AMRL-TR-75-125

AMRL-TR-75-125
AD A024899
Citation

PROCEEDINGS OF THE 6th ANNUAL CONFERENCE ON ENVIRONMENTAL TOXICOLOGY

21, 22 and 23 OCTOBER 1975

DECEMBER 1975

AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
Air Force Systems Command
Wright-Patterson Air Force Base, Ohio 45433

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FOR THE COMMANDER

Anthony A. Thomas

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER: AMRL-TR-75-125	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE *(and Subtitle)*: PROCEEDINGS OF THE 6TH ANNUAL CONFERENCE ON ENVIRONMENTAL TOXICOLOGY 21, 22, and 23 October 1975	5. TYPE OF REPORT & PERIOD COVERED
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s)	8. CONTRACT OR GRANT NUMBER(s): * In part under Contract F33615-76-C-5005
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio 45433	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 62202F; 6302; 630201; 63020115
11. CONTROLLING OFFICE NAME AND ADDRESS: Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio 45433	12. REPORT DATE: December 1975
	13. NUMBER OF PAGES: 527
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)*: Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

*Conference was arranged by the Toxic Hazards Research Unit of the University of California, Irvine

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Subject Field: 0620; 0615; 0606

Inhalation toxicology	Pathology
Environmental toxicology	Carcinogenesis, environmental
Cellular toxicology	Toxicology techniques

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Major technical areas discussed included measurement of lung toxicity in animals, applications and associated techniques of animal toxicology measurements, general toxicology, and problem issues in environmental carcinogenesis and mutagenesis.

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PREFACE

The Sixth Conference on Environmental Toxicology was held in Dayton, Ohio on 21, 22, and 23 October 1975. Sponsor was the University of California, Irvine under the terms of Contract F33615-76-C-5005 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. Arrangements were made by the Toxic Hazards Research Unit of the University of California, Irvine, and the papers presented at this Conference by personnel of the University of California represent research conducted under the cited contract. James E. Sterner, M.D., University of California, Irvine, California College of Medicine, Irvine, California served as Conference Chairman, and Mrs. Lois Doncaster, University of California, served as Conference Coordinator.

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OPENING ADDRESS

Brigadier General Howard R. Unger, USAF, MC

Commander
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Dr. Sterner, Dr. Doppelt, Ladies and Gentlemen, it is a great pleasure for me to welcome you to the Sixth Conference on Environmental Toxicology.

During this welcome, I want to reflect on Air Force concerns in the general area of Environmental Toxicology and my thoughts on the Aerospace Medical Division's research goals to protect the health of our greatest asset: the people who carry out the Air Force mission.

Although I have not had the pleasure of attending your previous conferences, I have looked at their proceedings and I am greatly impressed by the quality of the papers, the wide spectrum of scientific disciplines represented, and the spirited and candid discussions during the Open Forum sessions.

Since I was curious about the history of these conferences, I started by paging through the proceedings of the very first one held in April 1965, which was titled, "Conference on Atmospheric Contamination in Confined Spaces." This title reflected the main concern of the Air Force, NASA, and the scientific community at large in those years of planning for long duration manned space flight. Things have changed since then and, appropriately, the title of these conferences was also changed in 1970 to "Environmental Toxicology."

But in that very first conference proceedings of 1965, I read with moderate delight a paper by Dr. Harry Hays on "Problems in the Interpretation and Extrapolation of Animal Data to Man," which contains a "tongue-in-cheek" evolutionary account of the state-of-art in Toxicology. Allow me to quote just two paragraphs from his paper:

> In the beginning, it was customary to use a rat or two, an odd rabbit, and a few mice. Before long it was clear that toxicity in man could not be readily predicted in this way. So, the number of rats increased, and before long someone started statistics, so the number of rats increased still further. Dogs came in. Rabbits went out. Cats became scarce.
>
> Well, predictions improved but still there was a long way to go. So the number of rats increased, so did the dogs. So did the mice. More species were added -- monkeys, chimps, marmosets, quail, frogs and pigs. Longer tests were required, ten days, two weeks, six months, two years, to one life span. Still no closer to predictability in man. Once it was just toxicity, and then it was multigeneration tests, carcinogens came in, then co-carcinogens, and if you couldn't find a carcinogen, then you looked for a mutagen. If you couldn't find a mutagen, then you looked for a teratogen. We used not one species but many species. Not one strain, but many strains. Outbred. Inbred. Brother-sister mated. Random mated. Still no better predictability. Once you counted just the dead. This procedure was charged with fallacy, so everything that could be weighed was weighed, and everything that could be removed was sliced and examined histologically. The function of every organ was looked into. From the cellular, we went to the subcellular. Radioisotopes became a must. Physiology gave way to psychology, and now not even the rat doubts the results.

Times have indeed changed, and the Air Force toxicology program must now consider not only the microcosmos of the sealed habitable environment, but the total environment in which we work and on which our activities impact. The three "geneses" -- carcinogenesis, mutagenesis and teratogenesis -- have caught up with everybody in this country of ours and they are here to stay.

Commensurate with the increased sophistication of toxicological studies required by the regulatory agencies and the general concern over occupational and public health, the cost of safety evaluation of new chemicals is skyrocketing. As a result of these requirements, the cost of introducing a new drug, for example, has jumped from an average of 1.3 million in 1968 to 10.5 million today. A parallel situation exists for qualifying new pesticides and other widely used industrial chemicals. But even those older chemicals which have been used for decades may suddenly acquire the stigma of "suspect carcinogen" as a result of the currently increasing bioassay activities, as is the case e.g. with synthetic organic chemicals, industrial solvents, and the hydrazines. A recent list of suspect compounds published in the Federal Register contains more than 1400 chemicals, many of them well known and some of them as mundane as ethyl alcohol.

Other complicating factors to further confuse the issues are abundant. Very little has been done to resolve the controversies about methodologies, protocols and of what is acceptable animal data for the purposes of regulatory agencies. When there is a clear cut dose-response to a chemical carcinogen, can a threshold be postulated below which no oncogenic activity exists? How do we extrapolate animal data to humans? What is an acceptable model for extrapolation of risk? How do we go about risk versus benefit analysis on strictly military chemicals? These are just a few of the key issues, some of which may be answered scientifically, some on an ethical basis. Emotionalism is often a complicating factor.

But even the scientists disagree. The molecular biologist, on the basis of mathematical deduction, will argue that each reactive molecule hits a target molecule. That, of course, means that only an absolute "zero" dose is safe. The toxicologist, who deals with the whole animal, maintains that several "hits" are required to induce a biological effect resulting in the familiar sigma type curve. As you can see, the arguments become quite philosophical and sometimes even quite emotional.

On the further horizon lurks the question of animal experimentation. Since no humans can be used in carcinogenic exposure studies, what in vitro models are of scientific and predictive value? Which type of mutagenic studies will be acceptable? Can cell cultures eventually replace in vivo studies entirely?

These are very challenging questions to the scientific community, but they are equally directed to the bio-politicians and the legal profession.

What are the research needs then for the Aerospace Medical Division in the environmental toxicology of Air Force unique chemicals?

First, we have to find better ways to extrapolate animal data to man. This requires knowledge of the biochemistry and intermediary metabolism of suspect chemical carcinogens. Pathways involved in activation of compounds to proximate carcinogens are extremely relevant to explain species differences in response and to identify the laboratory animal species that most closely resemble man.

Second, in vitro assays for carcinogens and mutagens must be refined to exploit their full potential. Comparative evaluation of living cells from animals and humans should be pursued to identify their capacity to incorporate, metabolize or bind such chemicals in vitro. The same is true of microorganisms. Chromosome aberrations are a powerful tool which may serve dual purpose both as a biological indicator of a "hit" and perhaps even as a diagnostic tool.

Third, since most of our problems are related to potential exposures by the inhalation route, the state of the art must be advanced in experimental approaches to fully exploit the capacity of our exposure facilities. There are two major areas of thrust required here. More sensitive measures for pulmonary function and lung damage, preferably noninvasive, are needed so that differences in individual response due to total dose absorbed can be reconciled within the same animal species. Without this refinement, dose-response can become a very elusive issue. The other major area is the modeling of chronic toxicity. We must develop and validate accelerated test methods for compressing the chamber exposure time to something more manageable than lifetime exposure if we ever hope to get ahead of the power curve. Continuous exposure techniques may well be the most promising approach, since they can increase body burden of the chemical without exceeding the maximum tolerated dose as defined during interrupted exposure studies.

Much of this, of course, can be called basic research. The Air Force Systems Command (AFSC) strongly advocates a vigorous basic research program. As of 1 July this year, AFSC established a single manager for basic research in the Air Force Office of Scientific Research. This change of organizational command lines places basic research in a separate and distinct category from exploratory development. As the end result, research will not have to compete with development for its funds. This new program will devote 70% of its funding to contractual and grant programs with the remaining 30% dedicated to inhouse research at existing Air Force laboratories, to complete their full spectrum research and development role.

This reorganization in Air Force research programs is designed to respond to our technology needs and objectives, as I have discussed previously. It will provide an increased and more stable funding for research vital to us in the area of Environmental Toxicology if we are to meet tomorrow's challenges.

I wish you the best of success for another outstanding and stimulating conference.

KEYNOTE ADDRESS

THE NIEHS PROGRAM IN ENVIRONMENTAL TOXICOLOGY

Robert L. Dixon

National Institute of Environmental Health Sciences
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I would like to first express to you Dr. Rall's apologies and regrets that he is unable to be here. He is testifying today with regard to the Toxic Substances Legislation. As he indicated to me, with rather short notice, he has no control over the scheduling of testimony and when called, he appears.

However, it is my pleasure to be here today and I hope my overview of Environmental Toxicology and the role of NIEHS will be interesting to you. I am Chief of the Laboratory of Environmental Toxicology at the NIEHS, and although I already know many of you, I am anxious to become better acquainted. For those of you interested in the details of the intramural and extramural research program of the Institute, I have brought some descriptive material which I will leave in the back of the room.

The organization of the National Institutes of Environmental Health Sciences (NIEHS) is presented below:

Director .....Dr. David R. Rall
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Programs .....Dr. Cobert D. LeMunyan
Associate Director for Interagency Programs ...Dr. Philip E. Schambra
Associate Director for Program .....Dr. Hans L. Falk
Intramural Research Branches
Environmental Biology and Chemistry, Chief ..Dr. John A. Moore
Environmental Biometry, Chief .....Dr. David G. Hoel
Environmental Biophysics, Acting Chief.....Lyle D. Thomas
Environmental Mutagenesis, Chief .....Dr. Frederick J. de Serres
Environmental Toxicology, Chief.....Dr. Robert L. Dixon
Pharmacology, Chief .....Dr. James R. Fouts
Assistant to the Director--Contracts.....Dr. John L. Braun
Executive Officer.....George M. Kingman

Major research programs are associated with the Intramural Research Branches, Contract Program, and Extramural Programs.

The Intramural Research Branches are Environmental Biology and Chemistry, Environmental Biometry, Environmental Biophysics, Environmental Mutagenesis, Environmental Toxicology, and Pharmacology.

Contract research serves the important purpose of supplementing and complementing the Institute's intramural research programs. Research in this activity is supported through contracts and performed in collaboration with university investigators, industrial research organizations, and other Federal agencies. The Institute plans to provide continuing support for contracts concerned with the development of systems to detect mutations in mammalian cells, assessment of the effects of environmental components on reproduction and oncogenesis, and determination of the effects of chronic exposure to airborne environmental agents. Additionally, supported projects will assess the environmental toxicity of specific chemicals, survey environmental exposures to heavy metals, develop respiratory tract models to assist lung deposition studies, and provide for determination of the carcinogenicity of orally administered asbestos. The contract program will support studies designed to aid in the elucidation of adverse health and environmental effects associated with energy utilization and conservation.

The extramural program provides funds for support of research and research training activities within educational institutions, research institutes, and other public and private nonprofit organizations. NIEHS reaches out into the scientific community to those scientists and research teams which can make contributions to understanding problems in the environmental health field.

In addition to contributing to biomedical and clinical knowledge, this supported research is intended to provide health criteria for establishment of standards by those Federal agencies charged with regulatory responsibilities. Other beneficiaries of the basic knowledge developed are medical personnel concerned with etiology of new diseases peculiar to certain locales; research teams dealing with environmental components of cancer, heart and lung diseases, birth defects, and neurological disorders; and physicians responsible for health care delivery.

Fundamental data derived from research on environmental toxicants and their mechanisms of action will suggest new solutions to the complex and growing problems of environmental contamination. As the information base expands and new techniques of measurement and analysis are developed, new approaches to realistic control methods or standards may emerge.

Because of the numbers and kinds of environmental factors to which man is exposed, the scope of extramural activities is broad. Efforts are made to maintain an effective blend of problem oriented and fundamental research and training in a wide spectrum of the physical and biological sciences. These include biochemistry, biology, statistics, biometrics, chemistry, clinical medicine, engineering, epidemiology, pathology, pharmacology, toxicology, physics, physiology, radiobiology, and veterinary sciences.

For administrative purposes, research and training supported by NIEHS is divided into four primary program areas: Etiology of Environmental Diseases and Disorders, Environmental Pharmacology and Toxicology, Environmental Pathogenesis, and Environmental Mutagenesis and Reproductive Toxicology. None of these is rigidly delineated or mutually exclusive. Research and training may span one, several, or all program areas concurrently.

Support mechanisms in all program areas include Research Project and Program Project Grants and Research Career Development Awards. Research Training and University-based Center Grants also are supported. Grant Programs are administered through the Office of the Associate Director for Extramural Programs, NIEHS, in conjunction with the Division of Research Grants at the National Institutes of Health.

Research Project and Program Project Grants: Because of the lack of a broad base of fundamental knowledge in the environmental health area, NIEHS invests a large portion of its budget in support of highly meritorious investigator-initiated research project and program project grants. It is believed that supporting research by this method is the optimal way to broaden the nation's science base in environmental health. Dual considerations in support of projects are excellence and relevance to environmental health - specifically, to the effect of the environment on man's health.

Research Career Development Awards: The purpose of this award is to foster development of non-Federal individuals with clear research potential who require additional training and experience in a productive scientific environment in preparation for careers of independent research in the sciences related to health. Candidates must be nominated by a non-Federal public or private nonprofit institution engaged in health-related research and located in the United States or its possessions and territories. They must have at least three years of relevant postdoctoral experience prior to the beginning date of the award. Awards are made to eligible institutions on behalf of qualified candidates; each awardee is directly responsible to the institution to which the award is made.

Research Training Program: Grants and awards are of two types: Institutional Grants for National Research Service Awards and National Research Service Awards for Individual Postdoctoral Fellows. Institutional Grants may be awarded to domestic nonprofit private or non-Federal public institutions to support training programs in specified areas of environmental health research from which a number of awards will be made to individuals selected by the institution and program director. Pre- and postdoctoral trainees may be supported if either or both levels of training are justified in the application and approved.

