any difference. On the other hand, below a certain level of CO (2500 ppm), the addition of CO_2 (up to 17.7%) is not sufficient to make the combination lethal. There is, however, a range of CO concentrations (2500 to 4100 ppm) which when presented alone to the animals has a very low probability of causing death, but in the presence of certain levels of CO_2 (>1.5%) will act with a much higher probability to cause the death of the animals. The two gases act together by (1) increasing the rate of formation of COHb, (2) causing a severe degree of acidosis (which is greater than the metabolic acidosis from exposure to CO alone or the respiratory acidosis from CO_2 alone), and (3) prolonging the recovery period from this acidosis following exposure. The end result is that the animals die both during and following combined exposures to sublethal levels of CO and CO_2.

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QUESTION AND ANSWER SESSION

DR. YANG (NIEHS): My compliments on a very interesting and stimulating presentation. I have a couple of questions. In several plots you predict a safety zone. I was wondering whether the exposure-time element could be additional dimension such that you could predict not a zone, but a safety volume; that's question number one. Question number two; I was very impressed with the Maryland human victim data. When you mentioned changing the manufacturer and the supplier of animals you have different data. I was wondering, wouldn't it be prudent in this exercise to also use some 24-month-old animals and test them? So that your prediction of safety is not based on the joggers, the athletes, and so on, but also on some different age groups and different health conditions?

DR. LEVIN: I think both of those suggestions are well taken. When we first did the matrix of the safe zone we had only 30-minute data, and now that we have the other time data I am hoping to be able to fit that in in some kind of surface response curve that we could get out of it. I think your suggestion of using older animals might be a very good one.

EFFECTS OF IRRITANT GASES ON AVOIDANCE/ESCAPE PERFORMANCE AND RESPIRATORY RESPONSE OF THE BABOON

Harold L. Kaplan, Ph.D.

Department of Fire Technology, Southwest Research Institute, 6220 Culebra Road, San Antonio, Texas 78284

SUMMARY

The major toxicants in smoke are generally categorized as asphyxiants (hypoxia-producing agents) and irritants. Although the rodent appears to be an adequate model for evaluating the toxic effects of asphyxiant gases in man, the suitability of the rodent for evaluating the effects of irritant gases has not been established. In a study of the effects of irritant gases on escape performance of the baboon, exceedingly high concentrations of neither acrolein nor hydrogen chloride (HCl) prevented performance of the behavioral task; however, severe irritant effects were evident, even at lower concentrations. In a subsequent study of the respiratory response of the baboon to HCl, 15-min exposures to 5000 and 10,000 ppm (nominal concentrations) produced severe hypoxemia with a concentration-related increase in respiratory rate and minute volume. The difference between the rodent and the nonhuman primate in response to irritant gases suggests that the rodent may be an inadequate model for evaluating the toxicity of irritant gases to man and, therefore, the use of results of laboratory combustion tests to predict the toxicity of smoke in humans may lead to erroneous conclusions.

INTRODUCTION

When materials are combusted they produce smoke consisting of liquid and solid particulates and gases. The gases include a wide variety of chemicals and have the potential to act on many systems of the body. However, hypoxia and irritation are the predominant effects generally observed in laboratory combustion tests with rodents. Consequently, the major toxicants of smoke have often been categorized into two main classes, the asphyxiants (hypoxia-producing gases) and the irritants; a third class has been designated for those gases with other effects (1). The predominant hypoxia-producing gases in smoke are carbon monoxide (CO) and hydrogen cyanide (HCN), and irritant gases in smoke may include acrolein and other aldehydes, ammonia (NH_3), hydrogen chloride (HCl), and other acid gases (Table 1).

Several laboratory test methods have been developed to evaluate the toxicity of smoke produced by materials (2). These methods differ considerably in the combustion device, the combustion conditions, and other features, but all use either the rat or the mouse as the bioassay model and lethality as the primary index of toxicity. A major dilemma facing combustion toxicologists is how to use the lethality of the rat or mouse to evaluate the toxicity of smoke to man.

TABLE 1

CLASSIFICATION OF THE MAJOR TOXICANTS IN SMOKE

Asphyxiants (Hypoxia-Producing Gases)
• CO, HCN, CO_2
• O_2 Depletion
Irritants
• NH_3, HCl, HF, HBr
• Aldehydes
Other Toxicants
• Benzene, Toluene

The rodent appears to be a reasonable model for predicting the effects of hypoxia-producing gases in man, even though these species differ in the rate of uptake of these gases. However, the suitability of the rodent for evaluating the effects of irritant gases in man has not been established.

During the past four years, this laboratory has been investigating the effects of irritant gases in nonhuman primates and rodents. One of the purposes of these studies was to evaluate the utility of laboratory combustion toxicity tests with rodents to predict the toxic effect of smoke in man. This paper summarizes the results of two of these studies.

METHODS

Effects of Fire Gases on Avoidance/Escape Performance of the Baboon

The objective of the first study was to evaluate the effects of short exposures to CO, acrolein, and HCl on the escape performance of a nonhuman primate (3). The juvenile baboon was selected as the animal model, and a recirculating gas mixing/exposure system and a test apparatus were developed for measuring the effects of these gases on baboon escape performance (Figure 1). The animals were trained to discriminate between a red light and a white light above each of two levers, depress the appropriate lever identified by the white light in order to open an escape door, and then exit through the door into an adjacent chamber within 30 s. For each exposure, the gas bypass loop was closed, and the test gas was recirculated from the gas mixing chamber through the animal exposure chamber. After 5 min of exposure, the escape performance test was presented to the animal. If the animal did not exit within 10 s, shock was applied to the bars of the cage and maintained for 20 s (Figure 2). If the animal depressed the correct lever and exited within 10 s, the response was designated an "avoidance" response; if the animal exited after 10 s but within 30 s, the response was designated an "escape." Avoidance and escape responses were both considered successful escape performance. A comparable shuttlebox and behavioral paradigm were used to measure escape performance in the rat, except that the rat was not required to discriminate between red and white lights (3).

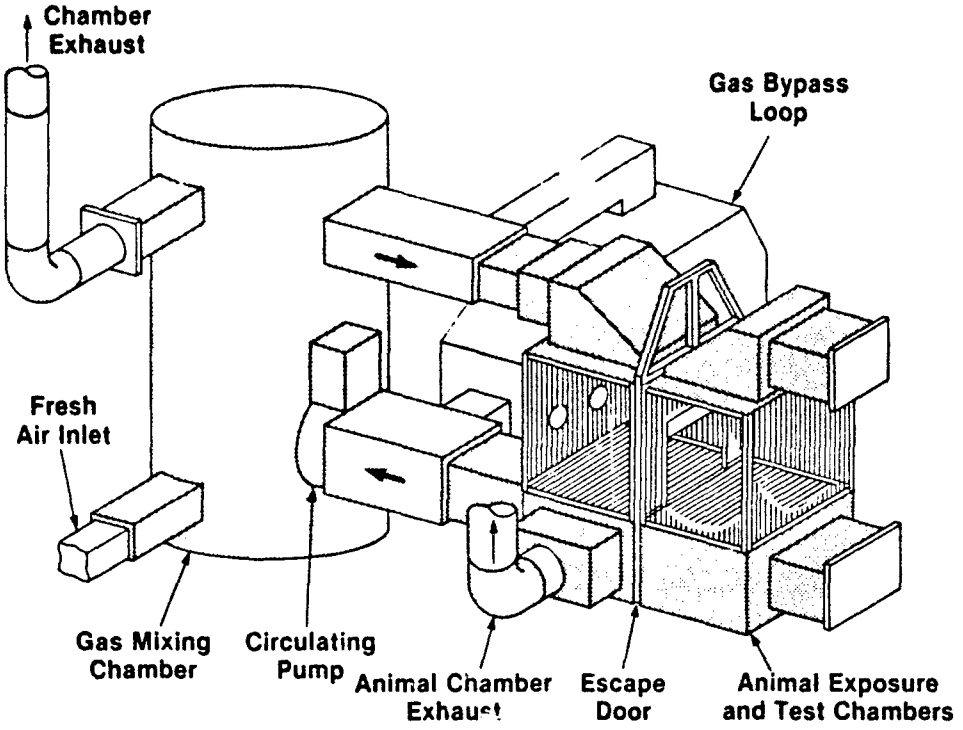


Figure 1. Gas-mixing/exposure system and escape performance test apparatus for primate tests.

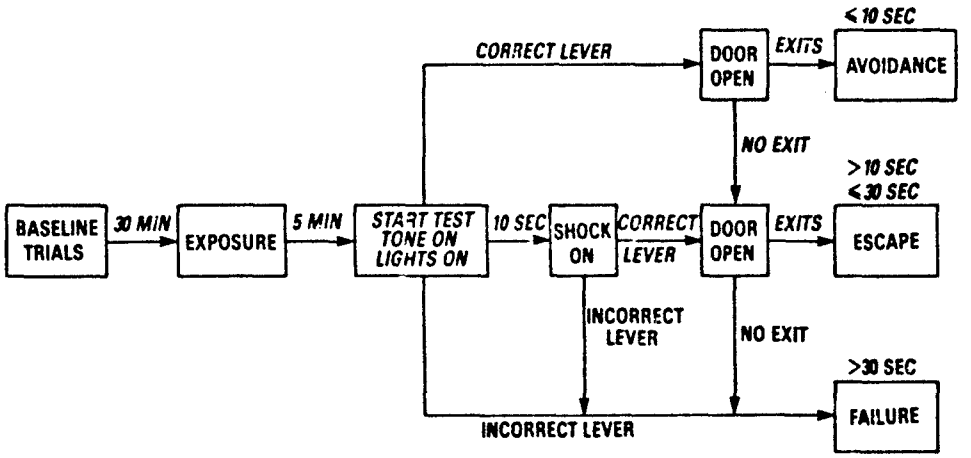


Figure 2. Behavioral paradigm for measurement of escape performance in the baboon.

A number of performance parameters were measured and recorded by the behavioral control system, which consisted of a Data General Nova 3 minicomputer equipped with a BRS/LVE Corporation INTERACT System. These performance data included (1) whether the response was an avoidance, escape, or failure response; (2) the time to first lever press; (3) the time to first correct lever press; (4) the time to exit the chamber; (5) the number of correct and incorrect lever presses;

and (6) the number of intertrial lever presses (ITIs), that is, the number of presses made prior to presentation of the escape performance test.

Effects of HCl on Respiratory Response and Pulmonary Function of the Baboon

The objective of the second study, which is in progress, was to evaluate the effects of HCl on the respiratory response and pulmonary function of the baboon. Nine baboons were lightly anesthetized with ketamine and, while in a supine position, were exposed to one of three nominal concentrations of HCl (500, 5000, and 10,000 ppm) for 15 min in a 200-l headbox. Three anesthetized control animals were exposed to air. Respiratory parameters were monitored by means of a Respitrace® System (Ambulatory Monitoring, Incorporated, White Plains, NY), which utilizes two transducer coils placed around the rib cage and abdomen of the animal. Respiratory movements change the inductance of each coil, and these changes are converted into measurements of respiratory rate (f), tidal volume (V_T), and minute volume (MV). Arterial blood samples were obtained from a catheter in the femoral artery prior to exposure, every minute during exposure, and every 5 min postexposure until the animal's appearance was normal. Blood samples were analyzed for pH, PaO_2, and $PaCO_2$ using an IL 1302 Blood Gas Analyzer (Instrumentation Laboratories, Incorporated, Lexington, MA).

Standard pulmonary function and carbon dioxide (CO_2) challenge response tests to 10% CO_2 were conducted with each control and each experimental animal the week prior to exposure to HCl or air and at 3 days and 3 months postexposure. For the CO_2 challenges, anesthetized animals were exposed to CO_2 for 10 min in the same headbox used for exposures to HCl and air. The CO_2 was metered into the headbox at a flow rate sufficient to reach a 10% concentration at the end of 5 min; this concentration was maintained during the next 5 min. Respiratory parameters (f, V_T, and MV) were recorded prior to, during, and after exposure to CO_2. Anteroposterior and lateral chest radiographs were obtained in conjunction with the pulmonary function tests.

RESULTS

Effects of Five Gases on Avoidance/Escape Performance of the Baboon

With CO, six baboons and four to six rats were exposed to each of four concentrations of the gas in order to evaluate concentration-response relationships for the escape performance measures. The EC_{50} values for escape failure were determined to be 6850 ppm (95% confidence limits: 6043-7773 ppm) for the baboon, and 6780 ppm (95% confidence limits: 6367-7271 ppm) for the rat (Figure 3). Exposure to CO did not affect any of the other performance measures in the baboon, except for a decrease in the number of intertrial responses per minute with increasing concentrations of CO (Table 2). In the rat, the ITi responses were not affected, but test escape times increased as the CO concentrations increased (Table 3).

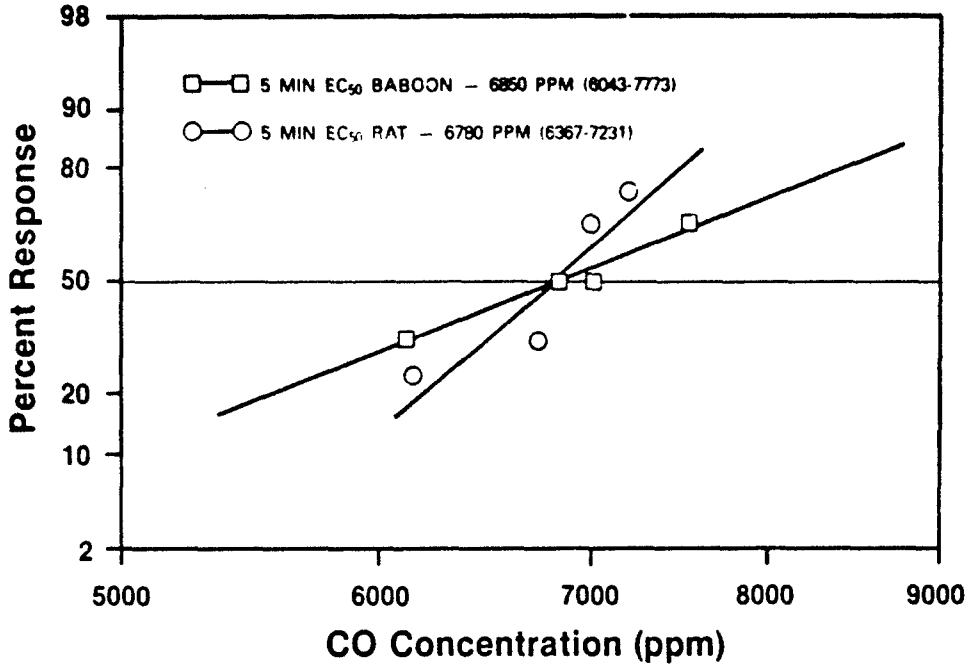


Figure 3. Concentration-response curves and EC_{50} values for escape failure of the baboon and the rat exposed for 5 min to CO.

TABLE 2

EFFECTS OF CARBON MONOXIDE ON ESCAPE PERFORMANCE OF THE BABOON

Effects	Average CO Concentration (ppm)			
	6120 ± 47	6840 ± 30	7010 ± 114	7520 ± 86
Number of Responses	4 Avoid 2 Fail	2 Avoid 1 Escape 3 Fail	2 Avoid 1 Escape 3 Fail	2 Avoid 4 Fail
Avoid/Escape Times (s)[a]				
Preexposure	7.4 ± 1.6	6.2 ± 0.5	6.6 ± 0.8	7.9 ± 1.8
Test	4.2 ± 1.5	8.5 ± 3.6	9.4 ± 3.1	5.0 ± 1.2
Time to First Lever Press (s)[a]				
Preexposure	3.0 ± 0.7	2.4 ± 0.4	2.2 ± 1.0	2.9 ± 0.9
Test	1.9 ± 1.3	2.6 ± 1.8	2.4 ± 1.1	1.7 ± 0.4
Time to First Correct Lever Press (s)[a]				
Preexposure	4.6 ± 1.3	3.1 ± 0.3	3.5 ± 1.0	3.6 ± 0.2
Test	1.9 ± 1.3	2.6 ± 1.8	5.4 ± 4.6	1.7 ± 0.4
Number of Incorrect Lever Presses[a]				
Preexposure	0.5 ± 0.3	0.4 ± 0.3	0.9 ± 0.3	0.6 ± 0.6
Test	0.0 ± 0.0	0.3 ± 0.6	2.0 ± 1.0	0.0 ± 0.0
Number of Lever Presses/min during ITI[a,b]				
Preexposure	1.0 ± 0.6	1.0 ± 0.8	1.7 ± 1.2	1.5 ± 1.6
Test[c]	2.5 ± 1.7	1.8 ± 1.9	2.4 ± 1.7	2.1 ± 1.7

[a] Values represent mean ± standard deviation (N = No. of avoid/escape animals)

[b] ITI = Intertrial interval

[c] Significant ($p<0.05$) negative correlation between test number of lever presses per minute during ITI and CO concentration with linear regression analysis of z scores

TABLE 3

EFFECTS OF CARBON MONOXIDE ON ESCAPE PERFORMANCE OF THE RAT

Effects	Average CO Concentration (ppm)			
	6150 ± 48	6730 ± 46	6990 ± 47	7220 ± 195
Number of Responses	3 Escape 1 Fail	4 Escape 2 Fail	1 Avoid 1 Escape 4 Fail	1 Escape 3 Fail
Avoid/Escape Time (s)[a]				
Preexposure	6.4 ± 4.3	6.3 ± 1.8	3.3 ± 1.6	11.3 ± 5.4
Test[b]	12.2 ± 1.2	18.6 ± 5.5	8.7 ± 1.6	18.9
Time to First Lever Press (s)[a]				
Preexposure	N/A	4.8 ± 1.9	2.2 ± 1.5	N/A
Test	N/A	14.4 ± 3.5	6.3 ± 5.4	N/A
Number of Lever Presses[a]				
Preexposure	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.2 ± 0.4
Test	2.0 ± 1.1	1.0 ± 0.0	1.5 ± 0.7	1.0
Number of Lever Presses/min during ITI[a,c]				
Preexposure	0.2 ± 0.3	0.1 ± 0.1	0.3 ± 0.6	0.1 ± 0.1
Test[c]	0.6 ± 0.6	1.2 ± 1.1	0.9 ± 0.6	1.6 ± 1.0

[a] Values expressed as mean ± standard deviation (N = No. of avoid/escape animals)

[b] Significant ($p<0.05$) positive correlation between test escape time and CO concentration with linear regression analysis of z scores

[c] ITI = Intertrial interval

N/A - Data not available due to equipment malfunction

In contrast to CO, neither acrolein nor HCl prevented performance of the escape task by the baboons. In eight experiments in which animals were exposed to acrolein in concentrations of 12 to 2780 ppm, all animals made avoidance responses; that is, they depressed the correct lever and exited within 10 s (Table 4). In one experiment in which the animal failed to exit the chamber, the apparatus malfunctioned; thus the result is not considered meaningful. Although differences between preexposure and test values for all performance measures were not statistically significant, test escape times were slightly less than the preexposure times in most of the experiments. The two animals exposed to the two highest concentrations of acrolein (1025 and 2780 ppm) developed severe respiratory complications and expired with severe pulmonary edema at 24 h and 1.5 h, respectively, following exposure. The effects of acrolein on escape performance were not investigated in the rat; however, Dr. C.R. Crane of the Federal Aviation Administration/Civil Aeromedical Institute observed that 5000 to 10,000 ppm of acrolein incapacitated rats in the motor-driven exercise wheel in 5 to 7 min, and that death occurred shortly thereafter (4).

TABLE 4

EFFECTS OF ACROLEIN ON ESCAPE PERFORMANCE OF THE BABOON

Average Acrolein Conc. (ppm)	Test Response	Avoid/Escape Time (s)[a]	Time to 1st Lever Press (s)[a]	Time to 1st Correct Lever Press (s)[a]	No. of Incorrect Lever Presses[a]	No. Lever Presses/min during ITI[a,b]
		Preexposure/Test	Preexposure/Test	Preexposure/Test	Preexposure/Test	Preexposure/Test
12	Avoid	6.3 ± 2.4/4.9	1.7 ± 0.9/1.1	3.0 ± 2.4/1.5	1.3 ± 1.7/1	1.4 ± 1.4/2.6
25	Avoid	5.0 ± 0.4/6.5	1.8 ± 0.4/3.2	1.8 ± 0.4/3.1	0.0 ± 0.0/0	0.0 ± 0.0/0
95[c]	Fail	d/d	d/d	d/d	d/d	d/d
100	Avoid	5.9 ± 1.2/5.8	2.7 ± 1.3/3.1	2.7 ± 1.3/3.1	0.0 ± 0.0/0	0.8 ± 1.1/0.6
250	Avoid	5.8 ± 1.4/5.2	2.3 ± 1.4/2.1	2.3 ± 1.4/2.1	0.0 ± 0.0/0	0.0 ± 0.00/0
505	Avoid	6.8 ± 1.8/5.1	2.6 ± 1.5/1.7	3.7 ± 1.9/1.7	1.3 ± 1.7/0	0.0 ± 0.2/0.4
505[c]	Avoid	6.6 ± 1.7/4.7	1.2 ± 0.6/0.9	1.7 ± 1.4/0.9	1.0 ± 0.9/0	1.9 ± 1.7/3.6
1025[c]	Avoid	5.9 ± 1.7/4.0	1.7 ± 0.9/1.1	2.6 ± 1.3/1.1	0.9 ± 0.9/0	2.8 ± 0.8/4
2780[e]	Avoid	6.6 ± 1.7/4.3	2.4 ± 1.8/1.8	2.4 ± 1.8/1.8	0.0 ± 0.0/1	1.5 ± 2.3/1.8
Mean ± S.D.		6.1 ± 0.6/5.1 ± 0.8	2.0 ± 0.5/1.9 ± 0.9	2.5 ± 0.6/1.9 ± 0.9	0.6 ± 0.6/0.3 ± 0.5	1.0 ± 1.0/1.4 ± 1.6

[a] Preexposure value for each concentration expressed as mean ± standard deviation (N = 10-12); test value (N = 1)
[b] ITI = Intertrial interval
[c] Subjects not previously exposed to acrolein
[d] Equipment malfunction, data not included
[e] Animal exposed to 100 ppm acrolein 5 weeks earlier

In eight experiments in which baboons were exposed for 5 min to HCl at concentrations of 190 to 17,290 ppm, six animals made avoidance responses and two made escape responses (Table 5). The two animals exposed to the two highest concentrations (16,570 and 17,290 ppm) expired several weeks later from bacterial pneumonia. The exposures did not affect any of the performance measures in the baboon, except that the number of ITI responses increased with increasing concentrations of the gas. With rats, 12 experiments were conducted with HCl at concentrations of 11,800 to 87,500 ppm (Table 6). All of the animals made avoidance or escape responses except for the experiment at 87,660 ppm in which the animal died after about 3 min of exposure. As with the baboon, the number of ITI responses made by the rat increased with increasing concentrations of HCl.