Individual Postdoctoral Fellowship Awards are made to individuals for specific training in environmental health and related sciences. Awardees are selected as a result of national competition.

Research areas in which fellowship and institutional training grant applications will be accepted by the NIEHS are: Environmental Biology, Environmental Epidemiology and Statistics, Environmental Pathology-Pathophysiology, and Environmental Toxicology. Emphasis in Environmental Biology is on development of methods of testing and evaluation of mutagenicity and teratogenicity of environmental agents in laboratory animals and human individuals and populations.

University-Based Center Grants: Supported by grant, and programmatically highly related to the Institute's intramural research program, the Environmental Health Sciences Centers are an integral part of the strategy of NIEHS in optimally serving the national needs in environmental health

sciences research and training. They serve to integrate the efforts of scientists in several traditional disciplines and to foster application of expertise across disciplinary lines to environmental health problems.

The Centers are made up of scientists who collaborate to develop fundamental and practical information about the causes and nature of environmentally-related diseases. The programs generally are broad, encompassing all or most of the NIEHS-program areas. Additionally, the Centers are located in teaching institutions selected for their ability to attract and train gifted students in environmental health and related sciences.

Center grants usually evolve within the University as a result of integration of a number of highly productive research or program projects and training activities relevant to the NIEHS mission. The Center is characterized by a unity of purpose and a central administration under a Center Director, though the specific research interests of participants may be diverse.

Because of the variety and complexity of environmental problems, several Centers with complementary spheres of interest are required to fulfill the national need. Accordingly, seven Centers, each with special expertise in one or more of the major program areas of the Institute, are supported. Among special areas emphasized at the Centers are interrelationships of exposures to heavy metals, pesticides, industrial by-products, naturally-occurring toxins, and air pollutants, singly or in combination, with respiratory and circulatory diseases, carcinogenesis, teratogenesis, mutagenesis, neural and behavioral aberrations, and other overt and subtle physiological anomalies. In addition, epidemiologic studies are being conducted at or near industrial sites, in large urban areas, and in a number of rather special life situations where some potential environmental risks have been identified.

A booklet (DHEW Publication No. NIH 76-932) which defines the mission and activities of the Institute in greater detail is available upon request.

This meeting is perhaps especially timely in light of the hearing scheduled next month by Congressman Brown and his Subcommittee on the Environment and the Atmosphere of the House Committee on Science and Technology. The hearings will consider the costs and effects of chronic, low-level environmental pollution. The aim is to consider the state of

knowledge in the areas of health, agriculture, climate effects, and other aspects of the biosphere. The purpose of the hearings is to assess the research needs in these areas, and how the state of knowledge relates to nation's ability to set reasonable environmental standards.

Because of limited resources in Environmental Health Sciences, as in other government sponsored research, it has been necessary to set research priorities in accord with perceived urgency. As a result, a few programs, such as environmental mutagenesis and carcinogenesis, have had high visibility while other important programs are not so well known or appreciated. There are two possible dangers in this. There is a danger that the consequence of other environmentally-related disorders will be underrated in policy and budgetary deliberations. Secondly, there is a danger that cancer will be looked upon as one disease and mutagenesis as a single phenomenon. A broader perspective is needed regarding the scope of health problems and the kinds of pollutants involved, and that is what I wish to stress today. For examples, I will depend largely, but not solely, on NIEHS-supported research.

Controversies often arise in evaluating health threats from environmental agents because of the socio-economic impact of regulatory decisions and the ethical framework within which health research must be conducted. The scientist is rarely able to experiment directly with man. Evidence is most often in the form of statistics which must be stated in terms of probabilities. The bulk of experimental evidence is obtained with laboratory animals and other models and must be extrapolated to man. Extrapolation is subject to controversy for several reasons:

1. Extrapolations are often made from effects of large doses used in experimentation to low doses encountered in real life situations, and they are often from short-term experiments to long-term effects.
2. Experimental data usually involve one or a relatively few factors. Real life exposure is seldom so simple or well-controlled.
3. Effects are often influenced by age, sex, diet, previous environmental history, state of health, etc. A great deal more research is required for critical evaluation of each factor in relation to individual agents or classes of agents.
4. Lastly, extrapolation from one species to another is laden with uncertainties because of genetic and physiological differences between species, or even between individuals within species.

Despite these shortcomings, experience shows that laboratory animals are useful indicators of threats to man, and prudence dictates that we consider positive indications to be applicable to man unless proved otherwise. Numerous disorders, traceable to noxious environmental factors, have been identified in organs in direct contact with the environment. These organs include primarily the respiratory tract, digestive tract, skin, auditory system, and visual system. I would like to mention these briefly first, and then proceed to discussion of other disorders which occur in organs not in direct contact with the environment, where establishment of etiologic relations is sometimes more difficult. Each of these are part of the Institute's research program.

As this audience knows best, disorders of the respiratory tract range from irritation and discomfort to emphysema and various forms of cancer. Lung irritation from SO_2 and sulfuric acid mists is well documented in both humans and experimental animals. In addition to discomfort, these substances induce stress with adverse consequences in individuals with preexisting respiratory and cardiovascular diseases.

Strong evidence has been generated over several years that NO_2 and ozone are contributing, if not primary, causes of pulmonary emphysema and that they play an important role in other chronic obstructive lung diseases. An experimental facsimile of human emphysema has been produced in rats with these gases at concentrations that exist in some urban atmospheres at peak traffic hours.

Significant lung cancer has been shown to occur among workers in the synthetic polymer industry. A notable carcinogen is bis(chloromethyl)ether, a compound used in large quantities in the manufacture of certain plastics. Other chlorinated compounds of this and related types have been shown to be toxic and to induce cancer in other organs. Oil mists, high molecular weight alcohols, and a number of ketones have also been implicated in various types of lung cancer.

The respiratory tract is a primary repository of environmental dusts, and a number of major lung diseases have been related to such deposits. The nature and site of disease varies with the size and chemical makeup of the particles. Lung tissue scarring and fibrosis is prevalent among talc, granite, and asbestos workers.

Other notable examples of overt lung disease associated with various dusts are coal miner's pneumoconiosis (black lung), which afflicts some 100,000 miners; brown lung, a disease of textile mill workers caused by inhalation of cotton dusts; pneumoconiosis in oil shale workers, which assumes greater significance as oil shale processing develops as a major source of fossil fuel.

Toxic gases and noxious dusts produce more subtle effects in the lungs also. Evidence from both human and animal experiments indicates impaired ciliary action, reduced production of lung surfactants (phospholipids) which assist in lung clearance, and retarded proliferation and activity of lung macrophages. Evidence is mounting that impairment of these defense systems leads to higher incidences of certain infectious diseases.

A second major organ system in direct contact with the environment is the gastrointestinal tract. Clearance of the respiratory tract through mucociliary action and coughing results in swallowing of deposits of foreign matter from the respiratory tract and transfer to the gastrointestinal tract. However, the bulk of the more important pollutants enter by direct ingestion of food and water. Residues of many industrial, agricultural and other chemicals have been identified in streams, lakes and water supplies and in many food staples. Among these are chlorinated hydrocarbon pesticides, PCB's (polychlorinated biphenyls), phthalates, bis(chloromethyl) ethers, and mercury compounds. The hazard of these agents at the levels they are ingested is uncertain at the present time but evidence is beginning to suggest that health problems may be real in some localities. Geographical disparities in peptic ulcer and in cancer of the gastrointestinal tract among human populations are best explained in terms of dietary and environmental differences.

Strong indications of the involvement of food contaminants and additives in gastrointestinal problems have been obtained from studies on experimental animals.

Because of its documented presence in water supplies in some localities, asbestos has become suspect as a cause of gastrointestinal cancer. The fibers have a propensity to embed themselves in the mucosa and to migrate into the tissues. Thus, experts in the physical and biological properties of

asbestos fibers feel that asbestos may be an important etiological factor in cancer of the stomach, intestines, and bowel. The NIEHS has just initiated a contracted study to study the effects of oral asbestos in rodents.

Skin disorders are a third category of problems associated directly with environmental factors. Everyone is more or less aware of the roles of ultraviolet radiation in cancer of the skin. However, many chemical agents, particularly halogenated compounds, also cause skin irritation and ulceration and evidence is accumulating that prolonged or repeated exposure to such compounds may give rise to skin cancer. The potential is verified by the fact that skin tests in animals is one of several kinds of tests used to determine the potential activity of carcinogens, cocarcinogens and cancer promoting agents. The carcinogenicity of bis(chloromethyl)ether was first detected in this way.

Allergenic sensitization of the skin has been encountered in products for home use. An outstanding example is toluic acid and related compounds used as whitening and brightening agents for home laundered clothes. In addition, the skin often provides the first overt indications of liver damage by certain environmental agents that produce photohypersensitization. These agents cause liver necrosis and release of pigments which are activated by light as they circulate in the capillaries near the surface of the skin. On reaction, the activated pigments induce skin rash, eruptions, and edema of surrounding tissues.

Another environmental factor impinging directly upon body organs is noise. Hearing defects are common in many industries and are particularly evident with advancing age. Hearing losses are characterized by permanent destruction of a portion of the sensory cells and nerve endings in the auditory system. There is evidence that a number of chemical agents (e.g., methyl mercury and certain ototoxic drugs) contribute to or synergize the damage.

A second broad category of disorders relates to those occurring in organs not in direct contact with the environment. Detection and establishment of etiologic relationships in these organs is often more difficult than in direct contact organs because of the many factors that modify the dose and chemical structure of the toxicant before it reaches the target organ. Nevertheless, a number of environmentally related diseases and disorders in essentially all major internal organ systems and processes have been established.

Liver. One of the organs affected by the largest number of environmental agents is the liver. Damage is generally manifested as hypertrophy, necrosis, fatty accumulation, cirrhosis, and various forms of cancer. Liver damage of one type or another is induced by a wide range of industrial compounds, natural products, and solvents. The PCB's and vinyl chloride, both highly visible in the news media and in the scientific community, are examples of large-scale chemicals causing liver necrosis and cancer, respectively. The mycotoxin, aflatoxin, a byproduct from the fungus Aspergillus flavus, is also a liver toxin and more importantly, a potent liver carcinogen in certain animals.

Kidney. Heavy metal compounds, as well as a number of organic compounds, induce various disorders of the kidney. Important renal toxicants are mercury, copper, uranium, cadmium, and lead. Recent detailed studies on cadmium show that the metal and its compounds induce the complete expression of the Fanconi syndrome in the rat, i.e., abnormal losses of water, sugars, amino acids, proteins, and certain ions such as sodium, potassium, calcium, phosphorus, and magnesium. The other metals produce similar disorders.

Lower Urinary Tract. Numerous studies have indicated the carcinogenic activity of certain food components and industrial chemicals in the bladder. At least presumptive evidence has been obtained of a relationship between excessive coffee drinking and cancer of the lower urinary tract.

In the 1940's, it was recognized that workers in chemical plants concerned with the manufacture of amines had an unusually high incidence of bladder cancer. Two compounds, beta-naphthylamine and benzidine, have been linked to cancer at this site. For many years, however, the linkage to beta-naphthylamine was an enigma because the compound does not induce cancer in the mouse or rat. It was shown later that the dog is susceptible and responds much like the human. The difference was traced to the way the compound is metabolized in different species.

Cardiovascular system. The mechanisms of contributions of environmental agents to cardiovascular diseases have been refractory to clear demonstration. However, there are many indications from experimental models and direct human studies of induction of disorders in the heart and the vascular system by a wide spectrum of pollutants. The additional stress

of NO_2, ozone, SO_2, and carbon monoxide on individuals in conjunction with preexisting cardiovascular disease has been documented by increases in morbidity and mortality during periods of atmospheric inversions and high pollutant levels.

Epidemiological studies involving cigarette smokers and others in situations where atmospheric levels of CO were high show that up to 35 or 40 percent of these individuals' hemoglobin may be in the form of carboxy-hemoglobin. This results in oxygen insufficiency and stress on the heart. Patients with preexisting circulatory insufficiencies and various forms of anemia are susceptible to morbidity or even mortality under these conditions.

CO effects other than reduction of the oxygen-carrying capacity of the blood have also become evident. CO has been shown to combine with muscle myoglobin, which is critical in arterial and heart muscle action. This may account for decreases in closing pressure of the heart and increased resistance to arterial blood flow observed in dogs after exposure to CO. These effects result in an increase in blood pressure and the work load on the heart.

Certain heavy metals have been found to have various effects on the cardiovascular system. An unusually high incidence of hypertension has been observed in workers exposed to cadmium fumes. Experiments with rats indicate a direct correlation between Cd intake ($CdCl_2$) and changes in renin, a component of the blood pressure regulatory system.

Experiments with animals also suggest that heavy metals may influence the level of fatty substances in the blood and perhaps fatty deposits in the arteries. Serum levels of fats rise upon exposure to toxic heavy metals but are reduced to normal levels upon administration of therapeutic amounts of copper or zinc in the diet. Copper and zinc are known to be displaced from essential metal-containing enzymes by toxic heavy ions such as mercury, lead, or cadmium. These studies suggest that toxic heavy metals may interfere with fat metabolism.

Recent studies show that fluorocarbons such as freons may also affect heart action. Experiments in animals indicate impairment of heart muscle action.

The brain and peripheral nervous system. Evidence has accumulated rapidly and convincingly during the past decade that many environmental agents are causative or contributory in neural and behavioral disturbances in exposed individuals. In some cases (lead and mercury poisoning) pathological damage to the brain and nerve fibers can be seen with the light or electron microscope. In other cases, however, damage can be detected only through behavioral aberrations and learning detriments.

Numerous types of chemical agents have been shown to produce one or several kinds of neurophysiological effects. The organochlorine insecticides DDT and dieldrin selectively affect response of the brain cortex to certain types of stimuli. Along with lindane, they also affect motor performance and muscular coordination in mice. Lead greatly enhances these effects when given to exposed animals at levels that would ordinarily have no effect.

Organophosphate pesticides also produce neurological distrubances. Parathion has been shown to cause distinct changes in behavioral responses of monkeys at doses that have no other detectable effects.

Some types of compounds in foods or in certain home care products have been shown to be capable of producing neurological disorders. Chlorinated bisphenols, a class of compounds to which hexachlorophene belongs, are highly toxic to mammals, and convulsions and neurological disorders have been reported in burn patients treated topically with preparations containing hexachlorophene.

There have been conflicting reports regarding neurological effects of monosodium glutamate (MSG), commonly used in foods as a flavor enhancer. It is well established that hypothalamic lesions are induced in mice fed MSG, but no such damage was found in neonatal monkeys given equivalent amounts per unit of body weight.

The most serious and best documented neurological disorders in man and in experimental animals are those caused by mercury and lead and their compounds. At high levels of intake both of these substances cause a variety of acute toxicological disorders in the digestive tract, liver, blood, kidneys, and other systems. However, at very low, chronic intake levels, toxicological symptoms may be virtually absent for long periods until they begin to be manifested as behavioral changes.

The effects of lead in children and in experimental animals are manifested through a slowing of conduction velocity of nerve impulses. Detectable changes in conduction begin to occur at blood lead levels greater than about 40 mg/100 ml of blood and the effect in severe cases of intoxication has been shown to persist for at least 25 years after intoxication. Changes in the brain and peripheral nervous systems are often expressed in children by recognizable aberrations in behavior (hyperkinesis, loss of motor control), and there is increasing evidence of learning deficits in such children.

Mercury and its compounds produce effects quite similar to those of lead. However, mercury is more insidious because it is inherently more toxic than lead to mammals, and is easily converted in the environment to methyl-mercury. Methylation greatly enhances accumulation in lipid-rich structures of the body, such as the brain and peripheral nervous system. Thus, the hazardous dose of methyl mercury is a small fraction of that of lead or inorganic mercury.

The immediate and long-term consequences of exposure to such ubiquitous agents which can affect the behavioral patterns and learning capacity of children are viewed as a special concern at the Institute.

Birth Defects. Effects on the reproductive system and on the developing fetus have been documented for many types of high volume industrial and agricultural chemicals and heavy metals. Parathion and a number of other organophosphate insecticides increase the number of fetal deaths in experimental animals. DDT and some of its isomers and homologs reduce ovulation and cause persistent vaginal estrus in female rats. Hexachlorophene, the active ingredient of Phisohex and similar cleansing agents, has been shown to produce brain damage in the fetus when given to pregnant rats.

Diethystilbesterol (DES), a synthetic hormone used as a morning after contraceptive and until recently as a livestock feed additive for fattening cattle, has been found to cause vaginal and cervical cancer in young women nearly two decades after their mothers used the drug to prevent miscarriage. It also affects ovulation in female and causes sterility in male rats.

A number of heavy metals and their compounds have notable effects on reproduction and development. Methyl mercury is a potent teratogen in humans as well as experimental animals. When ingested by pregnant females, it accumulates in the fetus to levels twice those in the maternal bloodstream. Chromium, lead, and cadmium are also embryocidal at high levels and at lower levels may induce defects in the skeleton and other parts of developing fetuses in mice.

Copper has some rather unusual effects. It interferes with blastocyst development, increases fetal resorptions, and causes fetal malformations in the rat. An unusual and specific malformation of the developing heart has also been observed.

Immunological disorders. The principle of immunosuppression is familiar because of the use of drugs to prevent rejection of foreign tissues in organ transplants. The danger of infectious disease upon use of such drugs is also well known. Unfortunately, suppression of the immune system is not limited to useful drugs but can be brought about by many environmental pollutants.

Effects of gaseous oxidants and certain particulates on lung macrophages have already been mentioned. Studies show that gaseous pollutants substantially reduce defenses against certain bacteria, due in part to immobilization of macrophages and the mucociliary escalator. Exposure of the small airways to NO_2 or O_3 results in sloughing of ciliated cells from the small airways and of the thin flat (Type 1) cells from adjoining alveolar walls. However, following short-term exposure and allowing time for replacement of the dead cells, new cells that appear are relatively resistant to the gases at the same concentrations. This is true also upon SO_2 exposure. Almost nothing is known about this mechanism for increasing tolerance in epithelial cells, but it is apparent that the phenomenon might be utilized for preventive and therapeutic purposes.

Certain heavy metals have marked effects on the cellular and/or humoral immune system. Lead has been shown to affect the antibody response in pigs and hamsters. Cadmium affects the humoral immune system well before overt signs of Cd toxicity appear. An unusual example of immunological effects is that encountered in berylliosis, a disease caused

by exposure to beryllium oxides. The disease is characterized by fibrosis. Beryllium apparently becomes bound to proteins and the altered proteins evoke defense mechanisms which form new fibrous tissues around the site of beryllium deposition. Fibrosis occurs in many diseases resulting from depositions of foreign particles and chemicals in the lungs and liver and in certain autoimmune diseases, such as schleroderma and cystic fibrosis.

Blood disorders. Many environmental agents have been shown to be hemolytic. Among the more prominent examples are hexachlorophene, phenols, and benzene. Benzene is one of the most widely used solvents and it is estimated that some two million people are exposed to it in various occupations. Its effects in high concentrations on red blood cells have been recognized for many years. Its primary effect at threshold toxic levels appears to be suppression of erythrocyte formation and maturation in the bone marrow, due to interference in the incorporation of iron into hemoglobin.

Lead under some circumstances causes iron deficiency anemia in children. Experiments in rabbits show that anemia can be induced in three to six weeks by administration of lead in the drinking water or diet. Basic studies indicate that anemia results from displacement of iron, or prevention of iron binding, in hemoglobin because of a competition between iron and lead for the binding sites.

Marine biomedical research. A new area of research recently undertaken by NIEHS is marine biomedicine and pharmacology. The oceans are important sources of food and of useful pharmacological agents. However, because of runoff of chemically contaminated surface waters and the use of lakes and streams as dumps for industrial byproducts, pollutants are entering the marine environment in great quantities where they are accumulated by marine organisms. A great deal of research is needed to learn more about the distribution of noxious agents in the oceans and how to preserve this important resource for human use.

Though they are valuable as a food source, the oceans also present some biomedical problems that need further examination. Red tide, a phenomenon due to massive aggregation of microorganisms called dinoflagellates, often renders portions of beaches useless for recreational

purposes. More importantly, the organisms may produce volatile toxins that can affect residents along the shorelines where the blooms occur.

In addition to their value as food, many simple marine organisms are useful models for toxicological and pharmacological research. They can often be used for studies in specific organs and tissues that are inaccessible or very complicated in higher organisms. Thus, study of the marine environment and its inhabitants can contribute a great deal toward understanding the terrestrial environment and environmental diseases.

Extrapolation of data. The problem of estimating human health effects of chronic, low-level pollutant exposure involves the extrapolation of both human and animal data from acute and chronic high-level exposures. High level exposure data are required because of the inability to statistically detect differences at the low dose level with reasonable size studies; and these differences may represent serious public health problems when the entire population is considered.

Generally, quality human data are not available for extrapolation purposes. This is primarily because of the lack of appropriate control groups, the poor quantification of exposures, and the confounding caused by extraneous environmental factors. The best example of a successful extrapolation from acute, high dose human data was the estimation of overall cancer mortality due to low dose ionizing radiation based upon follow-up studies of Japanese atomic bomb survivors.

Generally, the statistical extrapolation of high dose animal data consists of two steps. The first step involves selecting a mathematical function for the dose-response relationship and using it to estimate the response at a low dose level. This low dose estimate is then further extrapolated from the experimental animal to man. The errors involved with this procedure center around the lack of proper comparative pharmacological information and the lack of a solid understanding of the mechanisms of carcinogenesis. Recognizing these difficulties, overestimates of risk are often employed for standard-setting purposes by using upper bounds to the dose response function and applying safety factors for the species conversion. The potential errors and costs resulting from using these approaches have not been adequately studied.

In the broadest sense, the problem of gathering statistically significant data is two-fold: (1) quantifying the exposure, and (2) collecting appropriate data on response.

Any problems associated with the acquisition of meaningful mortality statistics are compounded when one attempts to investigate morbidity, which is generally poorly defined and not recorded unless it is so pronounced as to cause permanent disability and forced retirement.

Finally, it should be noted that not only is it difficult to acquire meaningful current data on environmental exposure, but it is often impossible to collect historical data of this nature. Yet, because of the long latency period associated with some chronic diseases and the relative lack of regulation of occupational exposures which formerly characterized many industries, these historical exposure data are often more important than current data on environmental exposure.

Unlike animals in classical laboratory experimentation, man is simultaneously subjected to a multiplicity of exposures which are often difficult to identify and quantify and are seldom amenable to externally imposed controls. When the health effect under investigation is both highly specific and relatively rare in the general population, it may be possible to isolate a common factor in the exposure history of the cases and circumstantially indict this factor as a probable cause of the particular effect under study. When the disease endpoint is more diffuse and/or when there is the potential for one or more factors contributing to its etiology, the role of any particular agent or exposure can be assessed only if adjustments for the effects of the confounding exposures can be made.

Obviously further research and greater financial support are needed for most areas of environmental toxicology. A great deal has been done but much remains, especially with regard to mechanisms of toxic effects, threshold definitions, data extrapolation from laboratory animals to man, and the development of more rapid and efficient predictive toxicity test systems.

Some of these recent advances and trends in Environmental Toxicology have been especially favorable, from the development of more reliable in vitro test systems to the increasing emphasis and awareness of chemically-induced

chronic disease. There is also increased communication between different scientific disciplines and fewer barriers between the basic and clinical scientist. Although sometimes discouragingly slow, there is increasing coordination between different government agencies charged with the scientific or regulatory aspects of Environmental Toxicology. It even appears that Congressmen are becoming better toxicologists and more aware of the problems facing those attempting to define human health hazards. The uncertainties in prediction of chronic, low-level pollution effects on health as well as problems involving repair mechanism, metabolic processes, and synergistic and antagonistic interactions of pollutants are increasingly recognized as are the problems in gathering epidemiologic data and defining effects in uncontrolled human populations.

The subcommittee on the Environment and Atmosphere is seeking opinions on avenues of research or organizational changes most promising in resolving problems in predicting and measuring human health effects. Changes in research emphasis, funding, or organization needed to pursue the most promising pathways and to permit more intelligent, and more legally and politically defensible standard setting will be discussed. These hearings also reflect an increasing realization that the basis of standards must move from proof of effects to probability of risk, and that standards must be provisional, allowing for further adjustment based on new information.

The people in this room have already contributed greatly to the information base and progress in environmental toxicology. New findings fill the remainder of your program. The scientific future of environmental toxicology is bright and the advances made to define and protect our environment encouraging. The National Institute of Environmental Health Sciences looks forward to playing an ever increasing role in the support and conduct of research in our important field.

ACKNOWLEDGMENT

The author wishes to acknowledge the assistance of Dr. Robert G. Owens and Dr. David G. Hoel who contributed a great deal in the preparation of this presentation.

SESSION I

MEASUREMENT OF LUNG TOXICITY IN ANIMALS

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AMRL-TR-75-125

PAPER NO. 1

MORPHOLOGICAL METHODS FOR EVALUATION OF PULMONARY TOXICITY IN ANIMALS

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INTRODUCTION

The mammalian respiratory system has a variety of important functions in addition to the primary one of gaseous exchange (Heinemann, 1969 and Fishman, 1974). The corresponding diversity of structural components of the respiratory tract, compounded by the inhomogeneity of morphologic responses of the lung to damaging agents, necessitates extremely careful selection and implementation of the several morphological methods required for its examination. Methods must be sensitive enough to reveal the presence and nature of subtle effects, and also provide information on which useful hypotheses of pathogenesis can be based.

This review is designed to present the important considerations in the choice of methods and is a guide to references describing them in more detail. It is not intended to be a detailed critique of methods or a complete laboratory protocol. The majority of the review will deal with the routine necessary for the staisfactory search for and documentation of toxic effects. Emphasis will be on the sine qua non for detecting subtle effects, which provide the most discriminating information relevant to pulmonary toxicity. The remainder of the review briefly addresses special methods for investigating various aspects of the pathogenesis of pulmonary lesions likely to be encountered and which are necessary for furthering the understanding of pulmonary pathobiology.

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ROUTINE EVALUATION

Gross Examination

The methods to be described in this and subsequent sections are post-mortem procedures, although most are applicable to surgical specimens. Radiographic studies, therefore, will not be discussed. They can provide indications of gross and subgross morphologic changes in vivo, however, and are particularly pertinent to chronic studies involving the larger species of experimental animals.

Tracheobronchial Tree and Parenchyma

The animal is deeply anesthetized by sodium pentobarbital and killed by exsanguination. The trachea and lungs are carefully exposed after the diaphragm is punctured, and search is made for abnormalities of the pleural cavity and its parietal and visceral surfaces (e.g., excessive fluid, adhesions). The trachea is transected 3-5 rings distal to the larynx, and the distal portion with attached lungs and other thoracic viscera removed. The surfaces of the trachea and lungs are examined for signs of abnormalities (e.g., indications of edema, hemorrhage, consolidation, emphysema, scarring, possible tumor nodules). These can be documented photographically or schematically in outline drawings. The partially collapsed state of the normal regions of the excised lung results in exaggerated appearance of the abnormalities and enables detection of small lesions that sometimes cannot be discerned in the inflated state. The extent to which the major airways and pulmonary parenchyma need be opened depends on the amount of gross damage. If there is no sign of edema or an exudative lesion, the examination of airways and parenchyma is left until after fixation. Even where major airways are opened, samples of lungs should be retained for perfusing fixation by the airways. The weight and fluid-displacement volume of the lungs can be obtained after tying off the major vessels and dissecting away the heart and mediastinum, if the degree and nature of the abnormalities observed indicates these would be useful quantitative parameters. The volume of fresh unfixed lungs is better measured from radiographs, however, as recommended by Dunnill et al., 1975.

Nasopharynx and Larynx

These structures should be surveyed for damage and the need for more extensive examination determined. In laboratory rodents, the nasal sinuses and turbinates can be examined by removing the overlying nasal bone with forceps or by sagittal section. In larger animals such as the dog, a sagittal section is made. Excepting in cases of tumors or severe upper respiratory irritation by inhaled materials, microscopic methods are usually necessary for detection of changes in these regions.

Fixation

Choice of Fixative and Method of Fixation

Criteria for suitable fixation are:

a) production of least artifact
b) reproducibility
c) simplicity and cost

a) The major aim with respect to production of least artifact is to retain as close as possible the in vivo appearance of the lung immediately proceding death. With pulmonary tissue, in addition to the usual fixation artifacts which have to be considered (e. g., shrinkage, mechanical distortion, changes in cellular organelles), there is the need to prepare pulmonary parenchyma for microscopic examination such that the correct configurations and relationships of airspaces are retained. Fixation by immersing small pieces of lung in various fluids is a common routine procedure. With the exception of severe exudative processes or where there are solid lesions such as tumors, however, immersion-filled lungs do not provide proper definition of either normal or abnormal components. The preferred method of distending the lungs with perfusion of fixative through the airways eliminates these disadvantages by returning the lung to a state similar to that in vivo.