Effects of HCl on Respiratory Response and Pulmonary Function of the Baboon

The actual concentrations of HCl to which the nine experimental animals were exposed (Table 7) differ from the nominal or "target" concentrations of 500, 5000, and 10,000 ppm. These deviations were primarily caused by inaccuracies inherent in the continuous analysis methods that were used to establish and maintain the HCl concentrations during exposure.

The animals responded to HCl with a concentration-related increase in respiratory frequency (Figure 4). Upon exposure to the two highest nominal concentrations, respiration ceased for approximately 10 to 20 s, after which the frequency rapidly increased and continued to increase over several minutes. In the 5000 ppm HCl-exposed animals, the average increase in the f-value was approximately 50% of baseline and, in the 10,000 ppm HCl-exposed animals, the increase was approximately 100%. The V_T was not significantly altered by exposure to any of the HCl concentrations (Figure 5). As a result of the increased f and unchanged V_T, respiratory MV increased in the HCl-exposed animals, and this increase appeared to be concentration dependent (Figure 6).

Despite the increased ventilation in response to inhalation of HCl, arterial PaO_2 values decreased rapidly in the 5000 ppm and 10,000 ppm HCl-exposed animals and reached levels of 40 to 50 mmHg by the end of the 15-min exposure (Figure 7). This hypoxemic condition persisted for at least 10 min after the exposure. Blood $PaCO_2$ values increased, and blood pH decreased, but the changes were not as severe as the changes in PaO_2.

At both 3 days and 3 months postexposure, blood pH and gases were within the normal range. The results of the pulmonary function tests at both 3 days and 3 months postexposure did not show a significant change from preexposure values in any of the pulmonary function parameters in the HCl-exposed animals. Also, HCl exposure did not cause significant alterations in the respiratory response of the baboon to CO_2 at 3 days or 3 months following exposure.

TABLE 5

EFFECTS OF HYDROGEN CHLORIDE ON ESCAPE PERFORMANCE OF THE BABOON

Average HCl Conc. (ppm)	Test Response	Avoid/Escape Time (s)[a]	Time to 1st Lever Press (s)[a]	Time to 1st Correct Lever Press (s)[a]	No. of Incorrect Lever Presses[c]	No. Lever Presses/min during ITI[a,b]
		Preexposure/Test	Preexposure/Test	Preexposure/Test	Preexposure/Test	Preexposure/Test
190	Avoid	5.4 ± 1.4/4.5	1.5 ± 0.9/0.9	1.8 ± 1.2/0.9	0.8 ± 0.7/1	3.0 ± 3.0/2.8
810	Avoid	5.1 ± 1.0/4.4	1.5 ± 0.2/1.5	1.5 ± 0.2/1.5	0.0 ± 0.0/0	0.0 ± 0.0/1.8
890	Avoid	7.7 ± 1.7/5.2	2.6 ± 1.1/1.8	3.5 ± 1.4/1.8	0.9 ± 1.2/2	1.0 ± 2.1/2.6
940[d]	Avoid	5.0 ± 2.4/4.2	1.4 ± 1.4/1.4	2.2 ± 1.9/1.4	0.9 ± 0.9/1	2.6 ± 2.2/1.2
2,780[e]	Avoid	6.0 ± 1.3/7.8	2.0 ± 1.3/3.5	2.8 ± 1.2/3.7	1.4 ± 1.0/1	2.8 ± 1.3/9.2
11,400[e]	Escape	6.7 ± 2.1/16.3	2.1 ± 1.0/10.0	3.9 ± 2.1/13.4	1.3 ± 1.3/6	8.2 ± 5.4/11
16,570[d]	Avoid	8.3 ± 3.6/5.9	1.4 ± 0.4/2.4	3.9 ± 3.8/2.4	2.4 ± 2.4/0	0.0 ± 0.1/1.4
17,290[e]	Escape	8.2 ± 3.5/10.9	4.2 ± 2.0/1.9	5.4 ± 3.4/7.1	2.2 ± 2.6/1	0.3 ± 0.7/9.6
Mean ± S.D.		6.5 ± 1.4/7.4 ± 4.3	2.1 ± 1.0/2.9 ± 3.0	3.1 ± 1.3/4.0 ± 4.3	1.3 ± 0.8/1.5 ± 1.9	2.2 ± 2.7/5.0 ± 4.2

[a] Preexposure value for each concentration expressed as mean ± standard deviation (N = 10-12); test value (N = 1)

[b] ITI = Intertrial interval

[c] Significant ($p < 0.05$) positive correlation between test number of lever presses per minute during ITI and HCl concentration with linear regression analysis of z scores

[d] Subject not previously exposed to HCl but exposed to acrolein several months earlier

[e] Subject not previously exposed to HCl or acrolein

TABLE 6

EFFECTS OF HYDROGEN CHLORIDE ON ESCAPE PERFORMANCE OF THE RAT

Average HCl Conc. (ppm)	Test Response	Escape Time (s)[a]	Time to 1st Lever Press (s)[a]	Number of Lever Presses[a]	No. Lever Presses/min during ITI[a,b]
		Preexposure/Test	Preexposure/Test	Preexposure/Test	Preexposure/Test
11,800	Escape	15.3 ± 5.1/25.7	11.6 ± 1.3/14.4	1.1 ± 0.4/1	0.0 ± 0.0/0.6
14,410	Escape	13.3 ± 1.2/13.9	11.3 ± 1.6/13.1	1.2 ± 0.4/2	0.8 ± 1.0/2.8
15,250	Escape	13.5 ± 1.7/15.3	10.8 ± 1.2/10.0	1.4 ± 0.5/6	0.0 ± 0.0/5.2
18,430	Avoid	10.0 ± 3.7/7.0	9.5 ± 3.8/6.3	1.0 ± 0.0/1	0.5 ± 1.0/4.6
22,260	Escape	13.5 ± 5.3/21.5	13.7 ± 5.5/13.0	1.1 ± 0.3/2	0.0 ± 0.0/ 2.4
25,300	Escape	13.6 ± 4.5/16.1	13.1 ± 4.5/7.9	1.0 ± 0.0/1	0.2 ± 0.6/3.2
25,850	Escape	15.1 ± 5.0/14.0	4.5 ± 5.2/13.4	1.5 ± 0.9/1	0.1 ± 0.3/0.2
27,690	Escape	16.8 ± 4.6/16.6	12.5 ± 3.8/10.2	1.5 ± 0.8/13	0.3 ± 0.8/3.4
50,910	Escape	12.2 ± 2.5/14.7	10.9 ± 1.1/14.0	1.5 ± 0.7/1	0.1 ± 0.3/7.0
53,900	Avoid	13.3 ± 2.3/2.9	12.7 ± 2.2/2.4	1.1 ± 0.3/1	0.0 ± 0.0/2.8
76,730	Escape	12.1 ± 3.9/21.4	10.6 ± 2.9/10.2	1.0 ± 0.0/11	0.2 ± 0.4/5.8
87,660	Died	–	–	–	–
Mean ± S.D.		13.5 ± 1.8/15.4 ± 6.4	11.0 ± 2.5/10.5 ± 3.7	1.2 ± 0.2/3.6 ± 4.4	0.2 ± 0 4.2 ± 3.3

[a] Preexposure value expressed as mean ± standard deviation (N = 10-12); test value (N = 1)

[b] ITI = Intertrial interval

[c] Significant ($p<0.05$) positive correlation between number of lever presses per minute during ITI and HCl concentration with linear regression analysis of z scores

TABLE 7

HCl CONCENTRATIONS (PPM) MEASURED DURING EXPOSURE OF BABOONS TO VARIOUS NOMINAL CONCENTRATIONS OF HCl

Nominal Concentration (ppm)	Animal Number	Exposure Time (min)					Average Conc. (ppm)[a]
		0-3	3-6	6-9	9-12	12-15	
500	5 HCl	610	610	570	570	570	586 ± 22
	6 HCl	590	560	590	600	570	582 ± 16
	7 HCl	540	600	480	500	470	518 ± 53
5,000	1 HCl	4,290	4,060	4,350	4,100	4,150	4,190 ± 125
	2 HCl	4,610	4,500	4,500	4,860	4,560	4,606 ± 149
	3 HCl	3,640	4,190	3,740	3,170	3,460	3,640 ± 376
10,000	4 HCl	11,980	11,580	11,890	11,140	11,240	11,566 ± 376
	8 HCl	8,270	8,720	8,610	8,460	8,440	8,500 ± 172
	9 HCl	10,290	10,580	10,260	10,720	10,370	10,444 ± 199

[a] Mean ± standard deviation concentration for 15-min exposure.

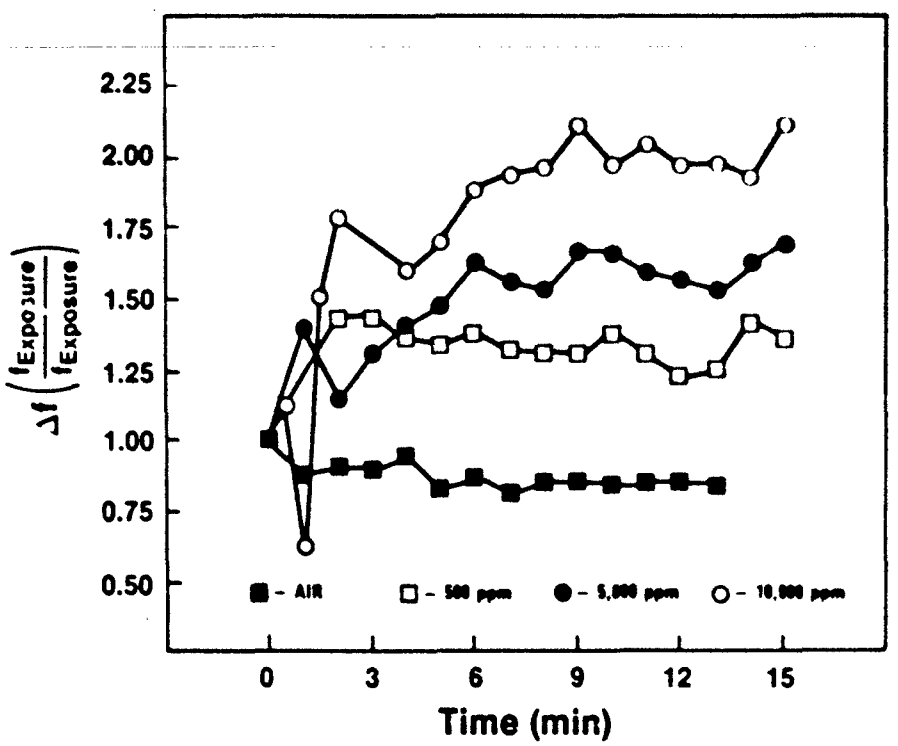


Figure 4. Change in respiratory rate (f) of the baboon during a 15-min exposure to air or various nominal concentrations of HCl. The f is the ratio of f during exposure to the baseline f during the 5 min prior to exposure. Data points are average values of three animals.

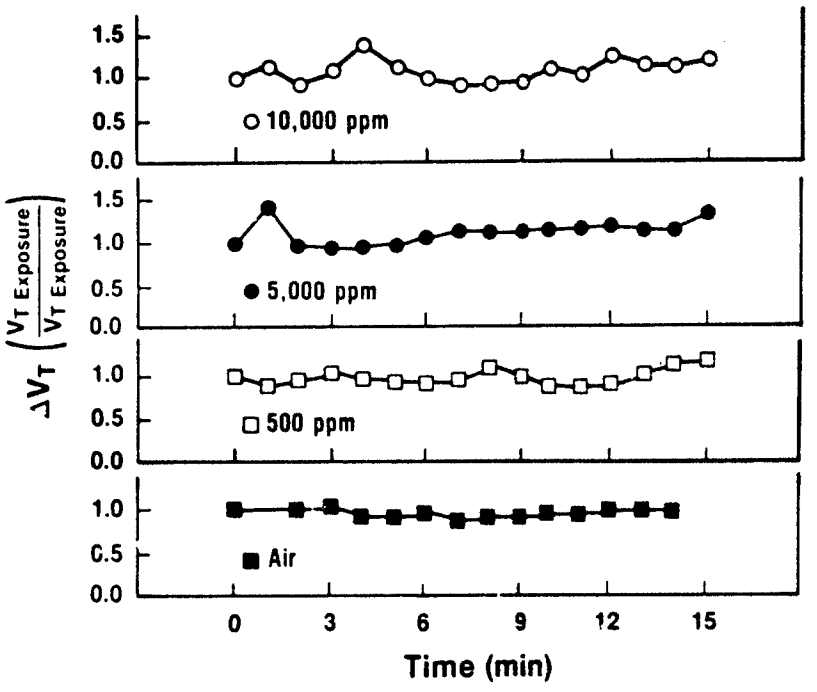


Figure 5. Change in Tital Volume (V_T) of the baboon during a 15-min exposure to air or to various nominal concentrations of HCl. V_T is expressed as the ratio of V_T during exposure to the baseline V_T during the 5 min prior to exposure. Data points are the average values of three animals.

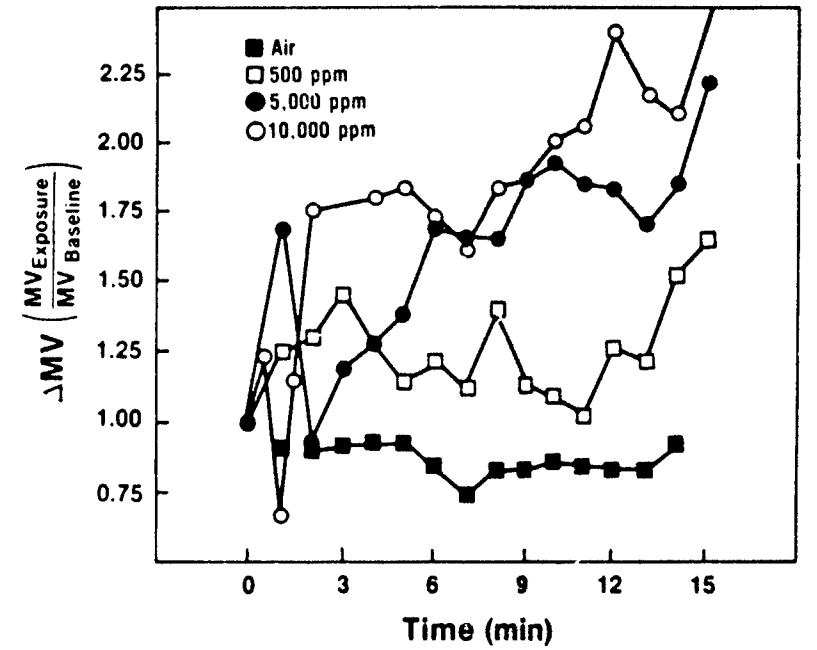


Figure 6. Change in minute volume (MV) of the baboon during a 15-min exposure to air or various nominal concentrations of HCl. MV is the ratio of MV during exposure to the baseline MV during the 5 min prior to exposure. Data points are the average values of three animals.

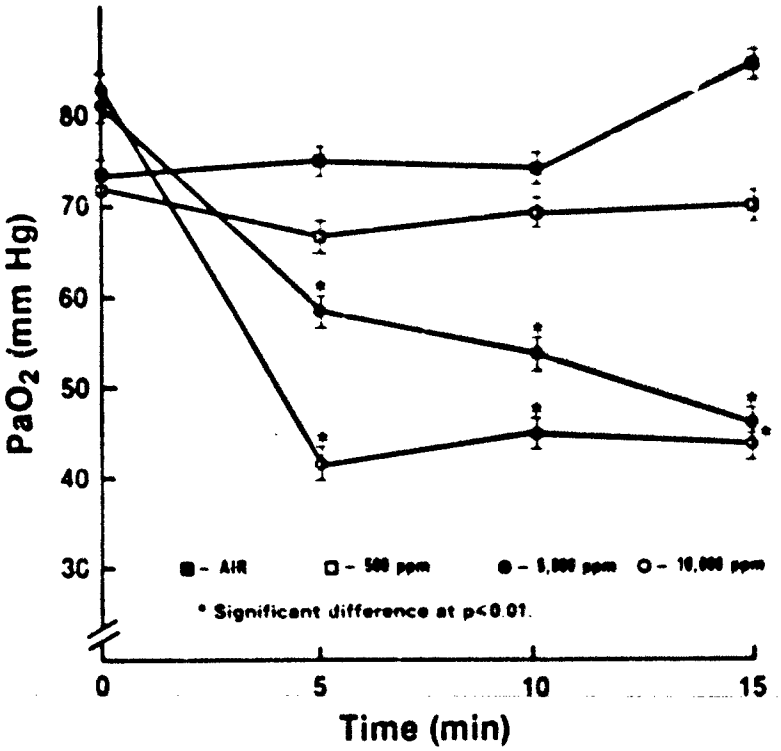


Figure 7. Arterial PaO_2 of the baboon during a 15-min exposure to air or various nominal concentrations of HCl. Values are means ± S.D. of three animals.

DISCUSSION

Before data from laboratory combustion toxicity tests are used to compare and rank materials, it must be shown that the rodent is a valid model for evaluating the toxic effects of smoke in man. Because the mechanisms of action of both CO and HCN are the same in the rodent and man, it is generally accepted that the rodent is a reasonable model for the effects of these asphyxiant gases in man (5). In the first study reported herein, approximately the same concentration of CO incapacitated both the rat and the baboon after a 5-min exposure, supporting the utility of the rat in predicting the toxicity of smoke in which CO is the principal toxicant to man. From the results of these and other reported studies it is evident that both the rat and the baboon can survive short exposures to high concentrations of the irritant gases, acrolein and HCl. However, there are considerable differences in the sensitivity of different rodent species to the lethal effects of irritant gases, and the mouse appears to be the most sensitive species (6). It has been claimed that a factor of 7 to 10 must be applied in extrapolating the acute lethal effects of HCl in mice to man (5). The results of the studies with the baboon do not support this claim and, in fact, suggest that the mouse may be considerably more sensitive to irritant gases than man.

Despite the similarity in tolerance of the rat and the baboon to the lethal effects of irritant gases, there appears to be a marked difference between all rodents and the baboon in the respiratory response to HCl and, possibly, to other sensory irritants. In the rodent, the typical response is a reflex inhibition of respiratory rate caused by stimulation of trigeminal nerve endings in the nasal mucosa (5). It has been shown that the decreased ventilation may slow the uptake of hypoxia-producing gases such as CO, thereby delaying the incapacitating and lethal effects of this gas (7). In contrast to the possible protective effect by sensory irritants in rodents, the respiratory response of the baboon to inhalation of HCl was a concentration-related increase in respiratory rate and minute volume. The increased ventilatory response appeared to be an attempt by the animal to compensate for the severe hypoxemia that was observed at the higher HCl concentrations. Although the cause of this hypoxemia was not determined, constriction of small airways or pulmonary edema may have been responsible for this effect. However, pulmonary edema, if present, could not have been severe because its presence was not detected by clinical observations or by chest X rays of the lungs taken within an hour after exposure.

This difference in response between the rodent and the baboon suggests that the rodent may not be a suitable model for evaluating the toxic effects in humans of smoke in which irritant gases are the principal toxicants. In rodents, the presence of irritants in smoke may delay the effects of other toxicants whereas the inhalation of irritant gases by man may cause a hypoxemic effect that could enhance the effects of hypoxia-producing gases. Therefore, the use of rodent lethality data from laboratory combustion tests to evaluate the potential toxicity of smoke in man, as required by New York State and New York City, may lead to seriously erroneous conclusions.

ACKNOWLEDGMENT

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REGULATORY ASPECTS OF FIRE TOXICOLOGY

Gordon L. Nelson, Ph.D

University of Southern Mississippi, Department of Polymer Science, Southern Station Box 10076, Hattiesburg, Mississippi 39406-0076

SUMMARY

Fire creates a complex toxic environment involving flame, heat, oxygen depletion, smoke, and toxic gases. The nature of that environment is dependent upon not only the materials present but on the fire event, that is, the fire scenario. Materials have different toxic gas profiles under different conditions; therefore, toxic fire gas generation is not intrinsic to any one material. Large fires in buildings constitute a severe toxic threat regardless of the materials being burned.

In the past, building codes in the United States included the phrase, "no more toxic than wood," in reference to fire gases from building materials. Such phrases have recently been deleted, because of the lack of either an accepted definition or test methodology to assess toxicity.

While several states have attempted regulatory activity, the most recent approach (taken by the state of New York) has been the establishment of a data bank on toxic potency of building and furnishing materials. The utility of such a data bank without available hazard analysis methodology is open to discussion, since toxic potency data are not directly applicable to toxic hazard assessment.

A number of small-scale animal exposure tests have been developed to assess the potency of the toxic combustion products from combustible materials. Criticism of these tests relates to the relevance of the combustion module (a smoke generation apparatus) and the appropriateness of the animal model, particularly for irritant gases. Recent data from more than 2000 fire fatality cases and carbon monoxide exposure cases are discussed in this paper to help put small-scale laboratory test results into perspective.

Toxicity is only one of the several fire properties related to materials. All fire parameters are interrelated, that is, they are not independent variables. Thus, predicting the toxicity of burning materials is a problem without a comprehensive solution. Measures have been taken, however, to reduce the number of fires and to reduce fire severity.

INTRODUCTION

This paper focuses on regulatory aspects of fire toxicity. The basic goal of toxicity testing in a regulatory context is to allow society to make judgments about materials that will reduce risk in an unwanted fire. While considerable discussion continues in the regulatory community, and laboratory toxicity tests find their way into liability litigation, these tests have not yet been accepted

for use in the regulatory process in the United States. The basic reason for this is that laboratory tests assess toxic potency, not toxic hazard, of the combustion atmosphere, the latter dealing with the rate and amount of smoke, heat, and oxygen depletion generated, integrated with toxic potency. Toxic hazard is not directly obtainable from toxic potency; fire hazard assessment methodology is required.