The work of Heard and colleagues (1958 and 1967) is the basis for most of the methods of perfusion via the airways used today. After the lungs have been examined grossly, they are inflated with fixative via the trachea at 30 cm of fluid pressure measured from the surface of the fixative bath in which the lungs are immersed. We have used pumps to provide the necessary height of fixative in the reservoir for large animals (e. g., horses) but have found the marriott bottle to be the most suitable device for lungs from animals the size of dogs or monkeys down to mice. We routinely use 30 cm of water pressure since this is clearly on the plateau of the pressure-volume curve for all of these species and does not result in tearing or rupture of any tissues. Fixation of dog lungs at 25 cm of water pressure has resulted in incompletely filled or distended alveoli. This is characterized by folds in the interalveolar septum which at total lung capacity should be straight. Specimens prepared at pressures which result in incomplete distension of the alveoli and airways are not suitable for morphometric analysis using stereological procedures, and are less suitable for scanning electron microscopy due to local variations in the degree of distension and therefore interrerelationships of the component parts. The airway perfusion method can be applied equally well to one lung or, as is sometimes necessary in large animals, to one lobe or bronchopulmonary segment. A more extensive discussion of general methods of fixation can be found in the report by Dunnill et al., (1975).

The perfusion method of fixation by the airways not only maintains the dimensions and configurations of the tissues at total lung capacity, but also provides the large volume of fixative in intimate contact with the various surfaces which is essential to rapid fixation. The distance the fixative must diffuse for complete penetration is minimal. This method has for general studies the additional advantage of providing a relatively unobstructed view of cell surfaces for scanning electron microscopy by flushing off mucous coat and alveolar lining material. It has the disadvantage of causing some translocation of exudates and particles and providing a specific artifact of increased tissue spaces around pulmonary vessels, the so-called edema artifact.

The choice of fixative is also a major consideration in view of the large numbers of fixatives which have been used on the respiratory system. The main components of these fixatives are usually one or more aldehydes, buffer, and various salts with high purity water so that the fixative has a constant pH and osmolality. Many investigators today use a mixture of glutaraldehyde and formaldehyde made from paraformaldehyde which results in rapid penetration and thorough fixation. Cacodylic acid is generally preferred as the buffer because it results in resilient lungs; that is, blocks of lung compressed by cutting rapidly resume their original fixed volume when placed in fresh fixative. A small amount of calcium is commonly added to the fixative to preserve phospholipids associated with pulmonary surfactant as well as those which are components of the various cell membranes. Although isoosmotic fixatives are used, we prefer a hypertonic fixative (approximately 550 milliosmoles). All of the above desirable characteristics are achieved using a modification of Karnovsky's formaldehyde/glutaraldehyde fixative with added calcium chloride (paraformaldehyde - 40 g/liter; glutaraldehyde 100 ml of 50% solution/liter; calcium chloride - 0.5 g/liter; cacodylic acid - 12.8 g/liter) which is diluted 1 to 4.5 before use with cacodylic acid (32 g/liter) and the pH adjusted to 7.2 with 1.0 N HCl (Nowell et al., 1972). The fixative is relatively simple to prepare and can be stored in the refrigerator for several months. It has the advantage of being a good room temperature storage fluid for fixed tissues. Using this fixative at 30 cm of pressure, fixation is rapid and complete. Fixation times of 2 and 4 hours are acceptable, but we prefer to maintain the 30 cm of pressure overnight or for 18 hours. Samples cut from these lungs are placed in fresh room temperature fixative where they may be stored without damage or deterioration for more than one year.

b) Fixation of lungs at a standard pressure of 30 cm of the fluid provides the most reproducible appearance for general purposes. Considerations of reproducibility and least artifact become more critical relative to morphometry. Here again, for purposes of pathology we find perfusion of the excised lung to be the method of choice. The alternative approach used for morphometry of normal lungs is perfusion via the trachea with the lungs in situ within the thoracic cavity (Forrest and Weibel, 1975).

c) Perfusion of excised lungs by trachea or major bronchus is a relatively simple procedure for rodents, once a series of delivery tubes leading from marriott bottle reservoirs is provided. Larger reservoirs are needed for lungs of larger species. Although the perfusion method cannot be performed as rapidly as immersion of samples in fixative, the greater effectiveness in enabling detection and evaluation of subtle or mild lesions more than outweighs the greater cost in time taken. Where large numbers of animals per treatment group are involved, at least a significant proportion of lungs should be fixed by airway perfusion.

Fixation of Lung by Perfusion Through Airways

As will be evident from the foregoing discussion, our preferred routine method of fixation is perfusion by the airways with modified Karnovsky's fixative at 30 cm of fluid pressure (Nowell, et al., 1972). We find that after partial collapse of the lungs has occurred on excision, no degassing is necessary to obtain complete distribution of the perfusate. Degassing is, in fact, contraindicated for most purposes because it increases the cumbersomeness of the technique, lessens the degree of reproducibility of reinflation, and makes redistribution of components of any lesion more likely.

Fixation of Lung by Immersion

Massively consolidated or edematous parenchyma, or large solid lesions such as tumors, have to be fixed by immersion in fixative fluid. For subsequent study by light microscopy Zenker-formol is preferable to formalin because it heightens the contrast of hematoxylin and eosin staining, especially the eosinophilia of proteinaceous transudates or exudates. The shrinkage caused by immersion in fixative is used to advantage in enumeration of tumor nodules in lungs of strain A mice which is the basis of a carcinogenesis bioassay system (Shimkin and Stoner, 1975). Tissue to be examined by electron microscopy is immersed in the modified Karnovsky's fixative described previously.

Immersion fixation is also used when the redistribution of intraluminal particles, cells or exudates might interfere with the objectives of the study, as in determining the fate of inhaled particles (Lauweryns and Baert, 1974; Sorokin and Brain, 1975).

Fixation of Nasopharynx and Larynx

After gross examination, these structures in small animals (i.e., rodents) can be fixed in toto in the modified Karnovsky's fixative after flushing surfaces with fixative to remove trapped air bubbles and mucous coat. Samples of tissues from recognized lesions and representative portions of the nasoturbinate region, pharynx and larynx need to be dissected out in large animals.

Sampling for Microscopic Examination

The size and diversity of components of the respiratory tract pose a considerable sampling problem in the thorough search for lesions. This is compounded by the inhomogeneity of morphologic responses of the tract to irritants as was mentioned in the introduction. These two features together require that sampling be both wide in distribution and specific in anatomic localization. The number of large blocks taken for examination by light microscopy and scanning electron microscopy will be determined by the compromise between thoroughness and the practical limit in terms of cost of preparation and examination. But there is a minimum below which the risk of spurious conclusions due to serious sampling errors becomes unacceptable. Any sampling of parenchyma must take into account vertical (gravitational) gradients affecting the distribution patterns of certain lesions and the difference between hilar and peripheral regions of lobes.

Tracheobronchial Tree and Parenchyma

The sampling procedure varies according to the size of the lung. In the case of rodents such as rats and hamsters, sampling of the trachea presents few problems other than to be aware of possible differences in the mucosa over the cartilagenous and intercartilagenous membrane portions of the trachea as has been found in the rat (Schwartz et al., 1976). A block containing a longitudinal section of distal trachea and the bifurcation into bronchi suffices for nonparenchymal regions. The preferred planes of section for rodents' lungs are illustrated in Figure 1. These are vertical sections in the sagittal plane for the left lung and from the hilus along the axis of major airways for the cranial, middle and caudal

lobes of the right lung. All of these blocks can be sectioned whole for histologic examination. Although the sagittal section of the left lung is a common section for major attention, we prefer the sections from the right middle and caudal lobes. One reason is that unless the section of the left lung is cut very close to the mid-line, most of the airways are cut transversely. The longitudinal sections of airways present in blocks from the right middle and caudal lobes reveal bronchial and acinar orientations of lesions much more readily, especially by scanning electron microscopy (SEM). A second reason is that the blocks from the right lung are a more convenient size to mount whole for light microscopy.

More care in sampling is required for lungs of larger animals such as dogs and monkeys because of the bulk of tissue to be surveyed and the increased likelihood of regional variations in response being manifested. To minimize sampling errors, standard parenchymal sampling sites covering both dorsoventral and hilarperipheral axes should be chosen. The 9 sampling sites we take from parenchyma of the 4 lobes of the right lung of the dog are illustrated in Figure 2.. Because we frequently use one lung of dogs and monkeys for biochemical studies or those requiring special fixation, such as freezing, we derive most morphologic information on the basis of one lung. If the two lungs are available, samples can be taken from both. Samples of major airways typically consist of proximal trachea, bifurcation of trachea, and lobar bronchus.

Evaluation of pulmonary toxicity invariably involves the comparison of lungs from two or more groups of animals. For this specific comparison, by qualitative or quantitative (morphometric) means, we use the same sampling sites for tissue blocks in all animals (Hyde et al., 1976) rather than the method of stratified random sampling using a random number table together with a numbered sampling grid (Dunnill, 1964). The latter method relates to statistical confidence with which the sample represents the lung from which the sample is taken rather than comparison among lungs where the lesion can be affected by specific anatomic location.

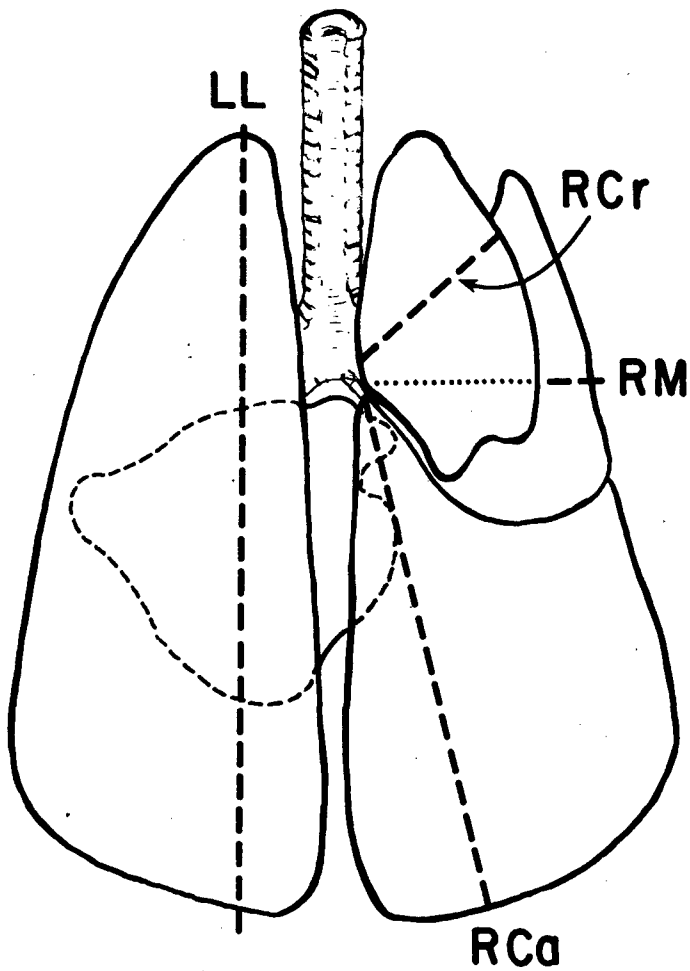

Figure 1. Schematic outline of the dorsal view of a rat's lung illustrating the vertical planes of section for sampling tissue. The contour of the accessory lobe is indicated by the narrow broken line. LL--left lung; RCr--right cranial lobe; RM--right middle lobe; RCa--right caudal lobe.

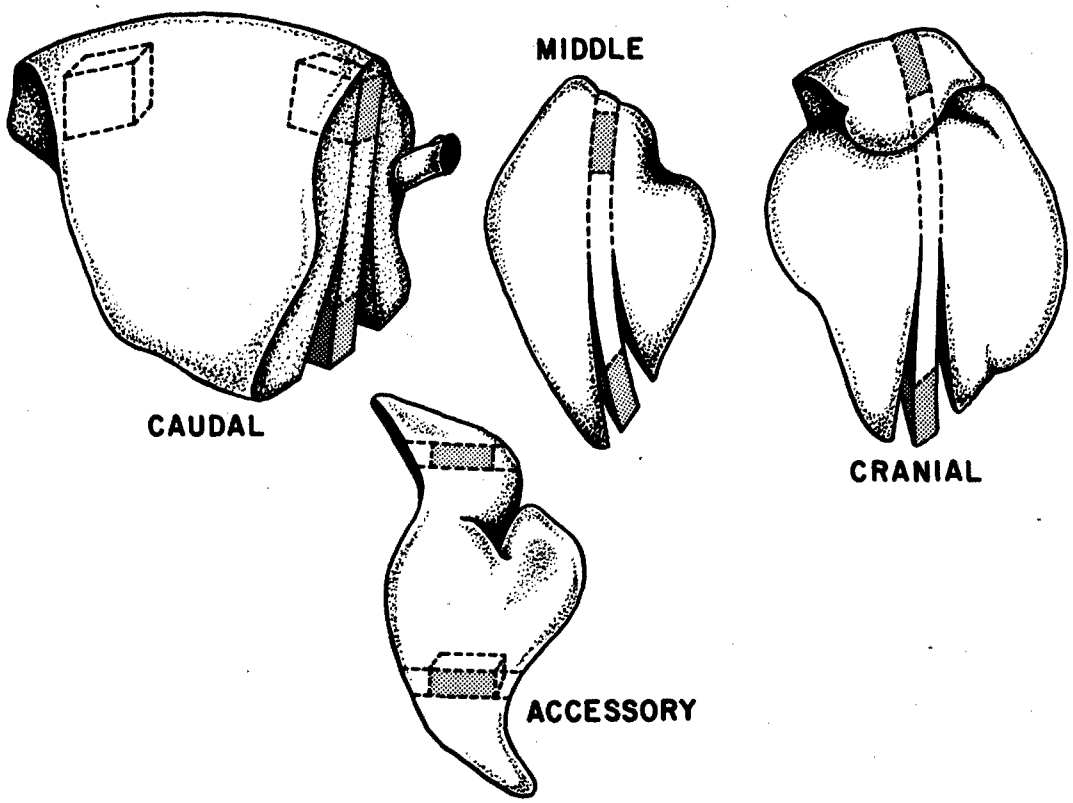

Figure 2. Schematic outline of the lateral view of the four lobes of a dog's right lung illustrating nine sampling sites.

When detailed comparisons are required within or among groups of animals, a useful approach is to select a specific bronchopulmonary segment of the lung for more specific study. Sections of segmental bronchus, terminal bronchiole, and more distal lobular tissue can be sliced out of the desired bronchopulmonary segment under a dissecting microscope. These sections, as well as those of trachea and lobar bronchus, can then be closely compared.

Any gross lesions not represented in the samples described previously must also be selected.

Nasopharynx and Larynx

Blocks are taken representing proximal and distal regions of nasal sinuses and turbinates, and the pharynx and larynx. Again, more are required for larger animals. For some studies it is desirable to dissect mucosa from the nasal septum or turbinates and prepare it as a whole mount for morphological examination. Further details of the use of whole mounts and sections of nasal regions can be found elsewhere (Bang and Bang, 1961; Adams, 1972).