Two actions on combustion product toxicity were taken, in 1986, by the States of New York and California. The New York State Uniform Fire Prevention and Building Code, in establishing a toxic potency data bank for building materials and some furnishing materials, includes the following disclaimer with those data.

> This test is a measure of acute toxicity of the thermal decomposition by-products of tested material using a specified procedure under controlled laboratory conditions. The test results do not constitute a characterization of the hazard, safety or risk of materials, products or assemblies under actual fire conditions. The results of this test, if used in any assessment of hazard or risk, should be considered in conjunction with all other factors which are pertinent to an evaluation of the fire hazard of a particular end use.

Representative product testing or classification of products is to be permitted. If approved by the New York Secretary of State, a test report on one of the products will satisfy the requirements for all products of a class. But what is a regulator to do with those data given this disclaimer?

In California, Bill AB 973, which was vetoed by the governor because of cost considerations, would have required a coroner's findings in fire deaths to be included with information submitted by local fire agencies to the State Fire Marshal. The California Department of Health Services would have established autopsy guidelines. The purpose of this legislation was to consolidate data on fire victims to permit fire fatality studies, a very worthy goal. This bill was reintroduced in 1987.

THE COMPLEX NATURE OF FIRE

Let us explore the complex nature of fire in more detail (1). The materials that burn are organic materials, of which the primary chemical element is carbon. When burned completely, carbon compounds evolve carbon dioxide (CO_2) and water. Combustion is seldom complete, however, even in furnacelike conditions; thus carbon monoxide (CO) is an ever present and ever expected toxicant. Of course, other species may be present in a combustion atmosphere. Nitrogen-containing materials, such as wool, may emit hydrogen cyanide (HCN) or oxides of nitrogen (NO_X), whereas chlorine-containing materials may emit hydrogen chloride (HCl). Therefore, the combustion mixture would, from a toxicological standpoint, be complex even if the materials were simple elemental compositions. Materials generally are not simple. Real materials are complex in chemical structure and may even be mixtures or alloys of complex polymer structures; and the surface may be different from the bulk material. The combustion atmosphere formed and the

toxicants present are based on material-specific chemistry and are totally dependent upon the specific conditions of smoke generation, conditions which change during a fire.

Fuel combustion has been studied for many years. Fuel mixtures and stack gases are routinely sampled by analytical techniques. The radical chain processes of combustion, even for a fuel as simple as methane, are known to be multistepped, with the generation and involvement of multiple species (Figure 1).

The simplest example of a combustion process is the burning of methane.

$$CH_4 + 2O_2 - CO_2 + 2H_2O$$

But even this simple combustion involves many free-radical production steps. Propagation steps that produce free radicals keep the burning going; chain-branching steps produce two free radicals, accelerating the reaction with explosive force; termination occurs when radicals are removed, quenching combustion. The flame-carrying radicals—H•, HO•, O•—occur in all flames, whether in methane or in polymers.

A Few Propagation Steps

$CH_4 + HO\bullet - CH_3\bullet + H_2O$
$CH_4 + H\bullet - CH_3\bullet + H_2$
$CH_3\bullet + O\bullet - CH_2O + H\bullet$
$CH_2O + HO\bullet - CHO\bullet + H_2O$
$CH_2O + H\bullet - CHO\bullet + H_2$
$CHO\bullet - CO + H\bullet$
$CO + HO\bullet - CO_2 + H\bullet$

Chain Branching

$H\bullet + O_2 - HO\bullet + O\bullet$

Termination Step

$H\bullet + R\bullet + M - RH + M\bullet$

where R is any organic radical and M is any surface. Heat is transferred to the surface, producing higher-energy M.

Figure 1. What happens when something burns?

In a classic experiment 150 years ago, Faraday showed that solid fuels such as candles evolve volatile fuels during burning and that these generated fuels are, in fact, burning (Figure 2). For many polymers, especially those that pyrolize readily, combustion is very much candlelike. For others, particularly those that are highly aromatic in character, oxygen (O_2) is involved in the fuel-generation step. For a few materials, such as polytetrafluoroethylene (PTFE) and carbon rod, direct surface combustion takes place. Making the process even more complex is the fact that not all combustion reactions lead to generation of smaller gaseous species. Some combustion processes produce larger solid chemical entities, that is, soot and char. Thus, the chemical species present in the combustion atmosphere are the result of fuel formation, combustion free-radical chain reactions, and soot- and char-forming reactions, all of which are interrelated and highly condition-dependent as well as sequence-dependent. The toxic atmosphere is described in Table 1. Each element may contribute to the life-threatening character of the fire environment.

The analytical chemist can use many tools to analyze a combustion atmosphere. These include gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), infrared (IR)

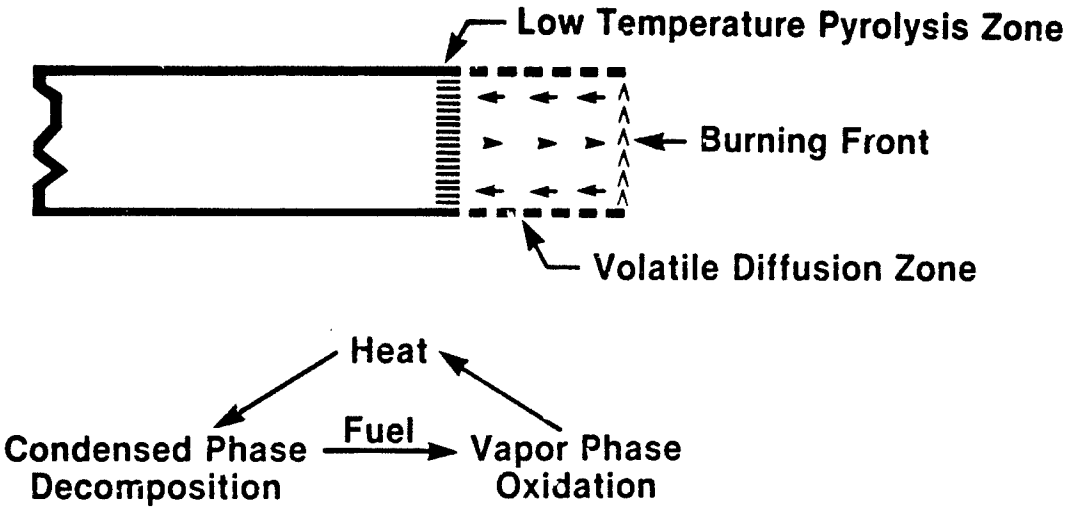


Figure 2. Burning process of a solid organic material. Heat decomposes the material at the surface, generating volatile fuel which burns generating heat which, in turn, generates more volatile fuel. This is the flammability cycle. Flame retardancy is achieved by disruption of the cycle in the solid or vapor phase.

TABLE 1.

PRODUCTS OF COMBUSTION

A. Smoke
Particulates
Fluid droplets
B. Toxic Gases
CO
CO_2
SO_2, NO_2, HCN, hydrogen halides, halogens
Irritants (aldehydes, organic acids)
Gases absorbed onto particles
C. Heat
D. Oxygen Depletion

spectroscopy, nondispersive infrared (NDIR) spectroscopy, gas chromatography/Fourier transform infrared (GC/FTIR) spectroscopy, and a variety of species-specific and class-specific instrumental and wet-chemical analytical techniques – such as ion-specific electrodes, electrochemical methods, calorimetric methods, pH measurement techniques, titrimetric procedures, and gas analysis tubes. Analytical techniques are available and have been used under many different circumstances. Stack or effluent sampling for products of incomplete combustion (PIC) for environmental studies, sampling in real fire studies, sampling in laboratory-scale tests, and even sampling in the flame itself can be performed. The results are a detailed profile of the chemistry and of the chemical species

present. Indeed, there is extensive literature presenting GC/MS data on decomposition products for literally hundreds of polymers under a multitude of generation conditions. For example, Boettner, Ball, and Weiss (2) analyzed the incineration of 19 common polymers for the U.S. Environmental Protection Agency.

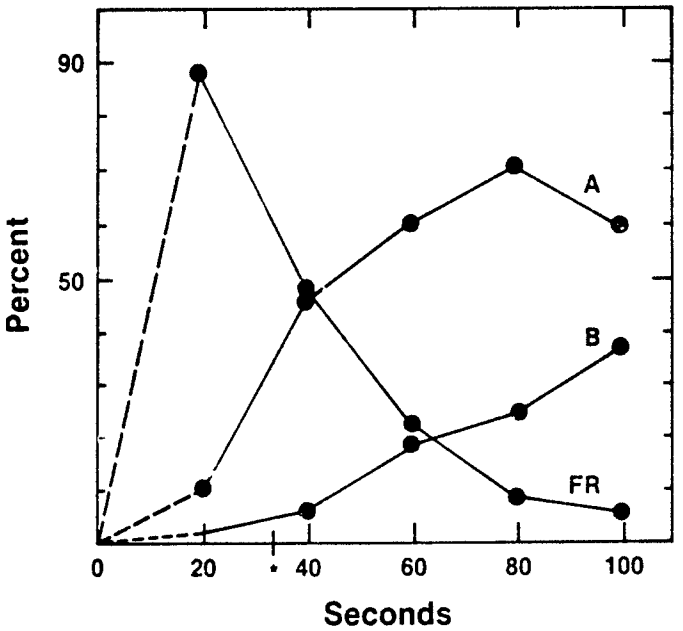
Tobacco has been the subject of most of the numerous analytical studies done on smoke. Nearly 400 compounds have been identified in cigarette smoke, with more than 6,000 additional minor constituents observed (3). Approximately 200 compounds have been identified in smoke from common woods. In each of our studies of decomposition, one to three dozen compounds were identified, and additional minor constituents were left unresolved. Clearly, smoke from burning polymers, whether natural or synthetic, is made up of numerous compounds.

Of great importance is the fact that analytical studies also show the dependence of products formed upon combustion conditions. For example, Figure 3 illustrates the dependence of pyrolytic gases on exposure time for a polymer alloy containing a flame retardant. The fire retardant agent is evolved first, followed by the breakdown of Polymer A, followed by Polymer B Depending upon the time cut taken, the chemical species present are quite different. Whether there is a flame present, and whether that flame is external or self-propagating, will have a critical effect. Thus, one must determine the atmosphere or set of atmospheres that represent a material. This is a key issue for laboratory tests and for the regulator.

Thus far this paper has presented a complex picture of smoke. One can, however, over-complicate that picture. While there may be traces of hundreds of compounds in a fire and gas mixture, most of these have little influence on the acute toxicity of that mixture. One is really only concerned with the primary toxicants, that is, those species present in sufficient concentrations to produce a toxic effect. Some of the toxicants most frequently cited are carbon dioxide, carbon monoxide, oxides of nitrogen, aldehydes, hydrogen cyanide, sulfur dioxide, ammonia, and hydrogen halides.

If one expects particular species, then clearly simplified analytical schemes are possible to search for, quantify, and even rate materials according to those gases. Here, again, there is extensive literature. The National Bureau of Standards smoke chamber has, over the years, been used in a semiregulatory context as a device for measuring toxic gases by analytical techniques. In France, regulatory activity includes restrictions on materials containing chlorine and nitrogen.

But again, laboratory toxicity test methods are only valid if their results are relevant to real fire circumstances. Several years ago, we reported results on an experimental program that compared laboratory animal exposure test data (toxic potency) using an LC_{50} approach with analytical data from large-scale fire tests (room corner tests) (4).



*Ignition point.

Figure 3. Dependence of pyrolysis gases on exposure time for a polymeric alloy (A + B) containing a flame retardant (FR).

LC_{50} data for some 16 materials were reported. A 740-L chamber was used for the laboratory animal exposure tests. Ten Sprague-Dawley rats, five male and five female, were exposed. A standard protocol of biological and analytical observations was followed. For all but one of the materials tested, CO was the primary toxicant. A range of a factor of 5 was observed for the LC_{50} data across the 16 materials. Wool was the only material for which CO was not the primary toxicant. In this latter case, the CO concentration at an LC_{50} was only 319 ppm. HCN was a major additional toxicant.

In room corner tests, two 4- x 8-ft sheets of material were mounted in the corner of a test room and subjected to a 10-lb wood crib ignition source. During large-scale fire simulation tests, analytical monitoring of CO, CO_2, O_2, SO_2, phosphorous, hydrocarbons, and visible smoke was performed. We had both room corner analytical test data and LC_{50} laboratory test data for five specific materials. LC_{50}, temperature, and CO analytical data were ranked and compared. For the five materials, CO was the primary toxicant. However, the CO present in large-scale tests was not ranked according to the LC_{50} (toxic potency) test results. The large-scale data suggest that the CO concentrations observed were largely a function of the flammability of the material (i.e., fire spread and growth). Clearly, for factors of 2-5 between materials, one would not wish to select one material over another on the basis of toxic potency differences.

FIRE RISK ASSESSMENT

It has been my experience that the choice of building materials does make a difference in fire safety. The appropriate choice of materials can prevent or retard ignition and flame spread. One assembly can withstand a given fire while a like assembly of different materials cannot.

Many recent discussions would lead one to believe that a number of separate fire parameters must be evaluated and incorporated into any assessment of fire risk, and that these parameters must be traded off or averaged in the comparison of one material or system versus another (1). Implicit in such a discussion is the risk equation:

$$R = f(\text{ignition}) + f(\text{growth}) + f(\text{smoke}) + f(\text{toxicity}) + \ldots$$

Yet we know that fire is a step function. First, an object must be ignited; second, fire must spread from the point of ignition; and third, as fire grows, smoke and toxic gases are generated in significant quantities (Figure 4).

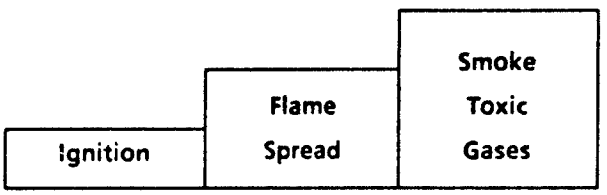


Figure 4. Fire step function. Fire is a sequence of events, one leading to another.

One must have ignition and spread of flame to create smoke and toxic gases. If ignition is prevented, spread of flame minimized, and smoke controlled, toxic gases are likewise affected. If the risk equation is viewed at its limits, one of those limits is that if the probability of ignition is zero, then the risk function must go to zero. This suggests that the risk function is not an additive function, $fA + fB + fC$, but is a product function, $fA \times fB \times fC$; that is,

$$R = Af(\text{ignition})^a \times Bf(\text{growth})^b \times Cf(\text{smoke})^c \times Df(\text{toxicity})^d \times \ldots$$

If f(ignition) is very low, R is low even if f(toxicity) is high. If f(ignition) and f(growth) are large, then R is large even if f(toxicity) is in a normal range. Such an equation also gives the expected response of external forces, such as sprinklers, on R; sprinklers reduce growth and R is reduced. Implicit in R are the probability and the severity of the event.

We also know that the exponent b in the growth term is not 1; in fact, for flame spread from a point, b is 2. It is not surprising then that ignition and growth terms will overwhelm toxic potency differences for differences less than one order of magnitude between materials.

Viewed in terms of toxic hazard, risk has the following form:

$$R_{toxic} = Df(\text{toxicity})^d = Df[(\text{amount})\,(\text{potency})]^d \textit{ and } \text{amount} \propto f(\text{growth})^b.$$

Thus, toxic hazard itself is influenced by other fire properties and is *not* an independent variable.

Clearly, one does not want to sacrifice ignition and growth performance for toxic potency performance. Ignition and growth performance determine the probability of a fire occurring and the magnitude of the fire if it occurs. All fire atmospheres are highly toxic. The smoke plume exiting a room for a fire of moderate size (i.e., extinguishable with a garden hose) has a CO concentration of 5000 to 50,000 ppm. A single object weighing in pounds can produce sufficient CO to be life-threatening in a home-sized building if it burns rapidly. A handful of charcoal can cause deaths if burned in a home-sized room.

In some fields of science, going from A to product B is the same regardless of path. Fire, however, is an irreversible process. Different paths give different events and different toxicities, which makes the use of small-scale tests so difficult in a regulatory context.

Smoke toxic potency tests involve four definable components, as listed below.

- A method for generating smoke
- A method for exposing the experimental animals to smoke
- A method for assessing the physiological and biological effects of the smoke
- A method for monitoring the composition of the smoke generated

The issues concerning the lack of relationship to actual fires or to human victims associated with each of these components have been discussed by other authors in this symposium. These issues alone render toxic potency rankings of materials of little value to the regulator.

Clearly, fire modeling is required to make regulatory use of toxic potency data. Current work on fire modeling is based upon detailed mathematical techniques to calculate fire behavior and growth. Unfortunately, whereas such work is important for research, it is unlikely that such techniques will be used by designers of buildings and manufacturers of products, at least not in terms of practical risk assessment, and certainly not within the next 5 to 10 years.

WHAT IS A NORMAL FIRE?

To regulate products by smoke toxicity would seem to suggest that there is an abnormally toxic fire. If we are to regulate a so-called abnormal fire, what, indeed, do we expect from a normal fire; that is, a fire for which CO is the predominant toxic agent? In literature on CO and CO fatalities, one frequently finds comments such as the following in texts and surveys.

> Carbon monoxide is present in significant amounts in virtually all fires. It is highly toxic when inhaled, and acts by combining with hemoglobin in the blood to form carboxyhemoglobin (COHb). Hemoglobin's function is to carry oxygen throughout the body, and it cannot do this if it is tied up as COHb and, therefore, unavailable for oxygen transport.
>
> The level of COHb in the blood for fire victims can be determined fairly easily. In the absence of other contributing factors, a COHb concentration of 50% or greater is generally considered lethal (5).

Most medical discussions of CO poisoning deal with normal, healthy individuals. But the real population is composed of a spectrum of individuals with varying degrees of health. While one frequently sees conclusions about a small set of victims without regard to other factors, conclusions that implicate particular materials for example, such conclusions may not be justified given the full spectrum of expected human behavior to CO poisoning.

Indeed, several issues can be raised. What are the lethal levels of CO in man? How are these levels related to COHb? What are the roles of age, disease, drug or alcohol use, sex, race, and physical activity? What are the mechanisms of CO toxicity? What is the relationship to the fatal event (fire, city gas, exhaust fumes)? Have fires changed over the years?

Literature Study. In a recent study, we reviewed more than 600 papers (6), from which several statements can be made in summary. Whereas CO exposure seldom occurs with CO in its ultrapure state, studies of human exposure to city gas and exhaust gas show that nearly 20% of exposed individuals die from CO poisoning at blood COHb levels thought by some to be less than normal (i.e., $<50\%$ COHb). While persons with cardiovascular and other diseases were included in that group, other individuals did not exhibit identifiable pathology, and persons with identifiable pathology did not necessarily die with concentrations of $<50\%$ COHb.

The length of time of CO exposure, level of victim activity, and the age of victim are all important variables. Why do we see the survival of young children exposed to the same atmosphere of CO that kills their parents? Clearly, CO poisoning is more complex than the percentage of COHb in the blood of the victim and more complex than the oxygen-carrying capacity of the blood.

Therefore, without additional information, a blood COHb value <50% does not provide conclusive evidence regarding a given victim's response. Indeed, for identical exposures, different individuals exhibit different symptoms and outcomes.

In the case of fire exposures, twice the number of victims (40%) are in the <50% COHb category than for automotive exhaust victims (40% versus 20%). However, despite the presence of different materials in the environment, the fire fatality/COHb profiles in Japan in the early 1960s, in Denmark in the late 1960s and 1970s, in the United States in the late 1970s, and in the United Kingdom in the late 1970s are very similar. Despite population and environmental differences, the role of CO in fire deaths is the same among different countries and over the years.

In CO-related deaths, male victims predominate; alcohol use is also a key factor. Many fire victims have burns. Clearly, exposure to heat and hot fire gases plays a role that is difficult to evaluate, yet is known to markedly alter COHb levels essential for death from fire.

A number of physiological conclusions can be drawn from an analysis of the published literature. These include the following.

- Under most circumstances the acute lethality of CO depends upon interruption of energy production (ATP) by cells.
- CO has the capacity to directly inhibit cellular metabolism and to affect other cellular activities, but the high CO:O_2 ratios required for this suggest that such actions are not the primary cause of acute toxicity, except under extreme or unusual circumstances.
- It is most likely that cellular production of energy is interrupted through interference with O_2 delivery to the intracellular sites of oxidative metabolism.
- Oxygen delivery is impaired by decreasing the oxygen-carrying capacity of blood and by increasing the O_2 affinity of hemoglobin, to which CO is not bound.
- Oxygen delivery is impaired by inhibiting the facilitated diffusion of O_2, which is essential to the heart and other active tissues. This inhibition of facilitated diffusion is most likely to result from the binding of CO to intracellular myoglobin.
- The physiological response to CO is largely cardiovascular, since the rate of blood flow must be increased to compensate for the reduced O_2 content and increased O_2 affinity of hemoglobin.
- Cardiovascular disease, particularly that involving the coronary and/or cerebral circulation, increases the risk posed by CO exposure.

Forensics Study. Although several previous case studies of CO poisoning have been reported, a larger data base is needed to answer some of the more difficult questions. We recently reported a preliminary discussion of those data (6).

A total of 2,240 cases was received directly from forensic laboratories. Of the 2,240 cases received, 1,800 were suitable for study. (Only cases with COHb values in excess of 20% were included.) Flash fire victims and complications such as cigarette smoking predominated below 20% COHb. Sixty-one percent of the data are for fire victims and 39% for nonfire victims.

Information was gathered on age, sex, method of CO analysis, percent COHb, percent ethanol, or drugs in blood, diseases, source of CO, and overall physical condition of the victim. Not all of the information requested could be provided in every case; the data base reflects that fact. A computer program was written in FORTRAN by the investigators to store, edit, sort, and analyze the submitted data.

The data obtained correlated well with previous studies but also provided several new observations. Data values showing the percentage rate of victims with <60% COHb on blood analysis for each category are given as a key indicator. Data representative of the entire data base are presented in Table 2 below.

TABLE 2

REPRESENTATIVE CARBOXYHEMOGLOBIN LEVELS IN FORENSIC DATA BASE. PERCENTAGE OF VICTIMS WITH <60% COHb GIVEN FOR EACH SUBCATEGORY.