Microscopic Examination

The need for examination of a wide sampling of pulmonary tissue has already been stressed. Requirements for cost effectiveness in the evaluation of lungs from large numbers of animals in toxicity trials means that the microscopic methods most useful are those that provide for examination of large samples, that is light microscopy (LM) and scanning electron microscopy (SEM). For initial detection and analysis of lesions we use correlated LM and SEM. The best way to do this generally is to take complementary blocks of tissue from the same sampling site, embed one in plastic suitable for large 1 μ sections and process the other for SEM. The surface and sectioned views can then be compared for interpretation. The advantage of the large 1 μ section is that it not only provides the best resolution for LM, but also enables precise selection of anatomic locations for thin sections to be examined by transmission electron microscopy (TEM).

This is a discussion of routine microscopic methods and we recognize that the word can take on shades of difference in meaning according to the objectives of the investigations. Often, most microscopic screening is by LM alone because of the bulk of specimens. Equally so, it must be realized that in the search for subtle effects or in the description of damage once it is found, at least a significant number of lungs from animals in the critical experimental groups should be examined by correlated LM, SEM and TEM.

Light Microscopy (LM)

Survey by LM of sections carefully prepared from vacuum-embedded paraffin blocks and stained by hematoxylin and eosin provides the basis for other modes of microscopic investiation. More definitive study of cellular components of lesions is made on the 1μ sections cut from large plastic-embedded blocks and these provide the essential link between LM and TEM (see segment on TEM below). The paraffin sections also provide the basis for a large variety of special staining methods (Luna, 1968).

Scanning Electron Microscopy (SEM)

The large, approximately 12 x 10 x 4 mm samples of tissue selected for SEM are the complementary halves of blocks used for LM and are cut so as to include longitudinal sections of airways in the surface to be examined. The tissue blocks are dehydrated in graded ethanol and then dried by the critical point procedure using CO_2 (Nowell et al., 1972; Anderson, 1951). The dried tissue is attached to standard SEM stubs, put in a high vacuum coating device on a tilting and rotating stage, and coated first with carbon then with gold palladium (Brummer et al., 1975). Such tissues can be stored in a dessicator for prolonged periods and still be useful for SEM.

Although not a routine procedure, to enable precise correlation between surface features seen by SEM and cross sectional features of selected areas, blocks can be removed from the SEM stub after evaluation and prepared for LM and TEM. They are placed in 100% ethanol, which is then substituted by propylene oxide, and are then embedded in

an Epon-Araldite mixture. The tissue is examined by LM of 1μ sections and specific regions can be selected for TEM (Brummer et al., 1975). Information on interior aspects of tissues and cells can be obtained by SEM after the tissue has been fractured either before (Humphreys et al., 1974) or after (Watson et al., 1975) drying. It can also be obtained from plastic-embedded tissue after iodine and acetone surface etching (Pachter et al., 1974).

Transmission Electron Microscopy

Because lesions in the lung are frequently focal and have a specific orientation relative to the acinar structure of the pulmonary parenchyma, it is essential to know precisely the anatomic location in the small airways or acinus from which the TEM blocks are taken. This precise location can be learned by several routes. The oldest is a modification of the procedure used by Grimley (1965) wherein large, 2 x 2 cm blocks of tissue are embedded as for TEM and alternate 30 μ and 10 μ sections cut on a large microtome commonly used for metal or bone. The 10μ sections are evaluated using light microscopy and the precise lesion area is dissected from the adjacent 30μ section, cemented on a block from a beam capsule and ultrathin sections cut (Plopper et al., 1973). It has the disadvantage of relatively low resolution for LM due to the thickness of the section. This can be avoided by embedding slightly smaller sections (i.e., 12 x 10 mm) and cutting 1μ sections on a Sorval JB-4 microtome using glass knives. These thin sections can be stained using various dyes and provide high resolution for evaluation of the tissue by LM. The areas of interest are selected in the one micron section, identified in the block, and the surrounding tissue removed leaving a plastic mesa containing the required region (Lowrie and Tyler, 1973). This mesa is sectioned in the usual manner and examined by TEM.

SPECIAL METHODS FOR GROSS AND SUBGROSS EVALUATION

Whole Lung Sections

The technique of preparing whole sections from human lungs was first described by Gough and Wentworth (1960) and was used in their studies of emphysema in man. The sections can be useful as permanent records or illustrations of whole lung involvement in certain types of

disease processes. Subsequent developments of the technique and their use in the measurement or grading of emphysema in human lungs is briefly discussed in a report by Dunnill et al. (1975). Preparation of lung macrosections and their permanent mounting by their lamination between sheets of transparent plastic film has also been described (Cote et al., 1963; Kory et al., 1966).

Vascular Injection Technique

A technique using thin slices of lungs in which the vessels have been injected with multicolored latex has been used in studies of the comparative subgross pulmonary anatomy of a variety of mammals (McLaughlin and Tyler, 1961; McLaughlin and Tyler, 1966). A major focus of attention in these studies was the comparative anatomy of the vascular tree. Vascular injection and casting has been used in investigations into the vascular changes accompanying emphysema in man (Wyatt et al., 1964).

Airway Casting

This is useful for development of mathematical models for behavior of inspired gases and particles (Phalen et al., 1973) and for the study of airway disease (Horsfield et al., 1966) and the pathogenesis of emphysema (Pump, 1973).

Replica casts of airways down to and including alveoli can be prepared in situ for large and small animals (Phalen et al., 1973). The method involves replacement of air by cyclic ventilation with CO_2 filling with degassed saline and slowly injecting silicone rubber through the trachea while allowing saline to drain from the thorax via slits between ribs. After curing (2-20 hours) the organ is removed from the thorax and the tissue digested away. Morphometric measurements that may be made on such casts include branching angles and dimensions of airways and alveoli. In some cases alveolar pores can be seen and their relative sizes determined via the scanning electron microscope.

The major limitation in using airway casts is that all but the simplest measurements made on them may require considerable time, effort and skill. On the other hand, a replica cast captures and preserves the entire airway structure, allowing precise determination of the spatial and structural distribution of lesions.

SPECIAL METHODS OF MICROSCOPIC EVALUATION

A variety of investigative methods is needed in the search for pathogenetic mechanisms underlying disease processes in the lung, as it is for any organ. These methods are relevant to studies of both cellular biology and pathobiology, and unavoidably investigations into the one have considerable impact on the other. The techniques in question have for the most part either been in use for a relatively short time, or are in the process of being explored. Only an introduction to these topics will therefore be provided.

Histochemistry

Histochemistry and cytochemistry are essential for the full elucidation of the pathogenesis of toxic changes in inhomogeneous organs such as the lung because it is necessary to localize biochemical changes to the specific cells or cell populations involved.

Many specialized methods of tissue preparation and incubation are required for the broad spectrum of histochemistry. Enzyme histochemistry and histochemistry for certain cellular components is best done on cryostat sections. Like all sections of the respiratory system, distended cryostat sections are much easier to evaluate and provide more useful information. Usually the tissue is distended with a cryostat embedding material, commonly 4% gelatin, as originally described by Tyler and Pearse (1965). This embedment has the double advantage of distending the lung and also providing a medium or embedment which permits much more complete sections than can be obtained if the lung is handled like other organs or tissues. Without such embedding, only fragments of sections are obtainable. These are extremely difficult to

evaluate in terms of total distal airway and parenchymal morphology. Freezing of the gelatin infiltrated section is generally accomplished using freon-22 cooled to near its freezing point of -160°C. Freezing directly in liquid nitrogen is considerably slower and frequently distorts the tissue blocks. While most cryostat sections tend to be thicker and therefore provide less resolution than paraffin sections, with appropriate equipment it is possible to serially cut frozen sections at 5 or 6 microns. Such sections are suitable for a wide variety of histochemical procedures which are commonly applied to the serial section in order to obtain correlated biochemical and morphological information at the cellular level.

Histochemical procedures have been diversified significantly in recent years (Pearse, 1968-72) and include methods for many enzymes as well as cell inclusions and intercellular material. Many of these procedures can be applied at both the light and electron microscopic levels of observation of the lung (Castleman et al., 1973; Goldfischer et al., 1967; Sorokin, 1967; Cutz and Conen, 1971; Schneeberger, 1972) and some are suitable for automated image analysis (Sherwin et al., 1973). The studies of Spicer et al. (1974) and Lamb and Reid (1968 and 1969) concerning toxic effects of inhaled gasses on respiratory mucopolysaccharides are especially noteworthy. Another example is the fluorescent amine technique of Falck that has been used in studies of seretonin-producing cells of neuroepithelial bodies present in respiratory mucosa, Lauweryns et al., 1973.

A promising new area of chemical analysis that can be applied to the respiratory system is that of analyzing X-rays, cathodoluminescence or backscattered electrons generated by the interaction of the electron beam of an SEM or TEM with the atoms of cellular components, inclusions, or histochemical final reaction products in situ, thus providing elemental analysis of endogenous or foreign materials in cells and tissues (Johari, 1972; Maata and Arstila, 1975; Funahashi et al., 1975; Yakowitz, 1975).

Autoradiography

Autoradiography has been used to determine the cytokinetics of pulmonary cells responding to damage caused by toxic environments, such as in the demonstrations that alveolar type 2 epithelial cells are

the precursors of type 1 epithelial cells (Evans et al., 1973; Adamson, 1974). The second major use of autoradiographic techniques is for tracing the intracellular pathways traversed by radiolabeled precursors of known or hypothesized cell products (Chevalier and Collet, 1972; Petrik and Collet, 1972; Kikkawa et al., 1975). A third use is in studying the deposition and fate of inhaled particles (Felicetti et al., 1975).

Morphometry

Morphometry is necessary for precise correlation of structure and function in both normal and diseased organs. It can provide accurate measurement of the severity of damage in diseased organs and it is the only means of confirming, by statistical methods, the existence of significant subtle lesions in a particular treatment group of experimental animals.

A systematic approach to a quantitative morphologic analysis of the architecture of the pulmonary system using manual methods has been provided by Dunnill (1962), Weibel (1963) and Thurlbeck (1967). Those authors established the formulae and methods necessary to obtain statistically reliable quantitative values for the pulmonary system. Recently quantitation of the pulmonary system has been automated by use of computed pattern recognition techniques (Levine et al., 1970) and automated measuring microscopes (Cole, 1966). The greatest application of automation has been with automated measuring microscopes. They have been used to quantitate selected features of conducting airways in normal and experimental bronchitis (Mawdesley-Thomas and Healey, 1973) and of distal airspaces in normal (Bignon and Andre-Bougaran, 1969), emphysematous (Anderson and Foraker, 1971), and experimental, pollutant-damaged lung (Hyde et al., 1976; Sherwin et al., 1973). Pattern recognition techniques have also recently been used to classify and measure the distal airways on an automated measuring microscope (Hyde et al., 1975).

Freeze-fracture

Freeze-fracture is a method of looking at replicas of fractured surfaces at very high resolution using TEM. Like the SEM, it provides a view of surfaces rather than cross sections. Thus for low magnification and low resolution of natural or fractured surfaces, the SEM is the most appropriate instrument, whereas for high magnification, high resolution freeze-fracture or freeze-etch is the most appropriate technique.

Freeze-fracture procedures avoid the necessity for including chemical interactions which may cause artifacts in the preparation of tissue and reveal an en face view of membranous surfaces. In the pulmonary system, the method has been used for study of cell organelles, particularly during secretion and phagocytosis (Lauweryns and Gombeer-Desmecht, 1973), for visualization of the alveolar lining layer (Utersee et al., 1971), and in the examination of endothelial cells relative to their capability for metabolizing circulating vasoactive agents (Smith et al., 1973). The method is also necessary for the study of normal and abnormal cell junctions (Hyde et al., 1976).

Tracer Techniques

These have been used in studies of the permeability of the pulmonary vasculature in both normal and edematous lungs. Horseradish peroxidase, hemoglobin, microperoxidase, ferritin and colloidal particles have been used (Pietra et al., 1969; Szidon et al., 1972; Schneeberger and Karnovsky, 1967; Williams and Wissig, 1975; Reese, and Karnovsky, 1967). The investigations on pathways of clearance of inhaled iron oxide aerosols (Sorkin and Brain, 1975) or intratracheally installed ferritin or colloidal carbon (Lauweryns and Baert, 1974) also involved the use of tracers.

Thick Histologic Sections

These were principally used in the study of human emphysema (Pump, 1974). To some extent they have been superseded by SEM, but they still hae an important role in documenting the pattern of collagenous and elastic fibers in interalveolar septa and determining their abnormalities during the pathogenesis of diseases such as emphysema.

ADDITIONAL SPECIAL METHODS OF FIXATION

No one fixation procedure is appropriate for all investigative purposes. To the extent that considerations of methods of fixation are intimately related to the techniques of evaluation for which they are to be employed, common fixation techniques have already been discussed. There remain several, however, that have special indications to be matched with the specific aims of the investigator.

Vapor Fixation

Methods have been developed for the use of formalin vapor (Blumenthal and Boren, 1959; Wright et al., 1974) or formalin steam (Weibel and Vidone, 1961) but have little to offer in the way of advantages and nothing at all in convenience. Air fixation likewise has no usefulness other than to provide a convenient gross anatomical reference. A recent method of vapor fixation using osmium tetroxide suspended in cooled fluorocarbon has been briefly reported by Kilburn and McKenzie (1975). The mixture was injected intratracheally into breathing hamsters to fix the lungs while inflated and to lessen the chance of translocation of cells and particles on luminal surfaces of airways.

Vascular Perfusion

The primary use of this method has been in the demonstration of extracellular lining layers of alveoli and bronchioles by electron microscopy (Gil and Weibel, 1969/70; Gil and Weibel, 1971). Translocation of cells and particles should be less than by intratracheal perfusion, which may make this method useful for localization of these components.

Rapid Freeze Method

This method was developed by Staub and Storey (1962). It provides an accurate representation of the morphologic state of the lung "frozen" at a point in time in its cycle of dynamic events. The animal's lungs are frozen while it is alive, at the desired phase of the respiratory cycle. The procedure does require thoracotomy with good exposure of the lungs. Carefully controlled ventilation is required to maintain physiological state with the ability to momentarily hold the lung at the desired degree of inflation or vascular perfusion. Freon 22 cooled to near its freezing point of -160°C or propane cooled to -175°C is used as the cryogenic agent for rapid freezing as each of them absorbs significantly more heat per unit volume of weight than liquid nitrogen which rapidly absorbs heat then boils, forming an air interface which effectively

reduces the transmission of additional heat from the specimen to the cryogenic agent. Only the first few millimeters of tissue under the pleura are extremely rapidly frozen; deeper tissue is frozen considerably more slowly. Tissues frozen in this manner may be freeze-dried or freeze-substituted for subsequent critical point drying followed by evaluation in the scanning electron microscope (Nowell et al., 1972) or followed by embedding in paraffin or plastic for light microscopy (Plopper et al., 1973) or TEM. In the SEM, the general architecture of the pulmonary tissues is well preserved and available for evaluation, but the surface detail of the cells is obscured by the mucous coat or alveolar lining layer in the airways or alveoli respectively.

ACKNOWLEDGEMENTS

This work was supported in part by N.I.H. grants LS4L-00628, RR00169, and ARB Contract Number 4-611.

We are grateful for the assistance received from M.E.G. Brummer, W. L. Castleman, D. M. Hyde, P. M. Lowrie and L. W. Schwartz.

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AMRL-TR-75-125

PAPER NO. 2

PULMONARY FUNCTION METHODS FOR RECOGNITION OF TOXICITY TO LUNGS

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INTRODUCTION

Impairment of lung function is often not apparent symptomatically or by clinical examination until substantial and largely irreversible damage has occurred (1, 2). Since many substances, toxic or potentially toxic to the lung, are present in our environment (3-5) and since clinically obvious lung disease may be evident only after prolonged exposure to these atmospheric contaminants, it is useful to have sensitive and noninvasive means for detection of pulmonary toxicity from inhaled substances.

Recent investigations have demonstrated that sensitive physiologic tests are capable of detecting abnormal lung function at an early and presumably reversible stage of development (1, 2, 6-12). These methods include such tests as forced mid-expiratory flow (FEF 25-75) (6), forced expiratory flow at 75-85% of vital capacity (FEF 75-85) (7), flow volume loops (MEFV) (8), single breath nitrogen washout (9), multibreath nitrogen washout (10), closing volume (11), frequency dependence of compliance (1), and ^{133}xenon regional ventilation-perfusion studies (12). Since some of the methods are invasive and/or require expensive equipment, they are not all equally suitable to be used as screening tests for abnormal lung function. However, a variety of physiologic tests are necessary since tests such as maximal expiratory flow rates detect airway obstruction whereas others such as single breath diffusing capacity for carbon monoxide (DLCO) are affected primarily by abnormalities of the pulmonary parenchyma or vasculature. Some methods, not suitable for screening purposes, are excellent confirmatory tests in specific circumstances.

In this review, a sequence is described for pulmonary function testing following experimental exposure of humans or animals to inhalants. In addition, a description of methods, their sensitivity and limitations, physiologic interpretation, and equipment needed for performance of the tests are presented in tabular form as a convenient reference for the inhalation toxicologist. The description of methods is divided into the following categories: (1) Tests of respiratory mechanics - analysis of the forces which overcome resistance to airflow and inflation or deflation of the lung; (2) Tests of distribution of ventilation - description of the degree of uniformity of alveolar ventilation. Maldistribution of the inspired gas is often associated with early lung disease even though overall ventilation measured at the mouth is normal or increased. Methods of detecting airway closure are also considered; (3) Tests describing the pulmonary circulation including measurement of vascular pressures, right to left shunting, and distribution of perfusion; (4) Tests describing regional ventilation/perfusion matching ($\dot{V}/\dot{Q}$). This includes direct evaluation of regional ventilation and perfusion as well as several indirect tests; (5) Test of diffusion - although impaired diffusion of oxygen across the alveolar-capillary membrane is not a common cause of hypoxemia (13-14), diffusing capacity is affected by a wide variety of lung diseases and therefore is a good screening test; and (6) Measurement of blood gas tensions

- these very important measurements are a function of abnormalities, single or combined, of altered respiratory mechanics, right to left shunting, altered $\dot{V}/\dot{Q}$ relationships, or, under special circumstances, impaired diffusion of oxygen across the alveolar-capillary membrane. Although not specific for any type of abnormality, arterial hypoxemia is a sensitive indicator of impaired pulmonary function from a multitude of causes, while mixed venous oxygen tension is more indicative of tissue oxygenation and more often reflects the state of cardiac rather than pulmonary function.

In the following pages we describe a sequential approach to pulmonary function testing (Part I) which includes suggestions as to screening and initial testing as well as later, more detailed investigation procedures. Different approaches are suggested for differing circumstances (awake vs anesthetized) and species (large and small animals vs man). The next section (Part II) consists of tables in which the limitations and applications of individual tests are critically reviewed.

I. Approach to Pulmonary Function Testing

A. Man (Unanesthetized):

Preexposure Testing for Control Values
↓
Exposure
↓

1. Screening
   a. Closing volume (CV)
   b. Single breath (SB) N_2 washout
   c. Diffusing capacity of the lung CO-single breath (DLCO SB)
   d. Maximum expiratory flow-volume (MEFV) curves
   e. Spirometry
   f. Airway resistance (R_{AW}) and thoracic gas volume (Vtg)

2. Follow-up testing
   a. Multibreath N_2 washout - 7 minute
   b. Arterial blood gases

3. Further analysis
   a. Compliance of the lung (C_L) - Static, dynamic (frequency-dependent)
   b. ^{133}Xenon regional ventilation-perfusion ($\dot{V}/\dot{Q}$) studies

For screening purposes following exposure to inhalants, closing volume (11) and SB N_2 washout (9) are noninvasive, easy to perform, and both tests may be calculated from the same expiratory maneuver. The test is moderately sensitive but is quasi-static since low flow rates are employed. Dynamic methods such as MEFV curves and spirometry, particularly if flow rates are measured at low lung volumes (FEF 25-75, FEF 75-85), may be abnormal when static tests are normal (15). Both procedures are noninvasive, easy to perform, and therefore well suited for screening purposes. All measurements can be made technically from a single maneuver although the tests are usually repeated to insure "best effort." The MEFV curve is probably best utilized with the subject serving as his own control since flow rates are highly variable from person to person (16). The sensitivity of the technique may be increased by comparison of curves obtained after inhaling air and helium-oxygen mixtures (17). Airway resistance is easy to perform but is sensitive to abnormalities primarily of large central airways (1, 18) and should not be used alone. Changes in maximal expiratory flow (particularly at low lung volumes) with no change in R_{AW} would suggest that the site of the lesion is in the small airways. DLCO SB is simple and easy to perform; this test and, possibly, SN N_2 washout might be the most sensitive detectors of early (interstitial) pulmonary edema or micro-atelectasis (19, 20).

Arterial blood gas analysis is a very sensitive parameter of change in respiratory status. The procedure is invasive and is best performed with local anesthesia; it therefore probably should not be considered a screening procedure. Multibreath nitrogen washout has a high degree of sensitivity to any pulmonary abnormality which changes the distribution of ventilation (10). The procedure requires more time than single breath tests and analysis of results is also time consuming. The procedure is best utilized in selected circumstances (i.e., confirmatory, to differentiate degrees of abnormality not apparent from screening tests, if an abnormality is suspected but screening tests were negative.)

^{133}Xenon regional ventilation studies require large equipment expenditures and quantitative analysis usually requires a computer. Compliance measurements are invasive, tedious, and uncomfortable, but they may be very valuable, especially frequency dependence of compliance which is a sensitive detector of early airway disease (1). This technique is best used for highly specific purposes in a small number of subjects.

B. Large Animals (Unanesthetized):

Preexposure Testing for Control Values

↓

Exposure

↓

1. Screening tests
   a. Spirometry - respiratory rate, tidal and minute volume
   b. Arterial blood gases - Pa O_2, Pa CO_2, pH
   c. Multibreath nitrogen washout - distribution of ventilation, functional residual capacity (FRC)

2. Confirmatory tests
   a. Dynamic compliance
   b. Dynamic resistance (airway)
   c. Total pulmonary resistance (oscillatory)
   d. Diffusing capacity for carbon monoxide (DLCO SS) steady state

C. Large Animals (Sedated or Anesthetized):

Preexposure Testing for Control Values

↓

Exposure

↓

1. Screening tests
   a. Spirometry - respiratory rate, tidal and minute volume

b. Lung volumes - total lung capacity (TLC), vital capacity (VC), functional residual capacity (FRC), residual volume (RV)
c. Arterial blood gases - Pa O_2, Pa CO_2, pH
d. Multibreath nitrogen washout - distribution of ventilation, FRC
e. Lung compliance - static (Cst), dynamic (Cdyn) including measurements at different respiratory rates (frequency dependence of compliance)
f. Dynamic resistance (airway)
g. Total pulmonary resistance (oscillatory)

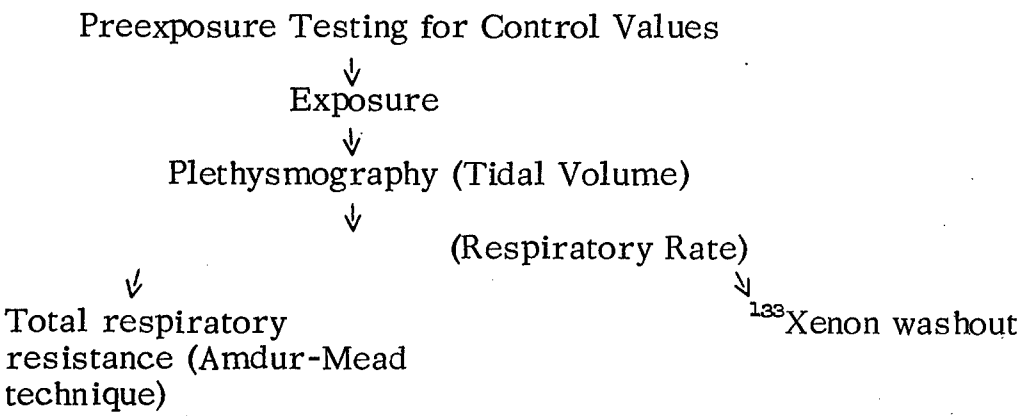
2. Confirmatory tests
   a. Closing volumes
   b. DLCO SS - steady state
   c. DLCO SB - single breath
   d. Maximal expiratory flow-volume (MEFV) curves
   e. ^{133}Xenon regional ventilation-perfusion ($\dot{V}/\dot{Q}$) studies

Unanesthetized large animals may be tested using either a face mask (21) or chronic tracheotomy (22). These techniques are used most frequently in relatively cooperative, trainable animals such as dogs. Alternatively, any animal may be tested using general anesthesia, although this method seems less desirable since the effects of anesthesia must also be assessed. Recently, however, Muggenberg and Mauderly (23) have shown that general anesthesia, using trifluprom azine HCl, is associated with minimal respiratory side effects.

Screening procedures, such as spirometry or multibreath nitrogen washout, are usually easy to perform, noninvasive, and require little equipment or training of animals (see tables). Arterial blood gases, although invasive, also require little time and equipment. They probably are best obtained from an indwelling catheter in a femoral or exteriorized carotid artery. Multiple samples can be obtained without causing pain and agitation which are often associated with reflex changes in respiration.

The confirmatory tests either require intubation and are, therefore, invasive (dynamic compliance, dynamic resistance, ^{133}xenon regional ventilation studies, MEFV curves), require expensive equipment (DLCO, dynamic compliance, dynamic resistance, ^{133}xenon regional ventilation-perfusion studies, MEFV curves), or are time consuming (dynamic compliance, dynamic resistance, ^{133}xenon regional ventilation-perfusion studies) and, therefore, are poorly suited for screening purposes except in anesthetized animals. However, many of these latter methods are also the most sensitive and yield the most information. MEFV curves, for example, have recently been used successfully in monkeys to evaluate the effects of coal dust upon the small airways (24). Although relatively elaborate equipment was required, individual subjects (monkeys) could be completely tested within 10-12 minutes following induction of anesthesia (24).

C. Small Animals:



1. Postmortem studies
   a. Histology
   b. Pressure volume curves
   c. Lung lavage for surface active material
   d. Pulmonary edema analysis (wet/dry weight ratios)

The most suitable technique for in vivo screening for response to inhalants is plethysmography. The plethysmograph is relatively easy to construct and small animals such as rats may be monitored (with difficulty) without anesthesia. The technique is sensitive and tidal volume and respiratory rate can be measured. Postmortem studies may be sensitive but are both time consuming and tedious and therefore poorly suited for screening purposes in large numbers of small animals. Pulmonary resistance as measured by Amdur and Mead (25) requires placement of an intrapleural catheter. Also required is a plethysmograph and physiologic recorder. The procedure has merit for in vivo testing of small animals but probably should not be considered a screening procedure. ^{133}Xenon washout requires expensive equipment as well as use of radioisotopes. The procedure has not been widely used in small animals and its utility is not definitely established. The technique is currently under evaluation.

II.
A. Ventilatory Exchange

Name of test	Physiological interpretation	Animal species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
Respiratory rate	Frequency of breathing	Any	Many	Low - large animals Moderate - small animals	Very few	Spirometer, face mask for large animals or plethysmograph, pressure transducer, recorder for small animals. Pneumograph and transthoracic impedance are noninvasive.	26
Tidal volume	Depth (volume of breathing)	Any	"	"	"	"	26
Minute ventilation	Total volume of breathing in one minute. (May measure inspiration or expiration.) Equals the product of respiratory rate multiplied by tidal volume.	Any	"	"	"	"	26

II.
B. Static Lung Volumes*

Name of test	Physiological interpretation	Animal species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
Total lung capacity	Elasticity of lungs and thorax, muscle strength	Any (see conditions)	Only man without external forces; animals 30 cmH_2O distending pressure.	Good	Requires maximal effort or external distending and/or withdrawing pressures.	Spirometer + helium catharometer, nitrogen meter, or other inert gas measuring device or body plethysmograph.	27, 28, 29, 30
Vital capacity	"	"	"	"	"	Spirometer	30, 31
Residual volume	"	"	"	"	"	Spirometer + inert gas measuring device or body plethysmograph	30, 31
Expiratory reserve volume	Expiratory force, diaphragm position	"	"	"	"	Spirometer	31
Functional residual capacity	Elasticity of lungs and thorax	"	Resting	"	Almost none	Spirometer, (body plethysmograph - with cooperation)	30, 32, 33
Inspiratory capacity	Inspiratory force, diaphragm position	"	Only man without external forces	"	See TLC	"	31

*In general, FRC is measured by inert gas dilution, nitrogen washout or body plethysmography; VC, ERV and IC by spirometry; TLC and RV are usually calculated.