Total Data Base (1,637 cases, with age reported):	
Overall:	34%
Male:	32%
Female:	36%
Age:	
11 to 50 years:	29%
61 to 80 years:	47%
Blood Alcohol >0.05% (33% of population: 38% of males, 23% of females):	
Overall:	31%
Male:	33%
Female:	27%
Alcohol >0.05% to 0.29%:	28%
Alcohol ≥ 0.30%:	46%
Alcoholics:	12%
Disease:	
Cardiovascular:	44%
Male:	46%
Female:	41%
Liver, pulmonary, kidney:	43%

The total data base comprises 68% males and 32% females. Overall, 34% of the CO victims died with COHb values <60%. Age was a factor; 29% of victims aged 11 to 50 years had COHb values <60% versus 47% of victims aged 61 to 80. Alcohol use is a pervasive factor in all CO studies. Approximately 33% of the population showed blood alcohol levels >0.05% (38% for males and 23% for females). For victims with blood alcohol >0.05% to 0.29%, 28% had blood COHb values <60%. By contrast, 46% of victims with blood alcohol ≥0.30% had COHb values <60%. Only 12% of alcoholic victims had COHb values <60%. The significant variations for victims with ≥0.30% blood alcohol and for alcoholics were not observed in previous studies. Disease was also a factor; victims with cardiovascular disease and with other reported physical disorders showed elevated percentages of the population with COHb values <60%.

Given the diversity of cases in the data base, one might draw erroneous conclusions looking only at the data base as a whole; source of CO is important, fire versus nonfire. Fire victims constitute 61% of the data base (63% male). Data for the fire segment are shown in Table 3.

TABLE 3

CHARACTERISTICS OF FIRE CASES IN FORENSIC DATA BASE. PERCENTAGE OF VICTIMS WITH <60% COHb IS GIVEN FOR EACH SUBCATEGORY.

Fire Cases [61% of Data Base (877), Blood Alcohol >0.05% (37% of population, 46% for Males, 23% for Females), Data Base 63% Male]:

Overall: 41%
Male 41%
Female 39%

Age:
- 11 to 50 years: 37%
- 61 to 80 years: 51%
- 1 to 10 years: 34%
- 1 to 10 years: 9% (>90% COHb)

Alcohol >0.05% (Subcategory 78% Male):
- Overall: 39%
- Male: 42%
- Female: 30%
- Alcohol >0.05% to 0.29%: 35%
- Alcohol 0.30 to 0.35 + %: 47%
- Alcoholics: 29%

Disease:
- Cardiovascular: 55%
- Liver, Pulmonary, Kidney: 57%
- Hydrogen Cyanide: 51%

Of the fire victims, 41% had COHb values <60%. Age differences were observed, with a substantially higher proportion of the over-60 age groups in the <60% COHb category. Hydrogen cyanide was measured in 142 victims. With 51% of the population in the <60% COHb category, some joint effect was present with CO and HCN.

Figure 5 shows the victim profiles for both fire and nonfire victims. Fire victims clearly include a larger number of <60% COHb victims, about twice as many as nonfire victims (41 versus 22%).

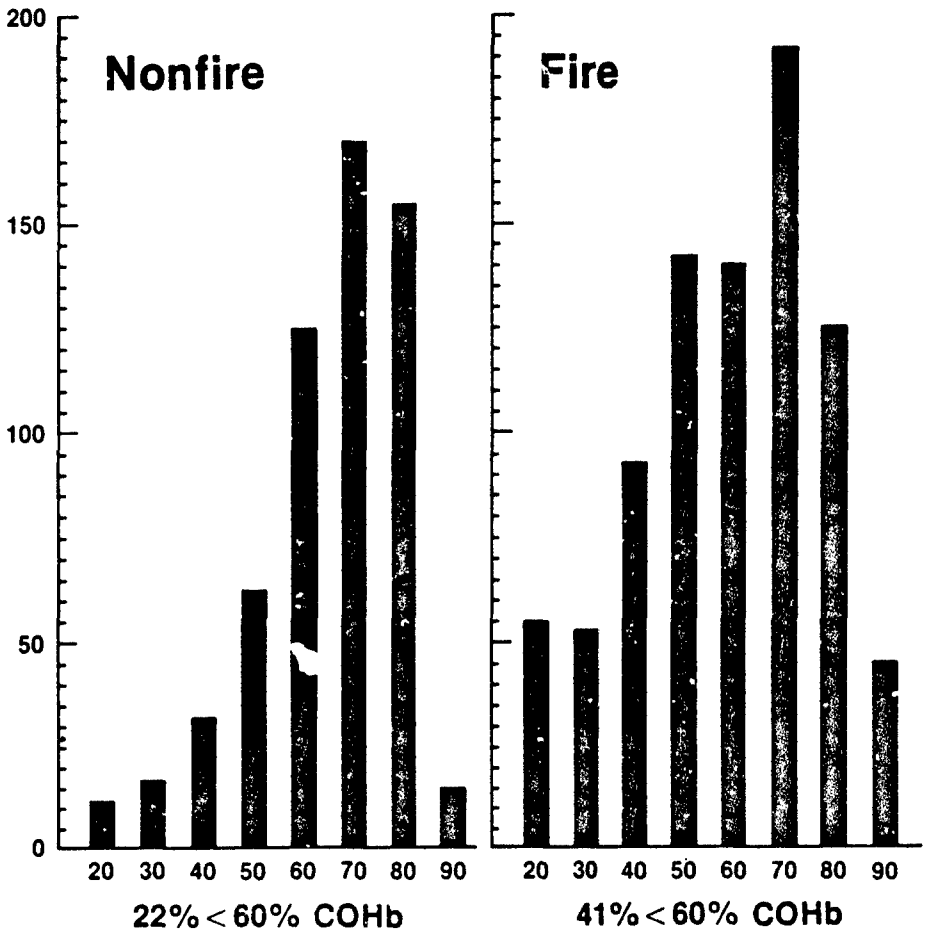


Figure 5. Victim COHb profiles for nonfire and fire segments of the data base. Fire victims include a larger number of <60% COHb victims than nonfire victims (41 versus 22%).

For the nonfire segment of the data base (Table 4), 85% were automotive exhaust gas victims and 75% were male. Despite the higher male population, the percentage of victims with >0.05% alcohol was similar to the fire segment.

TABLE 4

CHARACTERISTICS OF NONFIRE CASES IN FORENSIC DATA BASE. PERCENTAGE OF VICTIMS WITH <60% COHb IS GIVEN FOR EACH SUBCATEGORY.

Nonfire Cases [39% of Data Base (558), Blood Alcohol >0.05% (34% of population, 36% of Males, 29% of Females), 85% Exhaust Victims, Data Base 75% Male]

Overall: 22%
Male: 20%
Female: 28%
Exhaust Source: 19%
Natural Gas Source: 26%
Gas Heater Source: 76%

Age:
- 11 to 50 years: 20%
- 61 to 80 years: 31%

>0.05% Alcohol Subcategory
- Overall: 17%
- Male: 18%
- Female: 14%
- Alcohol >0.05% to 0.29%: 15%
- Alcohol 0.30% to 0.35 + %: 57%
- Alcoholics: 6%

Disease:
- Cardiovascular: 34%
- Liver, Pulmonary, Kidney: 27%

Overall, 22% of the nonfire population had COHb values <60%. Females showed a higher value, which originated in the portion of the population with <0.05% blood alcohol.

The source of CO is important because CO exposure does not occur with the gas in its pure state. Natural gas and gas heater exposures show larger populations with <60% COHb. Other constituents must have been present from these atmospheres. Overall, other observations for nonfire victims were similar to those for fire victims.

The foregoing observations are based upon the percentage of the population with COHb values <60%. Table 5 shows mean values, standard deviations, and highest peaks for a variety of data subcategories. The mean of all COHb values is 64.2%. For fire, that drops to 61.97%, versus 68.7% for nonfire victims. A smaller mean value and a larger standard deviation signify a broader population distribution (a higher <60% COHb population) in that subcategory.

The lowest COHb mean values are observed for ages 81-90 (56.8%), ages 91 and up (58.1%), and for victims with ethanol 0.30-0.34% (59.7%). The highest mean values are observed for nonfire victims with >0.05% blood alcohol (overall 70.8%, males 70.5%, and females 71.8%).

The interaction of CO with alcohol is worth further comment. One observation made from the data in Table 5 is that death occurs at a higher mean COHb value for nonfire victims with some blood alcohol (0.05% to 0.3%). Some blood alcohol appears to increase the amount of CO needed to cause death, yet the high proportion of victims with blood alcohol present indicates that alcohol increased the likelihood of death.

The mechanism of CO toxicity is not simple and is not understood in great detail. Yet the differences observed in different populations exposed to CO are to be expected, because it is known that more factors than hemoglobin's oxygen-carrying capacity are involved. Clearly, a greater percentage of fire victims die with COHb values <60% than nonfire victims; however, it is obvious that nonfire victims succumb at low COHb levels as well. Different segments of the exposed population exhibited different outcomes. Therefore, conclusions for given victims can be made only after thorough analysis of all factors present. Indeed, a victim with 30% COHb can be a case of CO poisoning without other agents required.

CONCLUSIONS

This paper has discussed the complex nature of fire, fire risk assessment, and the abnormal fire. The information given seems to present a bleak picture for the regulator, since 80% of fire deaths are the result of toxic gas exposure.

However, it is not as difficult for the regulator as one might think. The regulator is interested in risk from fire, not toxicity alone. In the fire risk equation,

$$R = Af(\text{ignition})^a \times Bf(\text{growth})^b \times Cf(\text{smoke})^c \times Df(\text{toxicity})^d \times \ldots,$$

ignition and growth terms dominate. Ignition and growth are readily evaluated and controlled in regulation by material performance specifications and engineering design requirements plus use of detection and suppression devices. And those regulations seem to be having an effect.

Data released by the Consumer Product Safety Commission (CPSC) show a 12% decrease in the number of residential fire deaths from 1983 to 1984 (4,840 to 4,260) and a 3% reduction in the number of fires (641,500 to 623,000) (7). The CPSC believes that this decline is a direct result of fire prevention/detection activities: flammability standards for mattresses, the voluntary standard for upholstered furniture, and promotion of smoke detectors.

TABLE 5

MEAN AND MODE FOR CARBON MONOXIDE DATA BASE SEGMENTS

Group	Mean%	Standard Deviation	Mode%*
All (Total Data Base)	64.20	17.47	70
Fire Segment	61.97	18.27	70
Nonfire Segment	68.68	15.00	70
Ethanol, ≥0.05%	65.76	17.61	70
Ethanol, ≤0.05%	63.82	17.39	70
Ethanol, ≥0.30%	61.21	19.40	50 (10 & 70)
Ethanol, 0.30 -0.34%	59.70	18.80	50 (60)
Ethanol, 0.35 -up	62.42	19.79	50 (70)
Fire, Ethanol >0.05%	61.77	18.54	80
Fire, Ethanol ≤0.05%	62.08	18.11	70
Nonfire, Ethanol ≤0.05%	67.65	15.37	70
Nonfire, Ethanol ≥0.05%	70.80	13.98	80
Males	64.49	17.28	70
Females	64.25	17.58	70
Males, Fire Segment	61.34	18.22	70
Females, Fire Segment	63.31	18.25	70
Males, Nonfire Segment	69.34	18.22	70 (80)
Females, Nonfire Segment	67.13	15.53	70
Males, Ethanol >0.05%	64.41	17.74	80
Females, Ethanol >0.05%	67.51	16.61	70
Male, Fire, Ethanol >0.05%	60.61	18.50	70
Female, Fire, Ethanol >0.05%	65.59	18.27	70
Male, Nonfire, Ethanol >0.05%	70.53	14.54	80
Female, Nonfire, Ethanol >0.05%	71.81	11.59	70
Male, Ethanol ≤0.05%	64.55	17.00	70
Female, Ethanol ≤0.05%	63.32	17.74	70

* Decade of highest peak values.

(continued)

TABLE 5 (Continued)

MEAN AND MODE FOR CARBON MONOXIDE DATA BASE SEGMENTS

Group	Mean%	Standard Deviation	Mode%*
Male, Fire, Ethanol ≤ 0.05%	61.92	17.98	70
Female, Fire, Ethanol ≤ 0.05%	62.67	18.19	70
Male, Nonfire, Ethanol ≤ 0.05%	68.46	15.14	80
Female, Nonfire Ethanol ≤ 0.05%	65.25	16.48	70 (80)
Age 1-10 Yrs	64.91	19.10	70
Age 11-20 Yrs	67.13	15.84	70
Age 21-30 Yrs	64.83	17.38	70
Age 31-40 Yrs	66.71	16.80	70
Age 41-50 Yrs	65.80	15.65	70
Age 51-60 Yrs	62.16	17.67	60
Age 61-70 Yrs	60.94	17.75	50 (70 & 80)
Age 71-80 Yrs	60.14	17.59	50 (70)
Age 81-90 Yrs	56.78	14.66	50 (70)
Age 91 and older	58.10	12.87	50 (70)

* Decade of highest peak values.

Fire risk assessment techniques, particulary Delphi techniques, also continue to evolve in the fire protection community.

Several general risk assessment formulae have been advanced.

$$\text{Risk} = \text{Probability} \times \text{Consequence (Hazard)}$$

$$\text{Risk} = \text{Probability of Occurrence} \times \text{Probability of Exposure} \times \text{Potential for Harm}$$

In the second equation, the last term is intrinsic to the product and is the result given ignition. This is somewhat akin to the Government Accounting Office (GAO) scheme introduced for rating buildings a decade ago – given ignition, what is the probability of a fire reaching the walls or barriers or going beyond the barriers? It is interesting how closely different experts rate buildings using the GAO Delphi approach.

In the insurance industry particularly, well-developed techniques have been utilized for risk assessment, most of which incorporate Delphi schemes. One such approach is Gretener's formula:

$$\text{Risk} = \frac{PA}{NSF}$$

where

P = Hazard

A = Activation

N = Normal Precautions

S = Protective Measures

F = Fire Resistance.

Through statistical evaluation, additional sophistication is possible.

In summary, fire creates a complex toxic environment involving flame, heat, oxygen depletion, smoke, and toxic gases. That environment is not only dependent upon the materials present, but on the fire scenario. Materials have different toxic gas profiles under different conditions; thus toxic fire gas generation is not an intrinsic material property. Large fires in the built environment constitute a severe toxic threat regardless of materials present. Toxicity is only one of several fire properties related to materials; all fire parameters are interrelated, that is, they are not independent variables. That makes predicting toxicology in fire a problem as yet without a comprehensive solution. Measures have been taken, however, to reduce the number of fires and to reduce fire severity.

While regulatory activity on fire toxicity has been present in several states, the most recent approach (in New York) is the establishment of a data bank on toxic potency of building and furnishing materials. The utility of such a data bank without available hazard analysis methodology is open to question, since toxic potency data are not directly applicable to toxic hazard assessment. Comparison of materials on toxic potency alone can lead to wrong conclusions relative to fire safety.

A number of small-scale animal exposure tests have been developed to assess potency of toxic combustion products of materials. Criticism of these tests relates to the relevance of the combustion module and the appropriateness of the animal model as a predictor for humans, particularly for irritant gases.

Rapid progress being made in mathematical fire modeling will eventually permit predictive fire toxicology to merge with fire science. This must be done if results of fire toxicology studies are to contribute to a reduction in fire fatalities. In the meantime, reduction in fires and fire deaths is being achieved, despite absence of apparent progress in toxicity regulation of fire.

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QUESTION AND ANSWER SESSION

DR. GRAND (SOUTHWEST RESEARCH INSTITUTE): You noted that the fire deaths went down from 1983 to 1984. Recent reports show that the number of fire deaths went back up in 1985 – back up to approximately the same level. So, 1984 may have been an unusually low year.

DR. NELSON: Or maybe the opposite in that since you are sighting the NFPA (National Fire Protection Association) data. The NFPA data for a number of years have been declining. So it may well be that this past year was an aberration.

DR. BURNS (NAVAL SUBMARINE MEDICAL RESEARCH LAB): As a former practicing forensic pathologist I was very interested in your human data. I was wondering, have you taken into account the bias that is introduced into your population by choosing death as an end point? Which is, that it is built by the fact that you are examining medical examiner cases.

DR. NELSON: We've certainly considered it. How to address it is another problem.

DR. BURNS: Secondly, did you take into account whether these victims had any type of cardiopulmonary resuscitation, either with or without prolonged survival time?

DR. NELSON: That we did take into account. Our forensics people actually go to the medical examiner's office and physically examine the records.

PANEL DISCUSSION V

Rosalind C. Anderson, Ph.D. – Rapporteur

Arthur D. Little, Inc.

DR. ANDERSON: I would like to say that this is the first time in quite a long time that I have been introduced as a member of an august body. For that I am very thankful to be here. Generally I get introduced with lesser adjectives than august. What I would like to do is essentially open the contest. Combustion toxicology always turns into a great contest. I would like to suggest that our appropriate subject matter for the next few minutes is essentially the one that Barbara brought up for us. How are we going to use the results of our work to make the world safer for rodents? You will notice that I have a much wider perspective than she has – I include many other categories, not just rats. We've got to be fair. Having chosen a subject matter, I would like to start by talking about two things that have been briefly addressed this morning. Essentially, there are two approaches to how we will use our data. Number 1 is the National Academy report which Barbara had a copy of. In that report the committee was addressing the question: Do we have any test methods, and if we do how can we honorably use the data in a way that is going to be to the benefit of somebody sometime? The Academy considered a relatively large number of methods and came to the conclusion that in spite of the fact that they are not necessarily in agreement, we do have test methods that can be used. Each one of them represents some aspect of some fire, some aspect of some exposure, and some arbitrarily chosen victim. We've got three arbitraries right there without even looking any further. Nevertheless, these are not worse than tests that we use for other difficult society problems. The National Academy Committee's recommendation was that the data are usable provided they are integrated into a larger context. Here the standard larger context is a complete hazard analysis and the 400-lb software that would allow you to know all things about what's going to happen in the third floor rear. The committee also felt that to use these data in an informal hazard analysis is equally responsible. It may not be as good, but it probably is not bad. In this informal context materials are already required to pass test A, test B, test C, and if you add test D you are perhaps improving the lifestyle of your rodent population. You are perhaps doing nothing for the rodent population. But it is highly unlikely that you are doing damage. In contrast to the National Academy approach, the New York State approach, which we have heard several times but which has never been fully explained, is a fascinating phenomenon; and some of the ways that it will be implemented are quite surprising. The regulation to be phased in over the next three years requires that manufacturers of electrical materials, plumbing materials, and finishing materials register toxicity data for their products in a publicly available data base. This data base, by the way, is essentially the same as that recommended by NBS (National Bureau of Standards) and NFPA (National Fire Protection Association) in their great effort to stave off the New York event. The

registration of the data can be by specific material (I have tested product 347 and here are the data), or it can be categorized (I represent all of my materials that are 80% cotton or more and less than 3% polyurethane with the following set of data) at the option of the manufacturer. This is going to drive those of us who have tunnel vision a little bit out of our standard pattern, because what we are looking at is each manufacturer having the opportunity to define his own set of categories, and my set is not likely to be equal to your set. Therefore, when we get into the great scramble called comparison of data, it is going to be truly strange. Because not only will the categories not be exactly superimposed. they may not even be similar. We will also find that the manufacturer may have chosen typical data representing the best of the category or the middle of the category. He has made a choice about what data to submit. This is going to be tricky data to use until you look at it again and realize that this regulation is not for the benefit of the fire marshall or data user. This regulation is aimed at the producer. The game here is to cause the producer to actively consider the products that are being put on the market, to actively stand up and say that these are my products and I know how they behave under one particular set of circumstances. It is a very, very interesting approach, and it certainly reflects another way to skin the cat. We've got more ways coming I'm sure, but this is a new one. With those two examples of how to use the data I would like to ask people in the panel and then the audience what your current perception is. And to the panel in particular, how could your data be used to improve the lifestyle of some species? I would like to start with Art Grand because he said if the only thing you know is that they are dead you haven't learned very much. So let's hop onto the chemistry and see how the chemistry data are going to be ultimately used to someone's advantage.

DR. GRAND: Well, as I indicated, I think the chemistry data have to be used along with the toxicity data. Some of the information presented by Gordon and by Barbara still leave some holes. We find deaths occurring at extremely low carboxyhemoglobin levels. Gordon suggested that we have to look at various mechanisms of carbon monoxide in action and things of that sort. I would suggest that perhaps there are some other effects there. Maybe, as Hal pointed out, irritants are affecting the blood O_2 level, which is then going to exacerbate the effect of carbon monoxide in the blood. Are there other species? For example, gas in automobile exhaust contains hydrogen sulfide, and this is a very toxic species. Do we look for that in the victims, do we account for that in any way? There are still a number of victims, actual fire victims who are dying at less than what we would expect in terms of just carbon monoxide. We can help in some of our analytical characterizations, whether on the laboratory or large-laboratory scale, by getting a feel for what other materials are in there and then doing some investigations to clarify this. I think we have to do the combination of the analytical and the animal experimentation.

DR. ANDERSON: Thank you. It still leaves us with the question: Is the actual diagnosis of cause of death going to help out? Which again is a magnificent research project. How is it going to help in controlling the show? But I'm not going to ask you that. I want to ask a question of Ghonda, who has three methods going. I am absolutely fascinated to finally see a lot of data from the DIN test because it is quite difficult. How might Mobay take advantage of one or all of these tests in order to perhaps improve products?

DR. SANGHA: Well, I should say that not just Mobay, our parent company Bayer, has been using the test methods all along in product development and also screening. For example, we were able to stop the development of some products based on these tests.

DR. ANDERSON: Barbara, you are all too obvious but I'll ask you anyway.

DR. LEVIN: Well, I think that the approach that we are taking to try to determine what the toxic species are and the interaction of those toxic species in the combustion products that cause the deaths will help the manufacturer to formulate his products such that they can now try to scrub out these toxic species. I think that some manufacturers are already taking this approach. For example, you could add something to PVC such that when the HCl is evolved it would react with that other species and potentially scrub out the HCl. I think that there are manufacturers who are now taking this approach. But you need to know what it is that is causing the effect. You need to know whether it is only HCl or other things. In the Biloxi jail fire, 27 died from smoke inhalation. The fire itself was confined to one cell. It was a very interesting fire because of the people in the same cell that were exposed to the smoke, some died and some didn't. In many cases, the ones that lived stood in showers; this is a real incapacitation test because to keep the shower going you had to keep your finger on the button and they stood in these showers. Essentially these people didn't die and the people that did not stand in the showers did die. We got some of that material, and we looked at it. It produced almost as much hydrogen cyanide as it produced CO, and hydrogen cyanide is scrubbed out by water. So in this case they were able to protect themselves from the hydrogen cyanide and there obviously wasn't enough CO to have killed them. If you know what the toxic products are that are coming off your product, you should be able to do something about it. I think that's the way to go.