II.
C. Respiratory Mechanics

	Name of test	Physiological interpretation (units)	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
1.	Compliance							
1a.	Static lung & thoracic cage compliance. C total	Stiffness of respiratory system $\frac{1}{C_{total}} \approx \frac{1}{C_{stat(l)}} + \frac{1}{C_{stat(W)}}$ (L/CmH_2O)	Any (see exp. conditions)	Results uncertain without anesthesia & paralysis of respiratory muscles	Moderate or less	Requires cooperation or relaxing anesthesia plus external forcing	Pressure & volume transducers, amplifiers & recorders. One method requires head-out body chamber.	34, 35
1b.	Static lung compliance. (Static volume pressure curves) $C_{st(l)}$	Stiffness of the lungs - quasi-static. A measure of distensibility, reciprocal of elastance (L/CmH_2O)	Mammals, any (see experimental conditions)	Only in man without relaxing anesthesia, for full curves. Animals: for $C_{st(l)}$ in tidal volume range in unanesthetized or full range with relaxing anesthesia.	Moderate	Requires cooperation or relaxing anesthesia plus external forcing (see experimental conditions)	Pleural (animals only) or esophageal balloon, pressure & volume transducers, amplifiers, recorder or CRT, or X-Y plotter, or tape	36
1c.	Static thoracic cage compliance $C_{stat(W)}$	Stiffness of chest wall (L/CmH_2O)	See Cst & C total	See Cst & C total	See Cst & C total	See Cst & C total	See Cst & C total	37

II.
C. Respiratory Mechanics

	Name of test	Physiological interpretation (units)	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
1d.	"Specific" compliance	Cst/V_{tg} where V_{tg} is usually at functional residual capacity (L/CmH₂O/L)	Mammals, any (see experimental conditions)	For Cst(l) in tidal volume range relaxing anesthesia may not be required	Moderate or less	See Cst(l) requires measurement of FRC	See Cst(l) See FRC	37
1e.	Dynamic lung compliance Cdyn(l)	Stiffness of lung at specified frequency. Frequency dependent if Cdyn is a function of frequency. (L/CmH₂O)	Mammals, any (see experimental conditions)	Usually only man without relaxing anesthesia for studies at a full range of frequencies.	Moderate or less	Usually requires cooperation or relaxing anesthesia plus external forcing.	Same as for static volume pressure curves plus a pneumotachograph measure flow	1, 38, 39
1f.	Static volume pressure curves of saline filled excised lungs	Tissue (quasi-static) distensibility (L/CmH₂O)	Mammals, any (at necropsy)	Saline filled excised lungs.	Moderate	Leakage and lack of uniform filling can create problems. Excised lungs.	Pressure and volume transducers, amplifiers, and recording devices	40
2.	Airflow							
2a.	Spirometry, forced expired volume vs time	Overall mechanical function of lungs & thoracic wall including flow rates at various parts of the expiratory curve (e.g. maximum mid expiratory flow in liters/sec) & FEV in liters at various times (e.g., .75, 1, 2, 3 seconds)	Any (see experimental conditions)	Usually only man without relaxing anesthesia with external forcing.	Good to moderate	Usually requires cooperation, a relaxing anesthesia plus external forcing.	Low resistance spirometer or pneumotachograph with integrator	41
2b1.	Flow volume maximum expiratory flow volume curves MEF	Overall mechanical function of lung & thoracic wall including Peak Flow Rates (PEFR) and flow rates at various volumes (e.g. MEF_{50} at 50% of vital capacity).	Mammals, any (see experimental conditions)	Usually only man without relaxing anesthesia. Measures with gases of different physical properties can be done (e.g. He or SF_6).	Good to moderate	Usually requires cooperation or relaxing anesthesia plus external forcing.	Storage oscilloscope or a photographic X-Y recorder or tape. Low resistance spirometer or pneumotachograph with integrator.	42

II.
C. Respiratory Mechanics

	Name of test	Physiological interpretation (units)	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
2b2.	Flow volume inspiratory maximum inspiratory flow volume curves MIF	Overall mechanical function of lung & thoracic wall including Peak Flow Rates (PIFR) and flow rates at various volumes (e.g. MIF_{50} at 50% of vital capacity)	Mammals, any (see experimental conditions)	Usually only man without relaxing anesthesia. Measures with gases of different physical properties can be done (e.g. He or SF_6).	Good to moderate	Usually requires cooperation or relaxing anesthesia plus external forcing.	Storage oscilloscope or a photographic X-Y recorder or tape. Low resistance spirometer or pneumotachograph with integrator.	42
2c1.	Lung and thoracic cage flow-resistance (total resistance) Rrs	Changes in total respiratory system ($cmH_2O/L/sec$)	Mammals, any	Can use several frequencies (e.g. 3, 6, 12, 24 Hz)	Good to moderate	Specificity of interpretation limited.	Oscillatory equipment, transducers for flow, pressure, amplifiers, recorders; stripchart, tape	43, 44
2c2.	Total lung flow-resistance Rl	Flow-resistance of airways and lung tissue Rl = Raw+Rlt ($CmH_2O/L/sec$)	Mammals, any [see Cst(l)]	See Cst(l)	Moderate	See Cst(l)	Pleural (animals only) or esophageal balloon, pressure & flow transducers, amplifiers, recorder or CRT or X-Y plotter, or tape	45, 46
2c3.	Airway flow-resistance Raw	Flow-resistance of airways $Raw=R_{peripheral}+R_{central}$ ($CmH_2O/L/sec$)	Mammals, usually only man (method #1) Any (method #2), see Cst(l) and Rl	(Method #1, body plethysmograph) usually only man. See Cst(l) and R for method #2 (esophageal or pleural pressure with assumed value for Rlt	Moderate or less	(Method #1) requires panting See Rl (method #2) must assume a value for Rlt	(Method #1) Body plethysmograph Equipment for Rl (method #2)	47
2c3a.	Central airways flow resistance Rc	Partition of flow-resistance to airways usually of < 2 mm in diameter ($CmH_2O/L/sec$)	Difficult with small animals.	Only animals or excised human lungs.	Good to moderate	Requires invasive procedure	Retrograde catheter, transducers for flow pressure, amplifiers (see Rl)	48
2c3b.	Small airways flow-resistance R_p	Partition of flow-resistance to airways usually of <2-3 mm diameter ($CmH_2O/L/sec$)	Difficult with small animals.	Only animals or excised human lungs.	Good to moderate	Requires invasive procedure	Retrograde catheter, transducers for flow pressure, amplifiers, recorders (see Rl)	48

II.
C. Respiratory Mechanics

	Name of test	Physiological Interpretation (units)	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
2c4.	Specific airway resistance SR(aw)	#1/SG(aw)	See SG(aw)					49
2c5.	Frictional resistance lung tissue Rlt	Subtraction of Raw from total lung flow-resistance Rlt = Rl - Raw (CmH$_2$O/L/sec)	See Raw and total lung flow-resistance					50
2c6.	Airway conductance Gaw	Gaw = ^{1}/Raw (L/sec/CmH$_2$O)	See Raw					28, 51, 52
2c7.	Specific airway conductance SG(aw)	(^{1}/Raw) V_{tg} where V_{tg} usually = FRC airway conductance per unit lung volume	See Raw See V_{tg}	See Raw See V_{tg}	Moderate	Need measure of V_{tg}	See Raw See Vtg	28, 51, 52
3.	Work of Breathing							
3a.	Work of breathing - lungs and thoracic wall	Work of moving lungs and thoracic wall (KgM/min)	Any (see experimental conditions)	Results uncertain without anesthesia and paralysis of respiratory muscles. Work of inspiration and expiration can be separated.	Moderate or less	Combined measure	See C total pressure, flow volume (recorder), body respirator	35, 53, 54
3b.	Work of breathing - lungs	Work of moving lungs (KgM/min)	Any	See Cst and total lung flow-resistance. Work of inspiration can be separated.	Moderate	See Cst and total lung flow-resistance.	See Cst and total lung flow-resistance.	35, 53, 54
3c.	Work of breathing - thoracic wall	Work of moving thoracic wall (KgM/min)	Any	Results uncertain without anesthesia and paralysis of respiratory muscles. Work of inspiration and expiration can be separated.	Moderate or less	See experimental conditions.	See C total pressure flow volume (recorder), body respirator.	35, 53, 54

II.
D. Distribution of Ventilation*

Name of test	Physiological interpretation	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
Closing volume (^{133}Xe bolus distribution)	Closure of dependent airways	Man, rabbit probably minimal size, larger animals better.	Requires cooperation. Only man without anesthesia. May be done in large animals with anesthesia, positive & negative pressure breathing.	Moderate; most sensitive if measured as closing capacity/TLC. Closing capacity = CV + RV)	See experimental conditions.	Spirometer; flow meter; scintillation counter; digital rate meters, physiologic recorder	55, 56
Closing volume (Helium bolus distribution)	"	"	"	"	"	Spirometer; flow meter; critical orifice helium analyzer; physiologic recorder	57
Closing volume (Argon bolus distribution)	"	"	"	"	"	Spirometer; flow meter; mass spectrometer, physiologic recorder	11
Closing volume (nitrogen dilution)	"	"	"	Moderate, may be slightly less sensitive than bolus techniques. Measure as CC/TLC.	"	Spirometer; flow meter; nitrogen analyzer; physiologic recorder	58
Nitrogen washout single breath	distribution of ventilation	"	"	Moderate; may be normal when dynamic measurements are abnormal.	"	"	9, 59
Nitrogen washout multi-breath	Distribution of ventilation	Man, large animal, beagle dog, Shetland pony, monkey, baboon	Only man and beagle without anesthesia; animals require tight-fitting face mask; restraints	High; sensitivity increased by washout at high respiratory rates and poor collateral ventilation.	See experimental conditions.	Spirometer or pneumotachograph; nitrogen analyzer; physiologic recorder.	60-62

*Distribution of ventilation may be defined as a description of the uniformity of distribution of inspired gas. In a hypothetical lung with perfectly uniform distribution of ventilation, the ratio regional tidal volume/regional lung volume is equal for all alveoli.

II.
D. Distribution of Ventilation

Name of test	Physiological interpretation	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
Regional pulmonary function - ^{133}Xe technique	Topographical distribution of ventilation.	Man, baboon, monkey	Only man without E-T tube; anesthesia, controlled ventilation.	High, man; low, animals (due to limited experience)	See experimental conditions.	Spirometer; multiple scintillation counters of Anger camera; computer or strip charts physiologic recorder ventilator air pump (animals)	12, 63
^{133}Xe washout multi-breath	Distribution of ventilation.	Man, baboon	Only man without E-T tube; anesthesia, controlled ventilation.	High, man; low, animals not yet well quantitated or subjected to compartmental analysis in animals.	See experimental conditions.	Spirometer; multiple scintillation counters of Anger camera; computer or strip charts physiologic recorder ventilator air pump (animals).	63-65
E. Pulmonary Circulation							
1. Cardiovascular pressures	Intravascular diastolic, systolic and/or mean pressures. Detects hypertension in vascular system. Necessary for calculations of vascular resistances and ventricular work.	Any (see conditions). Difficult in small rodents.	Awake animals following preparation of indwelling catheters. Anesthetized animals usual.	Can be done with good accuracy and reproducibility. Moderate to low sensitivity for pulmonary disease.	Few limitations if one has adequate training & equipment. Frequency-response of equipment & proper application essential. Animals should be studied under similar conditions and levels of activity.	Cardiac catheters, strain gauges and recorder. Surgical equipment, fluids, drugs and variety of stopcocks.	65-75
2. Cardiovascular volumes, flows, resistance and work	Cardiovascular (right and left ventricular load) and performance.	Same as above.	All can be done on reasonably tractable awake animal except pulmonary capillary blood volume and pulmonary capillary blood flow.	Same as above.	Calculated (derived) values. Each value depends upon several variables. Otherwise limitations as listed above.	As above. Dye dilution equipment body plethysmograph and appropriate strain gauges. Appropriate drugs and test gases.	65-81
3. Distribution of perfusion							
3A. ^{133}Xe technique	Regional distribution of pulmonary blood in the lung.	Meaningful only in animals the size of cats or larger	Animals usually anesthetized & positioned with limited movement.	Moderate to low	Expensive equipment. Useful only on larger animals. Requires use of radioactive material.	A. Four scintillation detectors & cylindrical collimetors. Magnetic tape recorder. Rate meter.	82-84

II.
E. Pulmonary Circulation

	Name of test	Physiological interpretation	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
3B.	^{136}I-macro-aggregated albumin technique	Same as A	Same as A	Same as A	Same as A	Same as A	B. Scanner. Radiological equipment.	82-84
4.	Right to left pulmonary vascular shunt during O_2 breathing.	The percentage of the cardiac output which is bypassing ventilated exchange area in the lung.	More conveniently done in animals at least the size of cats.	Animal must breathe 100% O_2 without rebreathing. Animals usually anesthetized. Must measure O_2 concentration in and expired and mix venous blood O_2-concentration.	Depends upon how rigorously each measurement is made for the shunt calculation. Moderate to good for advanced, chronic lung disease.	Accuracy of measure of O_2-content in mixed venous blood and alveolar-O_2	Blood gas analysis equipment; blood gas pressure analysis, Scholander and perhaps Van Slyke or gas chromatograph with gas-extractor.	85, 86
5.	Matching of ventilation & perfusion.	The regional distribution of ventilation relative to perfusion in the lungs.	As in 3 above.	As in 3 above.	Moderate to low.	As in 3 above.	As in 3A above	55, 87-93
6.	Reflexes							
6A.	Pulmonary vascular effects of breathing O_2	Measure of effect of high O_2-concentrations upon pulmonary vascular resistance (see 2 above) and distribution of perfusion (see 3 and 5 above).	All experimental animals.	Same as 1 above.	Moderate to low.	As in 1.	As in 1 and breathing equipment for giving O_2.	94-102
6B.	Histamine, fibrinopeptide B, bradykinin analysis	Chemical and/or biological analysis for concentration of pulmonary vasoactive agents.	All experimental animals.	Chemical and/or biological analyses of concentrations in plasma, blood or tissue.	Low	Availability of techniques.	As as 1 and breathing equipment for giving O_2.	103-104

Name of test	Physiological interpretation	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
Diffusion perfusion ratio studies	Measure of the distribution of pulmonary diffusion relative to pulmonary perfusion.	Animals at least the size of cats.	Animals anesthetized and usually terminal preparation. Requires special gas handling equipment.	Moderate of unknown	Requires excellent experimental control & measure of pulmonary and cardiovascular variables.	Respiratory gas chromatograph. Ability to handle & analyze labeled O_2. Breathing equipment.	105-107
Edema evaluation							
In vivo ^{113}Indium-transferrin	Estimate of extravascular fluid accumulation in the lung.	Animals at least the size of cats. Technique confirmed only on sheep.	Animal must be restrained by counters.	Moderate to unknown	New technique must be confirmed on species other than sheep.	As in 3 above.	108-111
In vitro wet/dry weight ratios	Measure of total lung H_2O.	All experimental species.	Study of lung tissue after death.	Moderate to good	Animals must be sacrificed.	Laboratory balance & dessicating oven.	108-111
In vivo pulmonary tissue volume	An estimate of tissue volume exposed to and in equilibrium with gas in airways.	As in A.	Anesthetized controlled airways in animals.	Moderate	Indirect measurement	Gas analysis gas-volume measurement	76
Post mortem pulmonary arterial & bronchial arterial casts	Relative distribution of pulmonary & bronchial circulations.	All experimental species & post mortem material from human beings.	Post mortem material	Moderate to low	Access to material. Tedious work requiring long hours.	Vascular canula, latex or other appropriate injection material.	112