DR. ANDERSON: Gordon, I almost get the impression that we don't have a problem. Would you like to play with that one?

DR. NELSON: We clearly have a problem. The problem is fire. Fire is a very toxic environment. It doesn't take a whole lot of material burning in your living room to create a life-threatening situation in the house. One burning television will do that. So, as I noted in my talk, in terms of fire risk assessment and trying to deal with that problem, the issue is whether one can utilize toxic potency

data or other data. I personally favor the end gas model approach as the only way that will allow us to improve safety for people in buildings, submarines, or aircraft. It seems to me that we need to be able to model the fire, and then through the modeling see what the gases are in the end and whether appropriate limits have been exceeded. That is going to be very difficult simply by having toxic potency data, because what toxic potency data does one use in the fire model given that the fire atmosphere is changing from each material over a time? I do however have considerable hope for the end gas model and fire model.

DR. ANDERSON: Would you have the regulators wait?

DR. NELSON: Well, you see, that was the point at the end of my talk. They are not waiting. That is, we are substitutively dealing with fire risk through regulating ignition, through regulating flame spread, and through additional detection suppression devices. So we are not out there all alone. In fact, we've made very significant progress over the years.

DR. ANDERSON: Hal, you are the last one. Your data is so complex that it takes me too long to assimilate it. So, tell me how we are going to use your rat and baboon study to improve lifestyle in Boston?

DR. KAPLAN (SOUTHWEST RESEARCH INSTITUTE): What is the saying? You can't please all of the people all of the time. Well, there is an exception. New York is the exception. Look, an LC_{50} is an LC_{50}. It was never developed the way it is being used. Every toxicologist, every trained toxicologist knows what the LC_{50} is. New York State and whoever else is making a life of developing LC_{50} data is just developing LC_{50} rodent data. Every toxicologist knows the way to evaluate something is to start off with the LC_{50}, determine the toxic effect, determine the target organs, and determine mechanisms. Then you can predict what is going to happen to man. I hope that our primate work will show, I think they already have shown, that the LC_{50} data are not adequate. You know, the LC_{50} for carbon monoxide for 30 minutes exposure is about 5000 ppm. It is the same thing for HCl, 5000 ppm. Now you tell me that man is going to survive in an atmosphere of 5000 ppm HCL in a fire and be able to escape?

DR. ANDERSON: I wonder how one could sponsor the other part of the toxicology evaluations given budgets. Do you see a way to use more elegant, better methods, more subtle methods on a large scale?

DR. KAPLAN: I think that research needs to be done with mixtures of fire gases. I think research needs to be done with some higher animals. I think you need to determine the mechanisms of action between these gases. Now it is being done at NBS and at Southwest in rodents, but that's where the field stands. It's in rodents, it's not in higher animals. I think this needs to be done and then when we know what the mechanism of smoke or the classes of the fire gases are in smoke, then we can use

the rodent data with some validity. But right now I don't think you can use the LC_{50} to predict toxicity in man. New York State, by requiring the LC_{50}, is implying that it can be used to predict toxicity in man. It's implying that – do you not agree?

DR. ANDERSON: I don't see it that way as a matter of fact. I think they are implying that there is information which probably will not hurt and could help someone. In the first place, LC_{50} is crazy; that means half of the people are dead already. So one doesn't use the number as a number Nor does one use it alone, because we already have however many fire tests there are – insofar as LC_{50}s are different by orders of magnitude, conclusions can be drawn. Gordon, you don't seem to have seen this but we certainly have. I think there is information, and there remains the very important question of how to get information out of the screening test. Every time we learn a little more, we've got 44 new questions. There is a big question of how we are going to balance screening and mechanism studies. We're probably almost out of time and I think we should probably let the audience in. Are there any questions?

DR. MEHENDALE: We have dealt with the components of smoke and what might cause death, but one of the things I noticed that we haven't touched upon is how to rescue that person who is inhaling smoke and at that moment. Are there things that we can do to prolong his chances of life? Should the firemen go and pull him out? I am alluding to such possibilities as putting things in the room, maybe in the wall; gadgets that will absorb carbon monoxide, perhaps cyanide. Those are the two major components. Perhaps introducing oxygen if hypoxia is a contributing factor in incapacitating the individual and perhaps eventually leading to death. Perhaps the members of the panel could deal with those aspects.

DR. GRAND: Can I just speak to that briefly from a chemical point of view? It is very difficult to absorb carbon monoxide. Among the very few materials is a catalyst called hopcalite that oxidizes the carbon monoxide to carbon dioxide. There have been a number of attempts to develop emergency escape smoke masks where you breathe the air and it just goes through a filtration device. A few of them can absorb the carbon monoxide but many of them are simply charcoal or other absorbants that absorb a reasonable amount of hydrogen cyanide and irritant gases and particulate, but don't absorb carbon monoxide. Also, the rate of evolution of these gases in a real fire situation is just enormous, and rather than attempting to absorb this as it's being formed, your better chance is probably to attempt to detect and suppress the fire in the very early stages before the high evolution of these materials is created.

DR. NELSON: I think that that's the point, that is, if one is going to add technology, then, depending upon the environment, let's get automatic suppression. One needs sprinklers wherever possible. We should look if those sprinklers are present. One doesn't see sprinklers in this room. Sprinklers work,

and we as citizens need to advocate, wherever possible, the use of sprinklers. Now there is an unfortunate issue with that. I am on the Board of Directors of the American Chemical Society and may, if all goes well on Friday, become president of the ACS. The ACS has a new office building which is under construction in Washington, DC. The District of Columbia doesn't require a building sprinkler. Every jurisdiction around the city of Washington would require that building sprinkler. The insurance companies, other people, the developers for that building said "no, no, there is no need to sprinkler the building." Well, because of one temperamental member of the ACS board of directors, who I won't name, that building is going to be sprinklered, but it won't be because of the regulations in the city of Washington. We have got to get sprinklers in buildings.

DR. ANDERSON: Certainly sprinklers are the future, and to some extent what we are worried about right now is the past. There is an enormous load of unsprinklered buildings, and they are not going to be retrofitted.

DR. LEVIN: I would like to answer that question from a different point of view. That is, what do we do with the fire victim who hasn't died? In the present, as far as I know, a person who is pulled out of a fire and who's maybe unconscious is given oxygen, and his throat is examined for soot, and his blood is examined for carboxyhemoglobin. Then, if after 6 hours he appears to be all right, he is sent home. Now I think that as the emergency personnel become more aware of the fact that there may be other toxic gases that that person has been exposed to, then they can get better therapeutic regimes for those people. Now one of the things that we have done in the past couple years was to give out some grant money to again look at what you would do if a rat were exposed simultaneously to CO and HCN. Now the antidote for HCN is not what you want to give a person who has been exposed to CO because the antidote for HCN raises the methemoglobin level, which means now you can't get as much oxygen in the body as well. So these two common therapeutic approaches are really counteractive to one another. What you really need to know is in every fire you are going to have CO and in some of those fires you are going to have HCN. One should really know if that person has been exposed to HCN, and then how would you treat that person if he has this dual exposure? That's a big question.

DR. ANDERSON: The medical approach to victims who have survived is almost uninvestigated, and the other thing that nobody in the field has been able to get to, of course, is the long-term effects of fire exposures. One of the reasons for that is that there has been a very small population having multiple fire exposures except for the fire fighters. The money does not flow so easily toward a very small subset of the population, so the starting point has been the single-fire victim. The single fire survivor may be next in line.

DR. LEVIN: But even for the fire survivor there is no information about what happens to that person after that six hours is up and the person goes home. My rats died postexposure, and if two weeks after this particular incident a person would come down with some type of respiratory problem, would they even equate that with the fire exposure that they had? If they went to a doctor, would they even mention the fact that they had been in a fire or had been exposed to some kind of smoke two weeks earlier? I don't think that's clear.

DR. ANDERSON: If they go to the doctor they might not. If they go to the lawyer they will.

DR. LEVIN: Exactly.

MAJ. CLEWELL (AAMRL/TH-WPAFB): Suppose that we were given some materials for a cockpit seat, and they wanted to know if they use this material and there is a cockpit fire, is the pilot going to become unconscious more quickly than if we use another material? I get the feeling from what I heard in the session that we are talking about a half dozen gases. We could just combust the materials, measure the gases, and get a feeling for the toxicity of the compound. But everybody that is trying to pitch their wares to me about what needs to be done says, "You've got to look for all these possible complex materials that might be produced in very small amounts but are extremely toxic, such as phosphorus." I would like to know how you feel about that.

DR. LEVIN: I would like to answer that question. The approach that I would advise you to take is to burn that seat and look at the primary toxic gases. And then, based on that, predict what you would think the LC_{50} should be, and then expose your animals to that LC_{50}. If some percentage of those animals die, you will be close to the LC_{50} and you can say "Okay, it's these primary gases that are causing the effect." If all the animals die, then there may be some other minor, more obscure gas that you have to worry about. Then find out what the other gas is.

DR. GRAND: If it's really an important problem, I wouldn't go with a lab-scale toxicity test at all. I would simulate a larger situation – actually construct a seat and create a fire as it might take place in real life – because, as Gordon pointed out, the ignition and the spread of flame and the development and rate of evolution of the smoke are really critical parameters. If you can simulate something more like real life, you can still analyze the gases; we still have mechanisms for exposing the animals to the smoke. But you simplify the fire so much when you take a little chunk weighing a couple of grams and stick it in a cup or put it under some fire scenario. You are not simulating that exact fire scenario in that small-scale apparatus.

DR. ANDERSON: I think a problem with the recipe that Barbara has given is that the data she has been working with have been primarily for CO and HCN. Both of these have the effect of compromising oxygen delivery to cells. The mechanisms are a little bit different, but there is a final common pathway. She has found that they add together in a rather impressive manner. Now it is

not clear, without doing some work beforehand, that if you are working with gases that do not add together at the same ultimate cellular function, that you would be able to get the same kind of elegant results that she is getting now. If you can't add together the effects of the toxic gases you have found, if you haven't demonstrated that they do add together to make one or more than one, then you are somewhat at sea, because we are not in a good position to take analytical data and play the so-what game. The toxicology data bases in the world tend to be single chemicals. When you try and add one-third of a lethal dose of X plus one-third of a lethal dose of Y plus who-knows-what-else, what you come out with does not necessarily appear rational. Nor does it necessarily work. The things that Barbara has done have worked beautifully. I should like to see that extended before I actually think a chemical analysis is going to put you into the position you want to be in.

DR. LEVIN: But that's why you still need to run your animal experiments. Because those animal experiments will tell you if you have explained the toxicity that you see or if something else is there. Right now, as Rosalind said, we only have three gases in this model. Hopefully, within the next two years or so we will add more gases to it and be able to explain a wider variety of material decomposition products. I don't see us getting rid of the animal tests completely, but I see where we may not have to do as many animal tests if we use a combination of analytical and animal.

MR. PATNODE (INDUSTRIAL COMMISSION OF OHIO): I recalled, during all of this, a fire aboard a commercial aircraft at Cincinnati about two years ago. I was surprised when I heard that the pilot never dropped the emergency masks that they have on board airplanes. But it seems to me if we have respirators of some sort available, and if fire deaths are primarily caused by oxygen deficiency, if the oxygen were available in the mask a lot of people could have been saved in a number of fires. Could anybody comment along that line?

DR. NELSON: In terms of the Cincinnati fire, it is my understanding that the reason the masks weren't dropped was that they were below the allowable altitude to drop the masks. There have been discussions within FAA over the years regarding the need for masks that could be used for that purpose. But the present oxygen masks are used solely to supplement oxygen, they are not to exclude other gases. Fire masks, may, for the aircraft situation, be a suitable additional solution. In fact the Cincinnati accident was the first major in-flight fire. Most fires have occurred as post-crash fires. So most of what FAA has been concerned about is the post-crash fuel fire scenario, and that's a totally different situation.

CAPT. HORTON (AAMRL/TH-WPAFB): Dr. Levin, in your slides you showed that when you used carbon dioxide and carbon monoxide that you could not induce an expected death rate. One of the things that I have been doing at Wright-Patterson is similar to that. I have used carbon monoxide and a pulmonary irritant. I can get the same blood gas chemistries or similar blood gas chemistries

that you were getting with your carbon dioxide and carbon monoxide study. Consequently, I think, instead of looking for another toxic chemical in your Douglas-fir nonflammable study, you might want to look at pulmonary irritation from the burning wood itself. From the compounding of the increased CO_2 in the atmosphere you might actually get the response that you wanted in terms of death in rats. From my work it looks as though when PCO_2 increases in the blood stream PCO_2 rapidly increases in the brain because it is easily diffused across the blood-brain barrier. That, in turn, causes a rapid decrease in the pH in the brain. The bicarbonate doesn't diffuse across the blood-brain barrier as quickly, and, consequently, in the brain you do not get the buffering effect that you would expect in the bloodstream. So, consequently, in your study, the pH of the bloodstream of your rats was pH 6.8. The pH may be even lower in the brain. Some of the studies have shown, in people, marked increases in PCO_2 will suddenly cause collapse, coma, and death. So, consequently, the effect may be associated with the PCO_2 more dramatically than you think. The other factor I think that we have to look at in terms of the human data that you presented was a group of people that showed a marked elevation of carboxyhemoglobin who didn't die as anticipated, compared to the other group. You explained the difference as possibly being athletes or younger people with better cardiopulmonary systems. I think the other thing we have to take into consideration in that group is that the athlete or the young child is capable of buffering metabolic acids at a greater level. Consequently, if they are able to buffer acids at a greater level in the metabolic form, when you put them in a fire, they are also going to be able to buffer the respiratory acids, such as carbon dioxide, in a greater level. Consequently, those people are going to be more resistant to a fire exposure than the elderly person who is obviously not very active and does not have the capabilities of buffering acids quickly and easily. So I think that we have to look at the bicarbonate ion as being an essential part or playing an essential part in why some of these people are capable of defending themselves in a fire mode, versus people who are in the same age group or in the same situation or activity level. One thing I wanted to look at was the possibility of having two groups of rats, one exercised and one not exercised, and see if there really is a difference in the rate of death. That would show that exercise, if there were less deaths, would be associated with the capability of buffering your respiratory acids at a greater level.

DR. ANDERSON: I think that is a splendid approach. The physiology of the process is sometimes neglected in the discussion of fire toxicity.

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ISOLATION AND CULTURE OF ALVEOLAR EPITHELIAL CELLS FROM INJURED RABBIT LUNG

Frank A. Barile

York College of the City University of New York, Jamaica, New York 11451

Type II alveolar epithelial cells are often subject to injury induced by pulmonary toxins. Acute alveolar injury (AAI) commonly occurs as a result of exposure to a variety of toxic stimuli and is characterized by widespread necrosis of the epithelium followed by regeneration and hyperplasia of epithelial cells. We studied the response of the lung to a chemical stimulus that provokes cellular proliferation in order to establish an *in vitro* system for studying epithelial regeneration. AAI was induced in rabbits by repeated daily injections of *N*-nitroso-*N*-methyl urethane (10 mg/kg body wt.). After four to eight days of treatment, the lungs were aseptically excised, minced, and immersed in Hank's balanced salt solution containing trypsin (0.125%), elastase (0.03%), and DNase (0.005%). Following enzymatic digestion and neutralization, the cells were pelleted, resuspended in medium, plated in tissue culture flasks, and incubated at 37°C using differential adhesion procedures. After three to seven days, a homogeneous monolayer of epithelial cells was established. Using phase contrast microscopy, the cells displayed typical cobblestone-like arrangement, cuboidal shape, and represented 90-95% of the cells in culture. To determine their *in vitro* life span, the cells were grown to confluency and passaged. They achieved passage level 5 ± 1 and population doubling level (PDL) 12 ± 2. At each level, they exhibited contact inhibition and maintained their cuboidal shape, but appeared flatter, larger, and contained numerous vacuoles with passage.

Ultrastructurally, the cells showed abundant rough endoplasmic reticulum (ER) with dilated cisternae, numerous surface microvilli, and occasional lamellar bodies early in culture. In order to provide further evidence for the identity of the cultured cells, the polyacid dichrome histological stain was used. The reaction is based on the classical Mallory trichrome and differentiates the yellow-staining keratinizing epithelial cell cytoplasm from the blue-staining cytoplasm of fibroblasts. In our cultures, the procedure confirmed that 95% of the population at each PDL was of epithelial origin. Under identical culture conditions, only fibroblasts were observed in cultures from control rabbit lung.

The results demonstrate that regenerating epithelial cells from injured lung can be isolated and propagated in culture as a relatively homogeneous population. This system, which is designed to understand the metabolic role of epithelial cells following acute injury, may serve as an alternative to animal models in pulmonary toxicity studies.

IN VITRO EFFECTS OF 1,1-DIMETHYLHYDRAZINE ON HYDROGEN PEROXIDE-INDUCED SUPPRESSION OF LYMPHOCYTE BLAST TRANSFORMATION

Don Frazier,* Melinda J. Tarr, and Richard G. Olsen

The Ohio State University, Department of Veterinary Pathobiology, Columbus, Ohio 43210

Previous experiments in our laboratory have demonstrated that exposure to 1,1-dimethylhydrazine (UDMH) enhances certain murine immune functions such as mixed lymphocyte response and Jerne plaque response. UDMH has also been shown to suppress murine macrophage functions of phagocytosis, microbiocidal capacity, chemiluminescence, and prostaglandin E2 synthesis. It is known that chemiluminescence by activated macrophages is associated with production of various oxygen metabolites, including hydrogen perixode (H_2O_2). It has also been shown that H_2O_2 *in vitro* can suppress certain immunoassays, hence it has been proposed as one of many natural immunoregulatory substances. We have found that H_2O_2 ($\geq 10\mu M$) suppresses mitogen (concanavalin A and lipopolysaccharide) stimulated lymphocyte blast transformation (LBT), and are beginning experiments to evaluate the effects of UDMH on this H_2O_2-induced suppression of LBT. Preliminary findings have shown UDMH to have additive suppressive effects with H_2O_2 on the LBT assay due to cytotoxicity. Experiments utilizing lower concentrations of UDMH with H_2O_2 added at various times during the LBT assay are now in progress.

These experiments should reveal if UDMH exerts part of its immunoenhancement by interfering with the "normal" suppressive activities of H_2O_2.

This work was supported in part by Grant No. 86-0129 from the Air Force Office of Scientific Research.

METABOLISM OF 4,4'-METHYLENE-BIS(2-CHLOROANILINE) IN EXPLANT CULTURES OF HUMAN AND DOG BLADDER AND DOG LIVER CELL CULTURES

Gary D. Stoner,[1] Narayan M. Shivapurkar,[1]* Herman A.J. Schut,[1] James E. Klaunig,[1] Marc J. Mass,[2] and Teresa A. Lehman[3]

[1]*Department of Pathology, Medical College of Ohio, Toledo, Ohio 43614*

[2]*Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711*

[3]*Comprehensive Cancer Center, Ohio State University, Columbus, Ohio 43210*

Studies were conducted to compare the metabolism, DNA-binding, and DNA-adduct formation of 4,4'-methylene-bis(2-chloroaniline) (MOCA) in cultured human and animal model tissues and cells. MOCA, an aromatic amine, is used for the curing of polyurethane elastomers and has been detected in the urine of exposed workers. It induces pulmonary, mammary, and hepatic carcinomas in rats; hepatic carcinomas in mice; and bladder carcinomas in dogs.

A comparison was made of the binding of MOCA metabolites to the DNA of cultured human and dog bladder explants and dog hepatocytes. Explants and cells were cultured for 18 h and then exposed to [^{3}H]MOCA (0.1, 1, and 10 μM, specific activity 24 Ci/mmol) for 24 h. The medium, explants, and cells were pooled and stored at -70°C. DNA was isolated by hydroxyapatite chromatography as described previously (Stoner, *et al.*, *Carcinogenesis* **3**:155-201, 1982). The binding levels were as follows:

Comparative Binding Levels of MOCA to the DNA of Dog and Human Bladder Explants and Dog Hepatocytes

Tissue	Concentration of MOCA (μM)		
	0.1	1.0	10
Dog bladder	0.25 ± 0.20[a] (N = 4)	1.28 ± 1.12[a] (N = 16)	12.35 ± 15.18[a] (N = 15)
Human bladder	1.01 ± 1.43 (N = 5)	8.59 ± 16.51 (N = 5)	22.01 ± 26.13 (N = 7)
Dog hepatocytes	0.56 ± 0.27 (N = 4)	6.03 ± 8.78 (N = 5)	19.11 ± 17.81 (N = 4)

[a]Binding is expressed as pmoles/mg DNA (mean ± S.D.).

As shown in the table, the data indicate that the binding of [^{3}H]MOCA to DNA is in the order of human bladder > dog hepatocytes > dog bladder. Studies are ongoing to identify and quantitate the adducts formed between MOCA metabolites and DNA and to identify MOCA metabolites in the culture medium.

These studies were supported by EPA Cooperative Agreement No. 812121 (GDS), NCI P-30-CA1605813 (OSU CCC), and NIH-NCI T32 CA09498-02 (TAL).

EXPOSURE-INTEGRATED PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS FOR RISK ASSESSMENT OF ENVIRONMENTAL CARCINOGENS

Kenneth T. Bogen,* Thomas E. McKone, and David W. Layton

Environmental Sciences Division, Lawrence Livermore National Laboratory, Livermore, California 94550

Physiologically based pharmacokinetic (PB-PK) models describing the metabolism of xenobiotic compounds in animals and humans are currently proposed for use in regulatory risk assessments of suspect human carcinogens (e.g., organochloride solvents). So far, the proposed regulatory application of PB-PK models to human risk assessment have involved only simplistic exposure scenarios, such as steady-state exposure via a single exposure route. More realistic exposure scenarios, for example, from a volatile groundwater contaminant, actually involve non-steady state exposures via multiple routes. We have developed time-profile models for human exposures to contaminated water supplies that include multiple routes. In addition to direct ingestion, these models account for the skin absorption during bathing and inhalation of volatile compounds released to household air by domestic water uses – showers, washing machines, toilets, etc. Here we present preliminary results from an application of PB-PK models to human risk assessment for two compounds of regulatory concern, perchloroethylene and trichloroethylene, where these models are integrated with more realistic time-profile models of human exposure in a domestic context. These results indicate that the use of exposure-integrated PB-PK (or EI-PB-PK) models that account for human metabolism under realistic exposure conditions may significantly improve the application of PB-PK modeling capability to regulatory risk assessment.

KINETICS OF TRICHLOROETHYLENE IN PREGNANT AND LACTATING RATS AND RAT PUPS

Jeffrey W. Fisher,[1]* Melvin E. Andersen,[1] Harvey J. Clewell, III,[1] and Douglas Taylor[2]

[1]Harry G. Armstrong Aerospace Medical Research Laboratory/Toxic Hazards Division, Wright-Patterson Air Force Base, Ohio 45433-6573

[2]Miami University, Zoology Department, Oxford, Ohio 45056

Recently, the Department of Defense (DOD) established a waste disposal site program, similar to the U.S. EPA's "Superfund," called the Installation Restoration Program. The goal of the program is to identify contaminants of waste disposal sites and remediate those sites, if necessary. One chemical that is commonly found in the environment near these sites (e.g., groundwater and soil) is trichloroethylene (TCE), a synthetic compound largely used as a degreaser. TCE has been shown to be an animal carcinogen and has adverse health effects in humans at high gaseous concentrations.

One aspect of toxicologic concern is transplacental exposure of fetuses and neonatal exposure via nursing as a result of maternal exposure to TCE. The purpose of this study is to develop a physiologically based mathematical model for the rat that describes exposure levels of TCE and one of its major metabolites, trichloroacetic acid (TCA), in fetuses and nursing pups as a result of maternal exposure to TCE by drinking water and gavage. Comparisons between simulated and experimental tissue burdens will be discussed.

THE USE OF FISH IN CARCINOGENICITY TESTING: TEMPERATURE EFFECTS

Henry S. Gardner, William H. van der Schalie, Karen L. Hoover, and Robert A. Finch*

U.S. Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, Frederick, Maryland 21707-5010

Fish are useful in chemical carcinogenicity evaluation for several reasons: they are sensitive to a number of known mammalian carcinogens; water is an excellent medium for continuous carcinogen exposure, and materials such as contaminated groundwater, effluents, and hazardous waste leachates can be directly evaluated; fish have a low background incidence of neoplasms; tests with fish are very inexpensive relative to mammalian tests; and the use of small fish species allows examination of whole body serial sections on a single microscope slide. Since fish are poikilothermic, a major variable in fish carcinogen testing is temperature selection. We have evaluated the effects of temperatures ranging from 20 to 35°C on diethylnitrosamine (DEN)-induced liver neoplasia in the Japanese medaka (*Oryzias latipes*). Two-week-old medaka fry were exposed to DEN at concentrations ranging from 20 to 200 mg/L for 48 h and then held in clean water for up to 26 weeks. Both neoplastic and pre-neoplastic liver lesions were found in medaka exposed and held at 25°C. These lesions were reduced in number or absent entirely in fish exposed and held at 30 and 35°C. Results of tests conducted at 20°C have not yet been fully analyzed, but it appears that 25°C may be the optimal temperature for development of DEN-related neopasia in the medaka.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

ANTIOXIDANTS, CAFFEINE, AND DIBUTYRYL cAMP PREVENT THE INHIBITION OF MOUSE HEPATOCYTE INTERCELLULAR COMMUNICATION BY LIVER TUMOR PROMOTERS

Randall J. Ruch* and James E. Klaunig

Department of Pathology, Medical College of Ohio, Toledo, Ohio 43699

Intercellular communication via gap junctions (IC) is thought to function in regulating cellular replication. Most tumor promoters are capable of inhibiting IC between cultured cells, and this effect may function *in vivo* to isolate initiated cells and permit their clonal expansion. In this study, the rodent liver tumor promoters, phenobarbital (PG; 20-55 µg/ml), DDT (0.1-10.0 µg/ml), and lindane (0.1-5.0 µg/ml), inhibited IC between male $B_6C_3F_1$ mouse hepatocytes in primary culture. IC between hepatocytes was detected by autoradiography as the passage of [5-^{3}H]uridine nucleotides from prelabeled "donor" hepatocytes to nonlabeled "recipient" hepatocytes. The addition of the antioxidants, superoxide dismutase (SOD; 100 U/ml), *N,N'*-Diphenyl-*p*-phenylenediamine (DPPD) (25 µM), and vitamin E (100 µM), to PB-, DDT-, and lindane-treated cultures prevented the inhibition of hepatocyte IC. Glucose oxidase (GO; 0.01-0.1 U/ml), which generates hydrogen peroxide, also inhibited mouse hepatocyte IC. This effect of GO was prevented by DPPD and vitamin E, but not by SOD. The inhibition of hepatocyte IC by PB was also prevented by caffeine (0.01-1.0 mM) and dibutyryl cAMP (0.01-0.1 mM), treatments that increase intracellular cAMP levels. These results suggest that tumor promoters inhibit mouse hepatocyte IC through the generation of activated oxygen species and that increased intracellular cAMP levels can override this effect.

RELATIONSHIP OF EARLY, CARCINOGEN-INDUCED, ATYPICAL CELL COLONIES TO *IN VIVO* MALIGNANT TRANSFORMATION IN THE RAT TRACHEAL EPITHELIAL FOCUS ASSAY

Vernon E. Steele,[1]* John D. Van Arnold,[1] and Marc J. Mass[2]

[1]*Laboratory of Pulmonary Carcinogenesis, In Vitro Toxicology Program, Northrop Services, Inc. – Environmental Sciences, Research Triangle Park, North Carolina 27709*

[2]*Carcinogenesis and Metabolism Branch, Health Effects Research Laboratory, U.S. EnvironmentalProtection Agency, Research Triangle Park, North Carolina 27711*

To define and study the initial stages of neoplastic transformation in respiratory epithelial cells, a series of morphologically transformed cell colonies from carcinogen-exposed rat tracheal epithelial (RTE) cell cultures were characterized. Tracheal epithelial cells were isolated from Fischer-344 rats, plated on collagen-coated dishes, and exposed to 7,12-dimethylbenz(*a*)anthracene on Day 1 for 24 h. Between Days 26 and 30, colonies of morphologically altered cells were isolated and classified into three major groups based on cell density: Class I – less than 1300 cells/mm^2, Class II – 1300-2500 cells/mm^2, and Class III – above 2500 cells/mm^2. Following plating, the cell populations were assayed for their ability to grow in various media and on various substrates. In general, Classes II and III had a higher colony forming efficiency when replated in various media and grew bettter on all substrates tested. The population doubling time generally decreased faster in Class II and Class III cells than Class I cells. The cells were placed into denuded tracheal grafts that were transplanted subdermally into nude mice. Untreated cells produced a mucociliary epithelium, whereas the progression from Class I to Class III in culture was reflected by a progression in the animal toward a more transformed phenotype. These studies show that early carcinogen-induced premalignant RTE cells differ in a variety of characteristics and that at least three classes initially exist that become heterogeneous with time.

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A RAPID *IN VITRO* BIOASSAY FOR THE DETECTION OF POTENTIAL CARCINOGENS AND FOR THE STUDY OF EARLY AND LATE NEOPLASTIC PHENOTYPES IN RETROVIRUS-INFECTED RAT EMBRYO CELLS

William A. Suk,*[1] Joan E. Humphreys,[2] and John D. Van Arnold[2]

[1]*National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709*

[2]*Laboratory of Cellular and Molecular Biology, Northrop Services, Inc. – Environmental Sciences, Research Triangle Park, North Carolina 27709*

A mammalian cell culture system using Rauscher leukemia virus-infected Fischer rat embryo ($2FR_450$) cells was used as a model to study the ability of chemical and physical carcinogens to induce early and late phenotypes of neoplastic transformation. When suspended in liquid media above an agar base, anchorage-dependent control cells showed a rapid decline in cell survival, whereas cells that had previously been treated with carcinogen survived in suspension as multicellular aggregates. The bioassay takes 11 days, and the early transformation end point, survival in cellular aggregates (SAg+), was measured by counting viable cells dissociated from aggregates that were suspended for four days. Chemical carcinogens (7,12-dimethylbenzanthracene, benzo(*a*)pyrene, *N*-methyl-*N*'-nitro-*N*-nitroso-guanidine, 4-nitroquinoline-*N*-oxide, and diethylstilbestrol), complex environmental mixtures, and the physical carcinogen, ultraviolet irradiation, induced SAg+ dose dependently. The bioassay discriminated between carcinogens and noncarcinogens, without the addition of a metabolic activation system. The induction of the end point was correlated with progression to neoplastic phenotypes in the same cells, as evidenced by their morphological transformation, growth in semisolid medium, and tumorigenicity in nude mice. The selection process of suspension of cells in liquid medium resulted in the rapid expression of transformed phenotypes. The SAg+ end point appeared to require the presence of the exogenous retrovirus, or the induction of the endogenous viral genome, because uninfected cells did not show a differential survival response when carcinogen-treated, noncarcinogen-treated, and control cells were compared. The results indicate that agents with carcinogenic potential can be assayed for rapidly by *in vitro* transformation of target cells selected by the integration and expression of retrovirus and that the formation in suspension of aggregated cells following carcinogen treatment selects for the expression of subsequent neoplastic phenotypes.

INFLUENCE OF THE TIME OF PHENOBARBITAL TREATMENT ON LIVER TUMOR DEVELOPMENT IN INITIATED $B_6C_3F_1$ MICE

Christopher M. Weghorst,[1]* Michael A. Pereira,[2] and James E. Klaunig[1]

[1]*Department of Pathology, Medical College of Ohio, Toledo, Ohio 43699*

[2]*Environmental Health Research and Testing, Inc., Cincinnati, Ohio 45245*

Previous studies have shown that infant male $B_6C_3F_1$ mice initiated with diethylnitrosamine (DENA) and treated with phenobarbital (PB) continuously from weaning until sacrifice (24-52 weeks) displayed a decrease in the number of liver tumors compared to mice treated with DENA only. It has also been shown that masculinization of young male mice was reduced by PB administration. The present study investigated the effects of varying the time of the start of PB treatment in neonatally DENA-initiated male $B_6C_3F_1$ mice. Mice were treated with either saline or DENA (5 mg/kg) in saline by i.p. injection on Day 15 of age. Mice were weaned at 28 days of age and divided into eight groups (Groups 1, 3, 5, and 7, DENA treated; Groups 2, 4, 6, and 8, saline treated). At weaning, Groups 1 and 2 received deionized drinking water for 24 weeks. Groups 3 and 4 received 500-ppm PB drinking water for 16 weeks followed by deionized water for 8 weeks. Groups 5 and 6 received deionized water for 4 weeks, PB water for 16 weeks, and deionized water for 4 weeks. Groups 7 and 8 received deionized water for 8 weeks followed by PB water for 16 weeks. Mice were sacrificed at 28 weeks of age and necropsied. Livers were processed for light microscopy. Hepatic lesions were histologically classified and quantitated. Hepatocellular adenomas and foci were observed in livers from Groups 1, 3, 5, and 7; however, no lesions were found in Groups 2, 4, 6, or 8. Groups 3, 5, and 7 had a significant decrease in the number of foci and the mean area of the foci when compared to Group 1. There was no significant difference between Groups 3, 5, and 7 in foci number, but the mean area of the foci in Group 5 was significantly larger than those in Groups 3 and 7. The incidence of adenomas in Group 1 was significantly higher than that of Groups 3, 5, and 7 with no significant difference between Groups 3, 5, and 7. The mean areas of the adenomas in Groups 1 and 5 were similar, whereas those in Groups 3 and 7 were significantly smaller. These results show that the inhibition of hepatocellular foci and adenomas by PB is maintained even if PB treatment of the DENA-initiated mice is withheld until 8 weeks after weaning.

BIOLOGICALLY BASED MATHEMATICAL SIMULATION OF CYTOTOXICITY AND MUTATION ACCUMULATION

Rory B. Conolly,[1]* Richard H. Reitz,[2] and Melvin E. Andersen[3]

[1]*Northrop Services, Inc. – Environmental Sciences, Dayton, Ohio 45431*

[2]*Dow Chemical Company, Midland, Michigan 48640*

[3]*Harry G. Armstrong Aerospace Medical Research Laboratory/Toxic Hazards Division, Wright-Patterson Air Force Base, Ohio 45433*

We have combined a well-validated, biologically based model for volatile toxicant pharmacokinetics with the cancer model of Moolgavkar and Knudson (*JNCI* **66**: 1037-1052, 1981), which states that cells become tumorigenic after accumulating "two discrete, specific, and heritable events." The resulting pharmacodynamic model simulates tissue concentrations, hepatic biotransformation, cytotoxicity of inhaled toxicants, and the accumulation of mutations occurring during cellular replication. The tissue dose of an enzymatically generated toxic metabolite of the parent compound is related to depletion (or alteration) of a hepatic macromolecule (MM) by a normal distribution centered about a set level of MM. The "liver" is a population of 10^8 cells with equilibrium birth and death rates of 10^{-6} h that can respond to an increase in the death rate, i.e., cytotoxicity, by increasing the birth rate until the steady-state population of 10^8 cells is regained. Every cell division incurs a 10^{-7} chance of "mutation." In the model, significant cytotoxicity greatly increases the rate of accumulation of mutated cells. Simulated one-year inhalation exposure (6 h/day) to a model cytotoxicant causing about 20% hepatic cell death/exposure with complete recovery of cell number between exposures led to a linear increase over time in the population of cells with one "mutation" and an exponential increase in the population with two "mutations." This work suggests that more sophisticated biologically based mathematical models will be useful in exploring the actual quantitative relationships between chronic toxicant exposure, cytotoxicity, DNA damage, genotoxicity, and tumor development.

AN IMPROVED FLOW-PASS NOSE-ONLY EXPOSURE SYSTEM

William C. Cannon,[1]* E.W. Bass,[2] and John R. Decker[1]

[1]Biology and Chemistry Department and

[2]Design and Drafting Department, Battelle Pacific Northwest Laboratories,
Richland, Washington 99352

An improved nose-only aerosol exposure system for rodents has been designed for efficient operation and low construction costs. The 48-port chamber delivers fresh aerosol independently to each exposure port ensuring uniform concentration of aerosol throughout the chamber. For small exposure groups, unused ports can be blocked off to avoid waste of scarce and/or expensive aerosols or to minimize use of hazardous materials. The chamber can be rotated for easy loading and disassembled to facilitate cleaning.

Use of O-ring seals and operation at negative pressures prevent escape of highly toxic aerosols from the exposure system. To avoid overheating of rats during exposure, a special provision is made to allow air cooling of their tails through a sealed thin-walled metal tube.

Rapid build up and clearance of aerosols and uniform aerosol delivery to ports help to maintain close control of inhaled dose during exposures.

Arrangements are being made with Harford Systems Division of Lab Projects Incorporated to have this equipment commercially available in the near future.

AN ACCURATE, WIDE RANGE, AUTOMATED HPLC METHOD FOR THE ESTIMATION OF OCTANOL:WATER PARTITION COEFFICIENTS

John E. Garst

Department of Surgery and Medicine, Veterinary Medical Center, Kansas State University, Manhattan, Kansas 66506

One physical factor governing interactions between xenobiotics and biological macromolecules is the hydrophobicity of the agent. A frequently used measure of hydrophobicity is the logarithm of the octanol:water partition coefficient, log P(o:w), of the chemical.

A high-performance liquid chromatography (HPLC) method has been developed that affords accurate and reproducible log P(o:w) estimates over the 0 to 8 log P(o:w) range. Correlation between literature shake-flask log P(o:w) values and the HPLC equivalent exceeds 0.9994, while high reproducibility is obtained for numerous organic compounds. Unlike other HPLC methods, this procedure uses commercial RP-8 columns, variable flow rates, and a dual solvent:buffer system to improve peak shapes and to speed elution. Data acquisition may be completely computer-controlled, enabling rapid, routine log P(o:w) determinations at costs comparable to the calculation of values, but with better results, possibly because this procedure better ensures the establishment of HPLC equilibrium.

Commercial, 10-µm particle size, RP-8 reverse-phase silica gel columns of varying lengths are used. The retention volume for the compound of interest is computed from the retention time at various MeOH:buffer concentrations by extrapolating the linear segment of the logarithm of the retention time versus the MeOH:buffer concentration to zero percent methanol. Use of the linear segment better ensures equilibrium because not all compounds afford a linear relationship.

A formula compensates the extrapolated value for different flow rates, temperatures, and column lengths, enabling the extraordinary 0-8 log P(o:w) range, while minimizing the time required for each measurement. This individual column-corrected log Vccr result is converted by linear regression methods to the log P(o:w) estimate. The resultant log Vccr appears independent of acid, but not of base concentration. However, TEA near 0.035 M became relatively independent of log Vccr and, accordingly, bases and neutral compounds afford a single line that is statistically indistinguishable from that with 0.004 M trifluoroacetic acid.

Using this methodology, phenyl-, methyl-, fluoro-, chloro-, and bromobenzene substitutents afford curvilinear plots of log P(o:w) versus substituent number indicating that substituent constants are not additive. This may question the validity of some calculated values. In addition, this procedure correlates binding of 34 compounds to serum albumin as well as a combination of shake-flask and calculated values, but is superior to other HPLC, shake-flask, and even calculated values in predicting environmental bioconcentration.

MOLECULAR PROBES TO DETECT POINT MUTATION IN MAMMALIAN SPERM

Sohaib A. Khan

Department of Anatomy and Cell Biology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0521

Occupational exposure of chemicals is a serious problem and can be responsible for a variety of toxic manifestations. A number of criteria are presently in use to monitor the population who are exposed to chemicals at work sites to determine if any deleterious effect might have occurred. Howeve:, there is lack of a test that can directly measure the effect of toxic chemicals at the genomic level in the exposed individual. The monitoring of mutation systems based on whole animals requires a pool of large numbers of animals that seems impractical and expensive for routine testing of mutagens. As an alternative to monitoring the pedigree, cells from exposed individuals may be considered for screening of point mutations through the use of an appropriate molecular probe directed towards a specific locus. Mammalian spermatogenic cells, which differentiate within a physiological environment during mutagenic treatment, are ideally suited for the detection of mutation. By using recombinant DNA techniques, I am developing a probe for the lactate dehydrogenase-X (LDH-X) locus. Toward this end, a mouse testis cDNA library has been constructed in an expression vector, λgt11. Results obtained concerning cloning of LDH-X cDNA, detection of LDH-X mRNA by Northern blot analysis, and *in vitro* translation data of mouse testis RNA are discussed. Furthermore, a strategy showing how the LDH-X probe can be used to detect point mutation at the LDH-X locus using RNA/DNA hybridization and *in situ* hybridization is presented.

CHEMICAL SCORING SYSTEM FOR HAZARD ASSESSMENT

Robert H. Ross,[1]* Terry O'Bryan,[2] and Po-Yung Lu[1]

[1]Chemical Hazard Evaluation Program, Information Research and Analysis Section, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

[2]Existing Chemicals Assessment Division, U.S. Environmental Protection Agency, Washington, DC 20460

To assist in the preliminary evaluation of compounds of toxicological and environmental interest to the U.S. Environmental Protection Agency (US EPA), a scoring system was devised as a collaborative effort between the US EPA and the Oak Ridge National Laboratory. The scoring system combines objective guidelines with professional judgment to evaluate chemicals and consists of 17 separate scoring parameters, seven of which pertain directly to toxicology, e.g., oncogenicity and genotoxicity. The remaining parameters are related to environmental fate and occupational and consumer exposure. This scoring system was designed to rapidly score chemicals in a minimal amount of time with readily available information. It is used by the Office of Toxic Substances of the US EPA as a tool to help set priorities in conjunction with other criteria. It is particularly useful in performing preliminary evaluations involving large chemical classes, such as petroleum distillates.

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CAN EARTHWORMS SUBSTITUTE FOR RODENTS IN DETERMINING ACUTE METAL TOXICITY?

S. Serda and Arthur Furst*

Institute of Chemical Biology, University of San Francisco, San Francisco, California 94117-1080

Earthworms have been known to accumulate heavy metals in their tissues. The posterior alimentary tract, specifically the chloragogenous tissue, appears to concentrate most of the heavy metals (Ireland and Richards, 1977). The chloragogen tissue seems to function as the invertebrate's liver (Roots, 1960). The LD_{50} values for metals can be determined by injecting the compounds into the hemocoel of the worms. This route is justified, for when worms were subjected to cadmium in soil, it was found that 68% of the cadmium was associated with the surface mucous (Flemming, 1981). The metal complexes formed from the interaction between surface mucous and the metal are soluble and are not thought to interfere with the cellular activities of the organism. The LD_{50} values for the sodium salts of selenite and selenate have been identified. This procedure for LD_{50} determination leads to reproducible toxicity data and may allow us to use earthworms as an alternative to the mammalian system.

APPLICATION OF FLOW CYTOMETRY IN TOXICOLOGY

Robert M. Zucker,[1*] Ken H. Elstein,[1] Robert E. Easterling,[2] Edward J. Massaro[2]

[1]Northrop Services, Inc. – Environmental Sciences, P.O. Box 12313, Research Triangle Park, North Carolina 27709 and [2]Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

Flow cytometry combines laser technology, optics, and electronics to analyze subtle changes in cell and nuclear structure. By utilizing fluorescent dyes and fluorochrome-conjugated probes, information such as cell surface characteristics and perturbation of the cell cycle is readily obtained.

Using fluorescence and light-scatter parameters, alterations were observed in the nuclear structure of murine erythroleukemic cells exposed to a variety of drugs and toxic metals. Also, it has been observed that either 90° light scatter or fluoresceinisothiocyanate (FITC, protein) fluorescence can be used to distinguish mitotic cells from G_2-phase cells. The resulting cytograms were remarkably similar, suggesting that a relationship exists between nuclear protein content and 90° light scatter. Using these flow parameters, treatment of cells with toxic chemicals or metals was shown to alter the cell cycle and increase 90° scatter, suggesting that alterations in the nuclear internal structure had occurred. Cytograms of 90° light scatter versus DNA fluorescence yielded more precise information than histograms of DNA fluorescence alone.

A helium-neon laser was used to obtain the forward red scatter signal, which is a seldom-used parameter. Changes in this parameter revealed alterations of the sperm nuclear structure as a function of chemically induced decondensation. Thus, it appears that laser-based flow cytometry is a highly sensitive method for the detection of nuclear structural changes resulting from the interaction of cells with toxicants.

AN AUTOMATED ASSESSMENT OF CARDIOPULMONARY FUNCTION IN SMALL MAMMALS

Jeffrey S. Tepper

Northrop Services, Inc. – Environmental Sciences, Research Triangle Park, North Carolina 27709

Evaluation of cardiopulmary function in animals provides the unique opportunity to examine the integrative effects of inhaled toxicant exposure on the intact animal. Many of the diagnostic pulmonary tests performed in humans can now be performed in small laboratory mammals. However, because the voluntary cooperation of the animal cannot be easily secured, anesthesia is required to test the structurally dependent and dynamic limits of lung volume and air flow. Such tests are especially useful for the evaluation of chronic inhaled exposure regimens that may produce subtle impairment and structural damage that manifests in functional disability. During acute exposure, however, it is often desirable to test cardiopulmonary function in unanesthetized animals. The major advantage of this latter approach is that unanesthetized animals can make dynamic adjustments to breathing during acute toxicant exposure that may be indicative of acute dysfunction. Such breathing alterations might be blocked or otherwise confounded with anesthesia. These procedures for testing cardiopulmonary function in anesthetized and unanesthetized mammals are amenable to automatic data collection and analysis using a digital computer.

Among the measurements that we use to assess pulmonary function in small anesthetized animals after chronic exposure include: lung volumes and their apportionment (TLC, VC, RV, EEV, etc.), static lung mechanics (respiratory system compliance), ventilation distribution and diffusion capacities (N_2 washout and DI_{CO}), as well as small airway integrity (FEF and FEV).

During or after acute exposure, our unanesthetized pulmonary function system allows us to obtain direct measures of tidal volume via plethysmography and intrapleural pressure using a surgically implanted catheter. From these primary measures, frequency of breathing, minute volume, dynamic compliance, and inspiratory and expiratory flows, times, and resistances can be computed. Furthermore, bronchoreactive substances can be administered to test airway reactivity or CO_2 can be used to test the animal's compensatory reserves, much like exercise is used in human pollutant studies. With the addition of a carotid artery catheter for blood pressure measurement, arterial PaO_2, $PaCO_2$, and pH data can be collected. Electrocardiogram (ECG) leads allow us to monitor heart rate as well as analyze arrhythmias and other cardiac abnormalities.

When combined, these techniques encompass a comprehensive assessment of cardiopulmonary function allowing an investigator maximum flexibility for the study of acute and chronic responses to pulmonary toxicants.

ANALYSIS OF POTENTIAL IMPACTS ON TERRESTRIAL WILDLIFE RELATED TO EXPOSURE TO DIOXIN FROM LAND APPLICATION OF WASTEWATER SLUDGE IN MAINE

Russell E. Keenan, Mary M. Sauer, and David W. Crawford*

Envirologic Data, Inc., Portland, Maine 04101

Potential impacts on American woodcock, robins, and meadow-jumping mice from 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in landspread sludges in Maine were examined. Computer-based, soil-loading models were developed for predicting dioxin concentrations in soil after repeated application of sludge. Habitat usage, migration patterns, and feeding characteristics were then considered to determine the number of individuals that might be exposed to TCDD, relative to local or regional populations of these species.

To evaluate the impact on those individual animals potentially exposed, models of exposure were constructed to predict the animal's daily intake of TCDD corresponding to soil levels that ranged from 6 to 150 ppt TCDD. Daily intakes were then compared with derived no-effect-levels to estimate allowable levels of TCDD in sludge and soil below which a threat to individual animal health does not occur.

The study indicates that because of life history and habitat characteristics, the impacts of spreading sludges containing TCDD on land will be inconsequential on local or regional populations of these species. In addition, exposed individual animals are not likely to be adversely affected by soil concentrations of up to 150 ppt TCDD. Based on the soil-loading model used in this analysis, these animals are not likely to be adversely affected by concentrations of TCDD in landspread sludges of up to 1 ppb.

HUMAN HEALTH RISK ASSESSMENT RELATED TO EXPOSURE TO DIOXIN FROM LAND APPLICATION OF WASTEWATER SLUDGE IN MAINE

Frank H. Lawrence, Russell E. Keenan, David W. Crawford,* and Mary M. Sauer

Envirologic Data, Inc., Portland, Maine 04101

A quantitative human health risk assessment of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in wastewater sludges applied to pasture and croplands in Maine was conducted. Computer-based, soil-loading models were developed for predicting dioxin concentrations in soil after repeated application of sludge. Human exposure to TCDD was estimated for a number of scenarios through routes of ingestion, inhalation, and dermal contact. Lifetime incremental cancer risks associated with each exposure scenario were extrapolated based on maximum loading of the site with sludge containing 50 ppt TCDD. Allowable levels of TCDD in sludge and soil that would result in no more than a 1×10^{-5} incremental lifetime risk of cancer were also calculated for each exposure scenario.

Based on the results of Envirologic Data's analysis and in light of the conservative nature of the risk assessment, it is concluded that landspreading of wastewater sludges containing TCDD presents little or no threat to human health under the exposure scenarios examined.

METHODS FOR ESTIMATING *IN SITU* OXYGEN REQUIREMENTS OF AQUATIC ANIMALS USING REMOTE AUTOMATED BIOMONITORING AND SATELLITE DATA RETRIEVAL

Eric L. Morgan,[1*] Richard C. Young,[2] and Jane T. Red[2]

[1]Biology Department, Tennessee Technological University, Cookeville, Tennessee 38505 and

[2]The Advent Group, Inc., Brentwood, Tennessee 37027

The objective of this study was to develop methods for deriving real-time oxygen utilization rates from breathing-rate activities of aquatic animals maintained *in situ* at remote locations. For example, coupling portable automated biomonitoring systems and computer-assisted data collection platforms, rainbow trout (*Salmo gairdneri*) breathing rates were continuously monitored at two remote sites on a mountain stream. At each site, six nonrestrained trout were individually isolated in tube-type holding chambers, and breathing events were retrieved in the laboratory four times daily by satellite data communications. These data were used in a mathematical model to estimate oxygen consumption on an hourly basis.

Utilizing similar configurations, respiration, and breathing activity rates may be continuously monitored from numerous aquatic animals, including insects and molluscs. These nondestructive multispecies biomonitoring methods provide an alternative approach to aquatic toxicity testing.

EFFECTS OF TISSUE CULTURE ENVIRONMENT ON TOXICITY OF PFDA IN L5178Y MOUSE LYMPHOMA CELLS

Nicholas J. Del Raso

Northrop Services, Inc. – Environmental Sciences, Dayton, Ohio 45431

In evaluating the toxicity of perfluoro-n-decanoic acid (PFDA) on the mouse lymphoma cell strains L5178Y/+/+ and L5178Y/+/- (i.e., +/- with respect to thymidine kinase activity), it was observed that varying the culture conditions had a significant effect on the sensitivities of the two cell strains to PFDA in concentrations ranging from 10 to 100 μg/ml. These concentrations had no effect on generation time. Cells grown in media supplemented with 2%, 5%, or 10% horse serum had a generation time of 12 h. Cell lysis was used as a measure of toxicity. Although the exposure of the lymphoma cells to PFDA did exhibit a dose response at all concentrations of horse serum tested, PFDA toxicity increased as the horse serum concentration decreased, and the L5178Y/+/+ cells were more resistant to PFDA. Thus, some component of horse serum seemed to protect against the action of PFDA. Cells maintained in media containing 10% horse serum were exposed to PFDA in serum-free media and media supplemented with 0.3 g/ml of Fraction V bovine albumin. Exposure of lymphoma cells in serum-free media resulted in a tenfold increase in sensitivity to PFDA over that observed for cells in media containing 10% horse serum. Exposure of lymphoma cells in media supplemented with albumin resulted in PFDA sensitivities similar to that observed for cells exposed in media containing 10% horse serum. Albumin appears to be the critical component of horse serum that alters the toxicity of PFDA in these cells *in vitro*.

PERFLUORINATED C10 SATURATED FATTY ACID IS TOXIC FOR BOTH EUCARYOTIC AND PROCARYOTIC CELL SYSTEMS

Alan Liss,[1]* Joanne C. Pfeil,[1] and Dan Levitt[2]

[1]Biology Department, State University of New York, Binghamton, New York 13901 and [2]Guthrie Research Institute, Sayre, Pennsylvania 18840

Exposure to perfluorinated fatty acids occurs from a variety of sources, including paints, commercial wetting agents, and waterproofing compounds. Once thought harmless, it now appears that some of these compounds can cause significant morbidity and even mortality in test animals. The interaction of the C10 saturated perfluorinated fatty acid, nonadecafluoro-*n*-decanoic acid (NDFDA), with a procaryotic cell system (the cell wall-less microbe *Acholeplasma laidlawii*) and an eucaryotic cell system (the human B-cell line, F4) was evaluated. Reactions in both cell types were similar. It was possible to observe both cytotoxicity and cytolysis of the target cells depending on the NDFDA concentrations used. At 0.5 mM or less, both cell types appeared normal. At higher concentrations, lethality was observed. At concentrations above 5 mM, brief exposure (15 min at 37°C) resulted in toxicity and an apparent lysis of target cells. This was noted by solubilization of the membrane-bound immunoglobulin in F4 cells and release of integral membrane proteins from acholeplasmas. Solubilization was equivalent to that observed when the target cells were treated with the tenside, sodium deoxycholate. Nonperfluorinated C10 saturated fatty acid (capric acid) was not lethal at a 0.5 mM concentration. At 10 mM, capric acid was lethal and also showed tenside activity. These data demonstrate that NDFDA acts on a membrane target. Parallel findings from procaryotic and eucaryotic cells suggest that a common mechanism may be responsible for the specific toxic event.

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TIME-COURSE OF PERFLUORO-*n*-DECANOIC ACID (PFDA)-INDUCED CHANGES IN [^{14}C]-PALMITATE OXIDATION MEASURED *IN VIVO* IN MALE FISCHER-344 RATS

Gary D. Pilcher,* Marilyn E. George, and Melvin E. Andersen

Harry G. Armstrong Aerospace Medical Research Laboratory/Toxic Hazards Division/Biochemistry Branch, Wright-Patterson Air Force Base, Ohio 45433

Perfluorocarboxylic acids and related compounds are commercially important because of their unusual surfactant properties and high chemical stability. Certain perfluorinated surfactants are found in aqueous film-forming foams used as fire extinguishants. One chemical in this class, perfluoro-*n*-decanoic acid (PFDA,) $CF_3(CF_2)_8COOH$, causes toxic signs similar to those of 2,3,7,8-tetrachlorodibenzodioxin (TCDD), a toxic contaminant of trichlorophenoxyacetic acid-based herbicides such as "Agent Orange." The mechanism of toxicity for these chemicals is unknown; however, both PFDA andTCDD are reported to cause alterations in lipid metabolism.

Studies were initiated to examine PFDA's effects on the disposition of a radiolabeled fatty acid substrate, [^{14}C]-palmitate. At 2, 4, 8, and 12 days after a single i.p. dose of 50 mg PFDA/kg body weight, rats were injected i.v. with [^{14}C]-palmitate and placed in a flow-through chamber system. [^{14}C]-CO_2 in the expired air was collected for a 12-h period. Results of this work indicate that palmitate oxidation as measured by cumulative 12-h [^{14}C]-CO_2 elimination was unchanged at 2 and 4 days postexposure in PFDA-dosed rats compared to pair-fed controls. At 8 and 12 days after dosing, [^{14}C]-CO_2 elimination in PFDA-dosed rats was significantly reduced by 39% and 33%, respectively, when compared to pair-fed controls. These data suggest that PFDA partially interferes with fatty acid oxidation over a delayed timecourse; however, the relevance of this observation to the toxicity of PFDA is unknown.

AUTOMATED INHALATION EXPOSURE SYSTEM FOR SMALL ANIMALS

John R. Decker,* H.S. DeFord, E.G. Kuffel, and R.D. Swannack

Biology and Chemistry Department, Battelle Pacific Northwest Laboratories, Richland, Washington 99352

An automated data acquisition and control system has been constructed for inhalation studies. By multiplexing monitoring devices and control elements, the system is capable of handling three separate test chemicals (vapors or aerosols) in three separate rooms using up to 30 Battelle-designed Hazleton 2000 exposure chambers (10 per room). The system monitors and controls chamber air flow, vacuum, temperature, and relative humidity, as well as test chemical concentration and generator functions. All data acquisition and control originates from a Hewlett-Packard Model 216 computer located in a control center. Experimental protocols reside in this computer and are entered into software tables accessed by menus. All protocol entries and changes are automatically recorded on the daily printout. Operators must enter passwords to access the menus.

Data from each of the three test chemical exposures are stored immediately upon completion of measurement on separate magnetic micro-floppy diskettes and are printed as a daily log by separate printers located in the control center. Operator comments may also be entered in the computer and printed in the daily log. At the end of the 24-h period, the daily data are automatically analyzed, and summary reports are printed.

A six-point alarm system with user-definable set points is available for each parameter and measurement location. Alarm conditions that may be a threat to the health of the animals alert security personnel who call the on-duty system operator.

HISTOPATHOLOGIC CHANGES IN THE LUNGS OF RATS EXPOSED TO INHALATION OF TOBACCO SMOKE OR MARIHUANA

Mohan Kumar,[1,2]* S. Poddar,[1] Louise Cardenas,[1] B.N. Rao,[1] and Mohan K. Menon

[1]University of West Indies, Faculty of Medicine, Kingston, Jamaica and [2]Cleveland College, Los Angeles, California 90004-2196

The histopathologic changes caused by tobacco and marihuana smoke in the lungs were evaluated by exposing two lots of healthy rats to smoke under controlled experimental conditions for periods ranging from 6 to 36 weeks. Two groups of 120 rats each were exposed to tobacco and marihuana smoke, respectively. The animals were placed in a compartmentalized grilled cage within a sealed polythene bag. The animals in one group were exposed to tobacco smoke, and the second group to marihuana smoke. Twelve cigarettes of either toxic substance were used for each lot over an 8-h period each day. The animals were exposed to smoke for 5 min, after which they were exposed to fresh air for 10 min, and the the experiment was repeated, in order to simulate actual human smoking conditions. Control animals for each group were subject to similar experimental conditions without the smoke. Lots of 20 rats in each group were sacrificed at 6, 12, 18, 24, 30, and 36 weeks, together with control animals. The lungs were dissected and processed by paraffin, and 6-μm representative sections were stained with H and E and Alscian blue. Changes caused by tobacco included vascular congestion, perivascular cuffing, inflammatory infiltrates, increased sulphation of mucus, early emphysematous changes, and moderate squamous metaplasia. Marihuana caused similar changes earlier and more severely, in addition to ulceration of the epithelium and focal hemorrhages.

MARIHUANA OR TOBACCO SMOKE EXPOSURE INDUCED AIRWAY HYPERREACTIVITY IN WISTAR RATS

Mohan Kumar,[1,2]* O. Parshad,[1] and Mohan K. Menon

[1]University of West Indies, Faculty of Medicine, Kingston, Jamaica and [2]Cleveland College, Los Angeles, California 90004-2196

The airway response to the chronic exposure of marihuana and tobacco smoke was assessed by exposing two groups of healthy Wistar rats to tobacco or marihuana smoke under controlled experimental conditions for periods ranging from 6 to 26 weeks. Two groups of 120 Wistar rats each were exposed to tobacco and marihuana smoke, respectively, in groups of 20. Twelve cigarettes of either toxic substance were used for each lot over an 8-h period each day. The animals were placed in a compartmentalized grilled cage within a sealed polythene bag. The unfiltered smoke of either marihuana or tobacco was pumped into the bag and the animals were allowed to breathe this smoke for a period of 5 min, after which they were exposed to fresh air and the procedure was repeated after a time interval of 10 min. Rats in the control group were exposed to air for the same period of time in the bag system. Airway resistance (Raw) and functional residual capacity (FRC) were measured at 12, 18, 24, 30, and 36 weeks by whole-body plethysmography, and specific airway resistance (sRaw) was calculated. The airway response to different doses of Ach, 0.25 µg, 0.50 µg, and 1.0 µg, was also evaluated. The smoke-exposed animals exhibited a significant increase in sRaw ($P < 0.01$) in response to smaller doses of Ach for which the control animals had no significant response. The effects were more severe in marihuana-exposed animals when compared to the animals exposed to tobacco. This airway hyperreactivity was found to be mediated mainly through the vagus nerve.

PARTICLE DEPOSITION IN RESPIRATORY TRACT CASTS FOR EXTRAPOLATION MODELING

Amit L. Patra,* Ali Gooya, and Nidal B. Shak'a

Northrop Services, Inc. – Environmental Sciences, P.O. Box 12313, Research Triangle Park, North Carolina 27709

In the present study, deposition of monodispersed particles in the different branches of a child lung cast and at different locations within a baboon nose cast was investigated. The hollow models were obtained by the lost-wax technique. Particles were dispersed by using a nebulizer into air flowing at a constant rate. Once the particles were deposited, the hollow cast was cut into different sections and the deposited particles were counted. The deposition efficiency was defined to be the ratio of particles deposited to the total number of particles entering the section. Experimental values were compared to available published data. Deposition efficiency in a child lung was found to be higher than that in an adult lung. In the nose, the maximum percentage of deposition occurred in the olfactory region. The qualitative similarities between child and adult lung deposition were noticeable, although nose deposition patterns differ from those in rat and guinea pig nose casts. Caution thus must be exercised in comparing the deposition characteristic results from one species to another and in extrapolating within the species.

COMBUSTION TOXICITY OF AIRCRAFT SEAT CUSHION MATERIAL IN FISCHER-344 RATS

Gerry M. Henningsen,[1]* Carl T. Olson,[2] Clifford J. Hixson,[1] and David L. Geiger[1]

[1]Harry G. Armstrong Aerospace Medical Research Laboratory/Toxic Hazards Division, Wright-Patterson Air Force Base, Ohio 45433 and [2]Battelle Columbus Laboratories, Columbus, Ohio 43201

Our laboratory was asked to provide information that would help determine the relative combustion toxicity of various candidate or existing seat cushion materials from USAF aircraft. Preliminary toxicity assessments were conducted on the thermal decomposition products from the seat cushions. Thermogravimetric analyses (TGA) were performed to determine the temperatures at which maximum weight loss occurred for each of the materials. Fischer-344 rats were then exposed to smoke generated by the materials heated to approximately the temperature of maximum weight loss determined by TGA. The exposures took place in a closed, FAA-style chamber and lasted up to 30 min. The chamber environment and clinical signs were monitored. Blood samples were obtained and analyzed for carboxyhemoglobin and clinical chemistry values. Rats were necropsied following acute exposure deaths or after sacrifice at 10 days postexposure, and tissues were processed for histopathological examinations. These studies indicated that acute lethality in rats could be attributed to excess CO or HCN, which are toxic gases generated above critical temperatures of decomposition. Certain exposures resulted in upper respiratory tract irritation or pneumonia, accompanied by body weight loss from the toxicologic stress. The potential for adverse health effects from exposure to the pyrolyzed materials could be compared from the biological data and used to recommend use or non-use of certain materials under specified conditions when fires may occur in USAF aircraft.

1,1-DIMETHYLHYDRAZINE MODIFIES THE PRODUCTION OF AND RESPONSE TO INTERLEUKIN 1 AND INTERLEUKIN 2

Richard M. Bauer,* Melinda J. Tarr, and Richard G. Olsen

The Ohio State University, Department of Veterinary Pathobiology, Columbus, Ohio 43210

Previous experiments have suggested that 1,1-dimethylhydrazine (UDMH) may act as an immunomodulator by affecting lymphocytes (e.g., suppression of lymphocyte blast transformation [LBT] response to mitogens) and/or macrophages (e.g., partial reversal of suppression of LBT induced by a macrophage activating agent, *Corynebacterium parvum*). Therefore, we have examined the effects of UDMH on interleukin 1 (IL 1) production by a murine macrophage cell line, P388D1, and on interleukin 2 (IL 2) production by a murine T-cell line, EL4. In addition the effects of UDMH on the ability of a standardized source of IL 1 to induce proliferation of C3H/HeJ thymocytes were evaluated. Furthermore, the effects of UDMH on the proliferative response of C3H/HeJ thymocytes in the presence of standardized IL 1 and on the proliferative response of CTLL-20 cells (an IL 2 dependent T-cell line) in the presence of medium containing IL 2 were determined.

The UDMH caused enhancement of IL 1 production by P388D1 cells depending on when it was added during the induction phase. However, UDMH severely suppressed the proliferative response of thymocytes to IL 1. This effect was not due to binding or inactivation of IL 1 by UDMH, but may have been partly due to cytotoxicity of the thymocytes.

The production of IL 2 by EL4 cells was reduced in the presence of UDMH. Also, the proliferative response of CTLL-20 cells to IL 2 was suppressed by addition of UDMH. The magnitude of suppression in both cases was concentration-related and not due to cytotoxicity of UDMH.

The results of this study indicate that UDMH may function as an immunomodulator by affecting both lymphocytes and macrophages, by interfering with their proliferative responses (lymphocytes) and altering their ability to produce cytokines (lymphocytes and macrophages).

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EFFECT OF UNSYMMETRICAL DIMETHYLHYDRAZINE ON CONCANAVALIN A AND PROSTAGLANDIN-INDUCED T CELL SUPPRESSOR FACTOR INDUCED SUPPRESSOR CELL ACTIVITY

Devraj J. Pillay,* Melinda J. Tarr, and Richard G. Olsen

The Ohio State University, Department of Veterinary Pathobiology, Columbus, Ohio 43210

Previous experiments both *in vitro* and *in vivo* in mice have demonstrated that exposure to 1,1-dimethylhydrazine (UDMH) results in enhancement of certain immune responses. One possible mechanism of immunoenhancement is interference with normal suppressor T-cell activity; hence it was decided to investigate the effects of UDMH on suppressor cell (SC) activity using two assays: (1) concanavalin A (Con A)-induced SC assay and (2) prostaglandin-induced T suppressor (PITS) assay.

Earlier experiments using the Con A-induced SC assay indicated that UDMH (25 μg/ml) interferes with SC activity when added to the cultures during the induction phase (with Con A). Furthermore, Con A-induced SC activity tends to be reduced in mice treated with UDMH. We are further investigating the *in vitro* effects of multiple concentrations of UDMH on the induction as well as the effector function of Con A-induced SC.

UDMH has also been shown to interfere with macrophage production of Prostaglandin E (PGE). We are presently studying the effect of UDMH on PGE-induced suppressor cells using the PITS assay. Nonspecific suppressor cells are generated by the PITS factor, which is produced by incubating glass wool adherent spleen cells with 10^{-5}M PGE_2 for 48 h, then harvesting the cell-free supernatant (containing PITS factor). Supernatants obtained from cells incubated in medium/vehicle served as controls. The effect of multiple concentrations of UDMH on the induction of PITS-induced SC and the effector function of SC induced by the PITS factor are being examined. The results from these experiments will provide further information regarding the effect of UDMH on SC circuits.

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EFFECTS OF 1,1-DIMETHYLHYDRAZINE ON *CORYNEBACTERIUM PARVUM*-INDUCED IMMUNO-SUPPRESSION

Melinda J. Tarr,* Richard M. Bauer, and Richard G. Olsen

The Ohio State University, Department of Veterinary Pathobiology, Columbus, Ohio 43210

Previous experiments have indicated that 1,1-dimethylhydrazine (UDMH) causes enhancement of certain immune functions upon *in vitro* or *in vivo* exposure. The experiments reported here as well as other experiments suggest that one possible mechanism of this immunoenhancement is interference with the "normal" suppressive immunoregulatory activity of macrophages, particularly activated macrophages. In one experiment, mice were injected with *Corynebacterium parvum* (Cp) to induce macrophage activation, and four groups of mice were treated daily with 0, 25, 50, or 100 mg/kg UDMH during the seven days following Cp administration. All mice were then sacrificed, and their splenocytes were evaluated for lymphocyte blast transformation (LBT) response to concanavalin A. Splenocytes from mice treated with Cp alone showed a marked decrease in LBT response compared to the response of untreated normal (control) mice (89% suppression and 97% suppression in 24- and 48-h LBT assays, respectively). UDMH treatment at all doses tested caused significant reversal of the Cp-induced suppression; the 24-h LBT response was restored to nearly normal, while the 48-h response was partially restored.

In the second experiment, splenocytes from Cp-treated mice were assayed for LBT response in the presence of varying concentrations of UDMH. This *in vitro* UDMH exposure partially reversed the LBT suppression resulting from Cp treatment at concentrations of 25 and 50 µg/ml, when added at the beginning of a 72-h LBT assay. These results indicate that the abrogation of Cp-induced immunosuppression observed with *in vivo* UDMH treatment is at least partly due to direct effects of UDMH on the splenocytes involved in the LBT response, and not to effects on other organ systems. Further experiments are under way to determine if UDMH is specifically interfering with the suppressor activity of activated macrophages, or if some other cell type (e.g., lymphocyte) is affected.

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COMPARISON OF MULTIPLE ASSAYS FOR SKIN IRRITATION

Wally E. Dalbey,* George Cruzan, M.L. Barth, C.J. D'Aleo, and Edward J. Singer

Toxicology Division, Mobil Oil Corporation, Princeton, New Jersey 08540

A composite model for skin irritation was developed for simultaneous evaluation of the influence of abrasion, occlusion, and duration of treatment on irritation and for fulfillment of requirements for labeling considerations under DOT, CPSC, OSHA, and EEC. Thus, the number of animals required to address submissions under multiple agencies is greatly reduced compared to performing each test separately. In the composite test, test materials were placed on six sites on the same rabbits: an intact and an abraded site each of which was occluded for 4 h, occluded for 24 hours, or left unoccluded for 24 h. Results are presented from 88 composite tests with 80 petroleum-related materials. For the materials tested, abrasion of the skin had no effect on the irritation response. Occlusion of the test site generally did not result in dramatic increases in response, except for petroleum refinery streams with a boiling point range below 500°F. Exposure for 4 h rather than 24 h generally resulted in less irritation; however, for individual compounds, the irritation from the 4-h exposure could not be predicted from the response to the 24-h exposure. Of the 80 materials tested, 12 would be labeled as skin irritants under FHSA guidelines, three under OSHA, and 20 under EEC. Of the 20 that would be labeled under EEC criteria, only seven would be labeled under CPSC criteria. Results from skin irritation studies performed under one set of conditions cannot be used to predict the degree of irritation that would be produced under a different set of exposure conditions.

PERCUTANEOUS ABSORPTION AND PHARMACOKINETICS OF PERMETHRIN IN YOUNG AND ADULT RATS

P.V. Shah,[1]* Henry L. Fisher,[2] Martha R. Sumler,[1] and Larry L. Hall[2]

[1]*Northrop Services, Inc. – Environmental Sciences, Research Triangle Park, North Carolina 27709*

[2]*U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711*

Percutaneous absorption of permethrin was determined in young (33 days) and adult (82 days) female Fischer-344 rats by employing *in vivo* and *in vitro* methods. Carbon-14-labeled permethrin was applied in acetone at a dose of 0.286 μmoles/cm^2 for both *in vivo* and *in vitro* experiments. *In vivo* percutaneous absorption in young rats ranged from approximately 10% at 6 h to 42% at 72 h, whereas in adult animals absorption ranged from approximately 12% at 6 h to 46% at 72 h. In contrast to this, *in vitro* results indicated lower percutaneous penetration. The static method (*in vitro*) resulted in penetration values of approximately 16% (young) and 12% (adult), and the continuous flow method (*in vitro*) resulted in approximately 15% (young) and 9% (adult) penetration values at 72 h.

At 72 h, urine and fecal excretion was greater than 90% of the absorbed dose in both ages, with approximately a 4:1 urine-to-fecal ratio. Approximately 10% of the body retention was in the liver and less than 1% in the kidneys.

A physiological compartmental model was fitted to the penetration, tissue, and excretion data. Results from the model fitting indicated a percutaneous absorption half-time of 105 h in adults and 138 h in the young. Equilibrium tissue-to-blood ratios ranged from approximately 0.8 to 4.5 in both age groups. The model indicated that the urinary excretion rate constant was 9 h^{-1}.

In summary, age-dependent differences in percutaneous absorption of permethrin were observed. Higher *in vivo* penetration values were observed for adults than the young. In contrast, *in vitro* methods underestimated permethrin penetration and indicated lower penetration values for adults than the young.

This is an abstract of a poster presentation and does not necessarily reflect EPA policy.

TOXIC EFFECTS OF INHALED DIMETHYL METHYLPHOSPHONATE (DMMP) ON THE TESTES OF FISCHER-344 RATS

David R. Mattie, Clifford J. Hixson,* Charles L. Gaworski, and Glenn R. Thorson

Harry G. Armstrong Aerospace Medical Research Laboratory/Toxic Hazards Division,
Wright-Patterson Air Force Base, Ohio 45433-6573

Dimethyl methylphosphonate (DMMP) is an organophosphorus compound used industrially as a viscosity depressant in polyester and epoxy resins and as a flame retardant and, militarily, as a potential nerve gas simulant. In order to investigate the toxic potential of subchronic inhalation exposure to DMMP, male Fischer-344 rats were exposed continuously to either 25 ppm or 250 ppm DMMP vapor for 90 days. Light microscopic findings consisted of segmental to diffuse degeneration of the seminiferous epithelium; the degeneration was present immediately postexposure and was still evident in rats killed one year postexposure. Ultrastructurally, the testes of rats exposed to 250 ppm DMMP vapor showed an increase in the number of lipid droplets in cells lining the seminiferous tubules, increased numbers of lysosomes in Sertoli cells, dilatation of mitochondria, presence of multinucleate giant cells, and a decrease in the number of spermatogenic cells.

MEASUREMENT OF PROTEIN DEGRADATION IN THE ISOLATED PERFUSED RAT HEART AS A PREDICTIVE TOOL IN TOXIC CARDIOMYOPATHY

David P. Thorne[1]* and Thomas D. Lockwood[2]

[1]Biomedical Sciences Ph.D. Program and [2]Department of Pharmacology and Toxicology, School of Medicine, Wright State University, Dayton, Ohio 45435

A major proteolytic process of myocardium is inversely coordinated with contractility on a minute-to-minute basis via β-adrenergic receptor occupancy (Lockwood, T.D., *Biochem. J.* **231**: 299-308, 1985). The normal function of this process serves to coordinate myocardial protein mass with functional demand (i.e., regulate hypertrophy) by β-adrenergic stimulation with epinephrine.

The intracell second messengers that link β receptor occupancy and decreases in protein degradation include increased calcium via its receptor protein calmodulin. Experimental calmodulin-blocking drugs such as trifluoperazine cause a massive stimulation of protein degradation in the perfused rat heart. These agents also cause profound structural damage and myofibrillar disorganization as observed by electron microscopy (Hull and Lockwood, *Tox. Appl. Pharm.*, in press).

A wide variety of agents are known to block calmodulin function, and many of these also cause cardiomyopathy (antipsychotic and antidepressant drugs, heavy metals, etc.). We have proposed a unifying theory of the mechanism of toxic cardiomyography based upon calmodulin blockage (Ibid.). We are investigating the measurement of stimulation of protein degradation in the perfused rat heart as a sensitive, rapid *in vitro* predictive tool to detect toxic cardiomyopathy from diverse agents and to predict ultrastructural damage under prolonged chronic exposure *in vivo*.

THE CYTOTOXIC EFFECTS OF DIETHYLNITROSAMINE ON THE LIVER OF MEDAKA

William N. Norton,[1]* Henry S. Gardner,[2] and Karen L. Hoover[2]

[1]Biology Department, Southeastern Louisiana University, Hammond, Louisiana 70401 and [2]U.S. Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, Frederick, Maryland 21701-5010

Fourteen-day-old medaka (*Oryzias latipes*), maintained at 25°C, were exposed to 200 mg/L diethylnitrosamine (DEN) for 48 h. The fish were subsequently held in clean water for 13 weeks. At sacrifice, the livers were excised and processed for transmission electron microscopy. Ultrastructural changes among exposed fish included a markedly vesicular and endoplasmic reticulum, abundant myeloid figures, and grossly swollen mitochondria. The latter contained distended cristae and a clear matrix. Glycogen concentration and distribution among hepatocytes appeared to be similar in exposed and control fish. Initial observations indicated no discernible structural alterations among lysosomes. Cholangiolar cells lining large bile ducts of exposed fish exhibited large blebs with varying amounts of cytoplasm. Such apparently acute cellular changes are particularly interesting in light of the extended period between exposure to DEN and sacrifice. We ultimately wish to determine the different cell populations present within the liver at the ultrastructural level and to differentiate individual responses of these cells to the carcinogen.

HYALINE DROPLET AND α_{2u}-GLOBULIN ACCUMULATION IN THE KIDNEYS OF MALE RATS AFTER GASOLINE ADMINISTRATION

Michael J. Olson,[1]* Bhagwan D. Garg,[1] F.H. Sarkar,[2] and A.K. Roy[2]

[1]Biomedical Science Department, General Motors Research Laboratories, Warren, Michigan 48090 and [2]Biological Sciences Department, Oakland University, Rochester, Michigan 48063

Saturated branched-chain aliphatic hydrocarbons, found in motor fuels, induce a unique nephrotoxicity in male rats. Unleaded gasoline administered to male rats (0.2- 2.0 ml/kg body weight, p.o.) for nine days caused a marked increase in the number and size of hyaline (protein resorption) droplets in epithelial cells of the proximal convoluted tubules (PCT) and increased cellular exfoliation at high dose levels. No other treatment-related pathological effects were observed in the glomeruli, distal tubules, or medulla. The renal content of α_{2u}-globulin, a protein synthesized in the liver and excreted in the urine of male rats, was determined by radioimmunoassay to be increased threefold after gasoline administration; accumulation of this protein was independent of gasoline dose. Subsequently, we observed that both hyaline droplets and α_{2u}-globulin were increased after a single dose of gasoline (2.0 ml/kg, p.o.). Immunoperoxidase staining of kidney tissue for α_{2u}-globulin revealed increased quantities of the protein in gasoline-treated rats, and large accumulations of antigen were localized in some PCT epithelial cells that contained hyaline droplets. The hepatic concentration of α_{2u}-globulin and the quantity of α_{2u}-globulin mRNA in liver were not altered by gasoline. Accumulation of α_{2u}-globulin was reversed within three days of terminating gasoline administration. A hydrocarbon-induced defect in the renal lysosomal degradation of low molecular weight protein, rather than increased synthesis or defective metabolism of α_{2u}-globulin, appears to cause hyaline droplet accumulation.

THE ISOLATION AND IDENTIFICATION OF THE URINARY METABOLITES OF ISOPROPYLCYCLOHEXANE FROM MALE FISCHER-344 RATS

Gerry M. Henningsen,[1]* Isabel Lopez,[2] Jacqueline Roberts,[2] Kyung O. Yu,[1] and M. Paul Servé[2]

[1]Harry G. Armstrong Aerospace Medical Research Laboratory/Toxic Hazards Division, Wright-Patterson Air Force Base, Ohio 45433 and [2]Wright State University, Dayton, Ohio 45435

Isopropylcyclohexane, a component of fuels and solvents, possesses a branched chain hydrocarbon substituent attached to a cyclohexane ring. Branched chain hydrocarbons containing 8 to 12 carbons are known to produce a servere hyaline droplet nephropathy in proximal tubular epithelium of male rats; whereas cyclohexane and methylcyclohexane, although toxic to the glomerulus at lethal doses, do not affect the proximal tubule.

2,2,4-Trimethylpentane and 2,3,4-trimethylpentane are two very nephrotoxic branched chain hydrocarbons that possess an isopropyl group in the molecular secondary structure, and which have been reported to yield alcohols and acids as urinary metabolites. Metabolism of cyclohexane and methylcyclohexane produces alcohols and diols as urinary metabolites.

In order to determine how the isopropyl moiety would affect the metabolic oxidation of the cyclohexane ring, male Fischer-344 rats were dosed with isopropylcyclohexane (0.25 g/kg) every other day over a 14-day period. Urine was collected for the first 48-h period of dosing and yielded the following metabolites: *cis*- and *trans*-4-isopropylcyclohexanol, 2-cyclohexylpropionic acid, 4-isopropyl-*cis*-1,2,-cyclohexanediol, and 4-isopropyl-*trans*-1,2-cyclohexanediol. At the completion of the 14-day exposure, kidney homogenate extracts contained *cis*- and *trans*-4-isopropylcyclohexanol and 2-cyclohexylpropionic acid. Unique stereoisomerism was observed. Histopathology of kidneys is pending that will allow comparison of metabolism to lesion characteristics.

SPEAKERS AND PRESENTERS

Dr. Bernard Adkins, Jr.
Northrop Services, Inc.
P.C. Box 12313
Research Triangle Park NC 27709

Dr. Rosalind C. Anderson
Arthur D. Little, Inc.
Acorn Park
Cambridge MA 02140

Dr. Frank A. Barile
Assistant Professor - Health Professions
York College of City University of New York
94-20 Guy R. Brewer Blvd.
Jamaica NY 11451

Mr. Richard M. Bauer
Department of Veterinary Pathobiology
Ohio State University
1925 Coffey Road
Columbus OH 43210-1092

Ms. Anne R. Beck
Northrop Corporation-Aircraft Division
One Northrop Avenue, 3874/62
Hawthorne CA 90250

Mr. Kenneth T. Bogen
Biomedical & Environ. Sci. Div.
Lawrence Livermore National Laboratory
University of California
P.O Box 808 L-453
Livermore CA 94550

Dr. William C. Cannon
Battelle Pacific Northwest Laboratories
P.O. Box 999
Richland WA 99352

Dr. Robert L. Carpenter
Northrop Services, Inc.
101 Woodman Drive, Suite 12
Dayton OH 45431

Dr. Lyman W. Condie
Toxicology Microbiology Division
U.S. EPA Health Effects Research Laboratory
26 W. St. Clair Street
Cincinnati OH 45268

Dr. Rory B. Conolly
Northrop Services, Inc.
101 Woodman Drive, Suite 12
Dayton OH 45431

Mr. David W. Crawford
Envirologic Data, Inc.
4 Milk Street
Portland ME 04101

Dr. Wally Dalbey
Toxicology Division
Mobil Oil Corporation
P.O. Box 1029
Princeton NJ 08540

Mr. Thomas R. Dashiell
Director, Environmental and Life Sciences
Office of the Secretary of Defense
Room 3D-129, Pentagon
Washington DC 20301

Mr. John R. Decker, Jr.
Battelle Pacific Northwest Laboratories
P.O. Box 999, LSL 2
Richland WA 99352

Mr. Nicholas J. Del Raso
Northrop Services, Inc.
101 Woodman Drive, Suite 12
Dayton OH 45431

Maj. Gen. Fredric F. Doppelt
Commander
USAF Aerospace Medical Division
HQ AMD/CC
Brooks AFB TX 78235

Dr. Robert A. Finch
Chief, Experimental Toxicology Section
US Army Medical Bioengineering
Res & Dev Lab
Fort Detrick
Frederick MD 21701-5010

Dr. Gerald L. Fisher
Toxicology and Health Sciences Dept.
Battelle Columbus Laboratories
505 King Avenue
Columbus OH 43085

Mr. Jeffrey W. Fisher
Miami University
Zoology Department
Oxford OH 45056

Mr. Don Frazier
The Ohio State University
Department of Veterinary Pathobiology
1925 Coffey Road
Columbus OH 43210-1092

Dr. John M. Frazier
The Johns Hopkins Center for Alternatives to Animal Testing
The Johns Hopkins University
615 N. Wolfe Street
Baltimore MD 21205

Prof. Arthur Furst
Inst. of Chem. Biol. - Univ. of San Francisco
San Francisco CA 94117-1080

Dr. Donald E. Gardner
Conference Chairman
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

Dr. John E. Garst
Associate Professor
Comparative Tox. Labs., Vet. Medical Ctr.
Kansas State University
Manhattan KS 66506

Dr. Arthur F. Grand
Southwest Research Institute
6220 Culebra Road
San Antonio TX 78284

LCDR Brian H. Gray
Naval Dental School, Naval Dental Clinic
NMCNCR
Bethesda MD 20814-5007

Maj. Gerry M. Henningsen
AAMRL/THT
Wright-Patterson AFB OH 45433-6573

Mr. Michael E. Hiteshew
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

Maj. Clifford J. Hixson
AAMRL/THT
Wright-Patterson AFB OH 45433-6573

Dr. William E. Houston
Northrop Services, Inc.
101 Woodman Drive, Suite 12
Dayton OH 45431

Dr. Harold L. Kaplan
Southwest Research Institute
6220 Culebra Road
San Antonio TX 68284

Maj. William C. Keller
USAF OEHL
Brooks AFB TX 78235-5501

Dr. Sohaib Khan
Univ. of Cincinnati
College of Medicine
231 Bethesda Avenue
Cincinnati OH 45267-0521

Dr. Loren D. Koller
Office of the Dean
Oregon State University
College of Veterinary Medicine
Corvallis OR 97331-4802

Dr. Raymond S. Kutzman
Northrop Services, Inc.
101 Woodman Drive, Suite 12
Dayton OH 45432

Dr. Barbara C. Levin
Center for Fire Research
National Bureau of Standards
Bldg. 224, Room A-363
Washington DC 20234

Dr. Alan Liss
Dept. of Biology
SUNY at Binghamton
Binghamton NY 13901

Dr. Larry K. Lowry
NIOSH
4676 Columbia Parkway
Cincinnati OH 45226

Maj. James N. McDougal
European Office of Aerospace Res. & Dev.
Box 14
FPO NY 09510

Dr. Mohan K. Menon
Research Director
Cleveland College
590 N. Vermont Ave.
Los Angeles CA 90004-2196

Mr. Eric L. Morgan
Department of Biology
Tennessee Technological University
P.O. Box 5187
Cookeville TN 38505

Dr. Owen Moss
Battelle Pacific Northwest Laboratories
P.O. Box 999
Richland WA 99352

Prof. Gordon L. Nelson
Dept. of Polymer Science
University of Southern Mississippi
Southern Station, Box 10076
Hattiesburg MS 39406

Dr. William N. Norton
Assist. Prof., Dept. of Biological Sciences
Southeastern Louisiana University
Box 814
Hammond LA 70402

Mr. Robert W. O'Connor
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

Dr. Michael J. Olson
Biomedical Science
GM Research Laboratories
30500 Mound Road
Warren MI 48090-9058

Dr. Amit L. Patra
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

Mr. Gary D. Pilcher
AAMRL/THB
Wright-Patterson AFB OH 45433

Dr. Devraj J. Pillay
Department of Veterinary Pathobiology
Ohio State University
1925 Coffey Road
Columbus OH 43210-1092

Mr. Robert H. Ross
Oak Ridge National Lab
P.O. Box X, Building 2001
Oak Ridge TN 37831-6050

Mr. Randall J. Ruch
Medical College of Ohio
Pathology Department
3000 Arlington Ave.
Toledo OH 43614

Dr. Ghona K. Sangha
Mobay Corporation
17745 S. Metcalf Avenue
Stilwell KS 66085

Dr. William J. Scott
Children's Hospital Research Foundation
Elland & Bethesda Aves., IDR-506
Cincinnati OH 45229-2899

Dr. P. V. Shah
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

Maj. Michael L. Shelley
AAMRL/THA
Wright-Patterson AFB OH 45433-6573

Dr. Narayan Shivapurkar
Medical College of Ohio
3000 Arlington Avenue
Toledo OH 43614

Dr. Thomas B. Starr
Chemical Industry Institute of Toxicology
P.O. Box 12137
Research Triangle Park NC 27709

Dr. Vernon E. Steele
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

Dr. Bruce O. Stuart
Director
AAMRL/Toxic Hazards Division
Wright-Patterson AFB OH 45433-6573

Dr. William A. Suk
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

Dr. Melinda J. Tarr
Dept. of Veterinary Pathobiology
Ohio State University
1925 Coffey Road
Columbus OH 43210-1092

Dr. Raymond Tennant
NIEHS
P.O. Box 12233
Research Triangle Park NC 27709

Dr. Jeffrey S. Tepper
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

Dr. Curtis C. Travis
Office of Risk Analysis, Health & Safety
Research Division
Oak Ridge National Laboratory
P.O. Box X, Bldg. 4500-S, S-204
Oak Ridge TN 37831

CAPT David E. Uddin
Naval Medical Research Inst./Tox. Detachment
Wright-Patterson AFB OH 45433

Mr. Christopher M. Weghorst
Medical College of Ohio
Pathology Department
3000 Arlington Ave
Toledo OH 43614

LCDR John F. Wyman
Naval Medical Research Inst./Tox. Detachment
Wright-Patterson AFB OH 45433-6503

Dr. Raymond S.H. Yang
NIEHS/NTP
P.O. Box 12233
Research Triangle Park NC 27709

Dr. Robert Zucker
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park NC 27709

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