II.
F. $\dot{V}/\dot{Q}$

Name of test	Physiological interpretation	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
Arterial PO_2		SEE BLOOD GASES					
$AaDO_2$	$\dot{V}/\dot{Q}$ or shunt	Any but larger better		Good	Requires arterial blood and measurement of alveolar gas (Scholander, gas chromatograph, mass spectrometer)		86, 113
$AaDN_2$	$\dot{V}/\dot{Q}$	Similar to $AaDO_2$					114
Radio-isotopes	Regional $\dot{V}/\dot{Q}$	Rabbit probably minimal size	Any	Fair	Restraint, cooperation or anesthesia required	Multiple probes or scintillation camera	83
	Regional ventilation	"	May be measured during breathholding or during breathing	"	Deposited aerosols do not measure ventilation; ^{133}Xe most convenient isotope.		115
	Regional perfusion	"	"	"	^{113}In, ^{131}I, or ^{99m}Tc, combined with albumin or other 30 μ particles most widely used; ^{133}Xe dissolved in saline useful when studies need to be repeated rapidly.		115
Single expiration PCO_2 & R	$\dot{V}/\dot{Q}$	Any, but larger better	Slow, complete expiration	Fair	Cannot quantitate	CO_2, O_2 meters of mass spectrometer	5, 116
V_D/V_t	$\dot{V}/\dot{Q}$ particularly high ratios	"	Need constant breathing pattern	Fair	Hard to quantitate	Analysis of CO_2 in mixed expired gas and arterial blood.	113
Lobar gas sampling	Regional $\dot{V}/\dot{Q}$	Large	Requires lobar catheters	Fair	Invasive, anesthesia	Catheter, gas analyzers	117
Multiple inert gas washout	Distribution of $\dot{V}/\dot{Q}$	Any, but larger better	Collection of expired gas, venous infusion, cardiac output measurement.	Good	Somewhat complicated	Gas chromatography mass spectrography; dye dilution or Fick cardiac output	118

II.
G. Diffusion

Name of test	Physiological interpretation	Species	Experimental conditions	Sensitivity	Limitations	Equipment needed	Refs.
$D_LCO(SB)$	D_M, V_C, Hgb (see below)	Larger better	Timed breath-holding at TLC	Good	Cooperation or anes-thesia	CO & He meters or gas chromatograph	119, 120
$D_LCO(SS)$	Above plus $\dot{V}/\dot{Q}$	Larger better	Regular breath-ing	Good	Cooperation or anes-thesia	Same plus measure-ment of V_D (physiol)	121, 122, 123
DM	Thickness and quantity of membrane	"	Timed breath-holding at TLC	Good	"	CO, O_2 & He mea-surement	124
V_C	Pulmonary cap-illary blood volume	"	"	Good	"	"	124
D_LO_2	D_LO_2	"	Regular breath-ing	Good	Computation of mean cap-illary PO_2 difficult	Measurement of $\dot{V}O_2$, $\dot{V}CO_2$ at 2 levels of oxygenation.	107, 125
$D_LCO(RB)$	Less affected by $\dot{V}/\dot{Q}$	"	Breath by breath analysis	Good	Complex method and computation	Rapidly responding analyzers.	126, 127

H. Blood Gases

Arterial PCO_2	Total alveolar ventilation	any, but larger better	Any	Good	Requires ac-cessibility of artery	Anaerobic blood col-lection, anticoagulant, (blood gas analyzer)	128, 129, 130
Mixed venous PCO_2	"	"	"	"	Requires mixed venous blood	"	131
Arterial pH	HCO_3/PCO_2	"	"	"	Arterial blood	"	129, 130, 132, 133
Mixed venous pH	"	"	"	"	Mixed venous blood	"	132-134
Arterial PO_2	Regional $\dot{V}/\dot{Q}$ R → L Shunt, alveolar ven-tilation	"	"	"	Arterial blood	"	128, 129
Mixed venous PO_2	Above plus cardiac output & $\dot{V}O_2$	"	"	"	Mixed venous blood	"	128, 129

DISCUSSION

Pathologic studies have confirmed that a surprisingly high percentage of autopsied nonsmoking adults have pulmonary emphysema (135, 136). Furthermore, there is a higher incidence of emphysema in both smokers and nonsmokers in areas with high atmospheric concentrations of sulfur oxides, nitrogen oxides, hydrocarbons, and particulates (136). Clinical chronic bronchitis is more common in urban areas (137), and children from urban environments have maximal expiratory flow rates lower than predicted (138). It seems reasonable that much of this data is explained by high atmospheric levels of common pollutants. However, air pollutants, particulates, viral respiratory infections, and cigarette smoking probably have additive deleterious effects upon lung function (136, 138).

The toxicity of all potential atmospheric contaminants, alone or in combination, as well as safe exposure limits need to be defined. One approach to this problem is to expose humans or animals, under strictly controlled experimental conditions, to varying concentrations, durations, and combinations of inhalants. The results of such investigations must be objective and reproducible. The methods must be sensitive and capable of large scale utilization. The use of the pulmonary function tests described would seem satisfactory for these purposes.

Similar considerations apply to the pulmonary toxicology of a variety of other inhaled (and, in some cases, ingested) substances. If a potentially toxic inhalant is to be investigated, certain tests are more valuable than others. The factors of reproducibility, sensitivity, and specificity will again be very important. The choice of specific pulmonary function tests, however, also depends upon other factors such as anatomic characteristics of the species to be tested. For example, in dogs, multibreath nitrogen washout would be expected to be a relatively insensitive detector of mild physiologic abnormality since the dog lung has a highly developed collateral ventilation system, a factor known to decrease the sensitivity of tests measuring distribution of ventilation. On the other hand, the pig lung has poorly developed collateral pathways. Therefore, multibreath nitrogen washout should be an effective means of detecting physiologic abnormalities in pigs. Multibreath nitrogen washout would also be expected to be a reasonably sensitive technique in man, whose lung has collateral pathway development intermediate between that of the dog and the pig. Thus, effective use of pulmonary function methods described in this report will depend upon the nature of the subject, the test itself, and the experimental conditions. These factors should all be considered in designing inhalation toxicology protocols.

ACKNOWLEDGEMENT

Preparation of this review was aided by U.S.A.F. Contract F33615-76-C-5005 and by California Air Resources Board Contract 4-611 with the Department of Community and Environmental Medicine, California College of Medicine, University of California, Irvine, California.

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AMRL-TR-75-125

PAPER NO. 3

PARTICLE DEPOSITION AND CLEARANCE AS A TEST OF TOXIC EFFECT*

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INTRODUCTION

The field of inhalation toxicology is currently in an intriguing active phase characterized by two features: (1) compilation, description and quantitation of effects other than mortality that include physiological, morphological and biochemical parameters, and (2) development of an understanding of biologic responses in terms of the physical and chemical properties of inhaled materials. Further development in both of these areas seems necessary for inhalation toxicology to become a predictive discipline as opposed to merely a descriptive one. The phenomena associated with deposition and clearance of inhaled particles have recently been recognized as important aspects of the defensive mechanisms of the lung. The purpose of this paper is to justify the use of deposition and clearance tests in the inhalation toxicology laboratory.

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* Supported in part by the Air Resources Board of the State of California under Contract Number 4-611.

** Affiliated with the Neutron Generator Facility, San Diego State University, San Diego, California, 92182.

Particles, or aerosols as they are called when airborne, can be broadly classified with respect to their origin. Naturally occurring aerosols include spores, pollens, microorganisms, inorganic dusts, ash, degradation products of various plants, liquid condensation droplets and various solids formed from naturally occurring volatile materials. In and about areas populated by humans additional aerosols include those produced from combustion of fuels, a multitude of industrial processes, erosion of machinery and building and household materials, spraying devices, and again, reactions of gaseous materials. Aerosols in and about localized workplaces form another practically innumerable subcategory. A common belief, probably justifiable, is that virtually all aerosols are capable of producing toxic responses in humans if inhaled in sufficient concentrations over a sufficient time (Christie, 1967).

For purposes of scientific elucidation, the fate of many inhaled aerosols can be analyzed into two phases: (1) deposition on surfaces of the respiratory tract, and (2) clearance (or lack of) after deposition has occurred.

The patterns of deposition of inhaled aerosols are becoming understood in terms of forces that act on airborne particles, the air flow characteristics of breathing, and the geometric properties (morphology) of the respiratory tract. Mammalian respiratory systems have geometric and air flow properties such that particles within a given range of size and shape tend to deposit preferentially in characteristic locations. For example, the human nose is known to be highly efficient in collecting particles with aerodynamic diameters greater than a few micrometers. The deep lung can only collect particles that have eluded the nose (or mouth) and the tracheobronchial tree; that is, particles with aerodynamic diameters below a few microns.

It appears that clearance mechanisms at various levels in the respiratory tract are efficient in handling particles in the size ranges that preferentially deposit at a given site. For example, alveolar macrophages seem to exhibit efficient engulfment for particles in the one micron diameter size range; this is just in the size range of high deposition probability in alveoli. Infectious organisms are often in the size range for deposition in the deep lung, where conditions are usually favorable for their rapid reproduction. Fortunately, macrophages can inactivate many infectious organisms. Similarly, the nose effectively clears the largest inhalable particles via sneezing, blowing and mucus movement, and the moving mucus of the tracheobronchial

tree is efficient in transporting large quantities of solid or liquid particles of various sizes, shapes and densities. The point is that deposition and clearance phenomena appear to be somewhat matched or balanced with respect to particle size characteristics. This being the case, one might suspect that alterations in either the deposition or clearance patterns could predispose one toward future injury from inhaled aerosols. In some instances, of course, shifts in deposition or clearance patterns might act to afford increased protection.

Fortunately, despite differences in size and morphology, most animals appear to have clearance phenomena that are remarkably similar with respect to rates and mechanism; for example, they commonly have mucociliary clearance in the nose and tracheobronchial tree and a macrophage response in the alveolar spaces. Also, though correspondence is not as close here, basic similarities exist in aerosol deposition characteristics and in responses to toxic materials. Thus, it is reasonable to consider tests of aerosol deposition and clearance in animals in toxicologic evaluations of injury.

INHALED AGENTS THAT ALTER DEPOSITION OR CLEARANCE PATTERNS

Several materials are known to alter deposition or clearance (including killing or inactivation of microorganisms) of inhaled particles. A few examples will serve to illustrate. Cigarette smoke, an almost ever-present co-insult in human inhalation exposure situations, has understandably been well studied. The controlled studies of Albert et al. (1969, 1970, 1974) show effects in humans and donkeys that depend on dose and exposure time. Low single doses or early effects of repeated exposure to smoke were associated with acceleration of clearance rates in the tracheobronchial tree of both species. Heavier doses and long-term repeated exposures were associated with sporadic clearance, intervals of clearance stasis, and even retrograde movement of deposited particles (again in both species). Cigarette smoke exposures (220 mg/m^3) have been shown to increase deposition and delay clearance in rats (Garver, 1968) and to increase the survival of inhaled viable bacteria in hamsters (Henry et al., 1970). In the hamster study, excess deaths due to bacterial infections were seen in animals exposed to cigarette smoke for 2 hours ("3% v/v").

Preexisting influenza infection has been shown to impair both upper and lower respiratory tract clearance. Studies by Green (1965) with P-8 virus infected mice that were exposed to viable staphyloccocus bacteria showed that infected animals did not effectively kill the bacteria. Similarly, Creasia et al. (1973) found that P-8 virus-infected mice had drastically impaired clearance of radioactive "insoluble" particles. In humans, Camner (1973) found that influenza infection could impair tracheobronchial clearance for up to one month after disappearance of the familiar clinical symptoms.

Elliot Goldstein and coworkers (1971, 1974) reported work in which mice were challenged with radiolabeled viable staphyloccus both before and after exposures to relatively low levels of ozone and nitrogen dioxide. Prior exposure to ozone (0.6-2 ppm, 17 hours) or ozone plus nitrogen dioxide (0.1-0.1 and 1.5-4.2 ppm, 17 hours) lead to: (1) decreased overall deposition of bacteria, and (2) impaired killing of deposited bacteria. In the same series of studies, exposures of ozone plus nitrogen dioxide (0.4 and 4-6.8 ppm, 4 hours) after inhalation of bacteria, caused increased survival of the bacteria. Ozone alone at 2 ppm (4 hours) was observed to cause increased survival and increased clearance of the inhaled bacteria.

Sulfuric acid mist exposures by Fairchild et al. (1975) at 3 mg/m^3 (1.8 μm CMD) in guinea pigs caused increased total deposition of inhaled streptoccocus.

Sulfur dioxide (1 ppm, 7 hours, 5 days to 25 days) has been shown to diminish the clearance of inert particles in both the lung and tracheobronchial tree in the rat by Ferin and Leach (1973). A similar effect was seen in donkeys after brief exposure (300 ppm SO_2, 30 min) by Spiegelman et al. (1968).

Many other agents have been reported to change deposition and/or clearance patterns in various species.

EXPERIMENTAL DESIGN FOR DEPOSITION AND CLEARANCE TESTING

A successful test of deposition and clearance implies that certain criteria have been met. These criteria apply to the test aerosol, animal subjects, experimental plan, and analysis of data. Recommendations can be made in each of these areas.

The Aerosol

For deposition and clearance studies, the aerosol should be inhaled by the subjects and have an aerodynamic size that permits significant deposition beyond the nose. For most studies an aerosol smaller than about 5 μm in diameter is appropriate. Monodisperse aerosols, or at least those that have diameters distributed with geometric standard deviations less than about 1.3, should be used. Aerosol characteristics should be reproducible from one run to the next. The aerosol should be detectable in the lung, either by external radiation detection or chemical/biological assay in excised lung. Radioactive aerosols should have a tag that does not have excessive leaching; a few percent per day in the lung environment is sufficient. Initial activity of labeled aerosol should be on the order of one microcurie to allow for precise external counting. An aerosol that fits the above criteria is radiolabeled polystyrene-latex. The basic particles are available commercially (Dow Chemical Company, Midland, Michigan) in several sizes, and methods for labeling with radioisotopes are available (Szende et al., 1975 and Black and Walsh, 1970).

The Animal

Unanesthetized animals should be used when possible in order to avoid variable and often uncertain effects associated with anesthesia. In most studies two species should be used, especially when one is either the guinea pig or the rat. Rats tend to have respiratory infections and guinea pigs appear to have unusually reactive airway musculature. Individual subjects should serve as their own controls to reduce variability in the data. Healthy individuals usually have relatively repeatable deposition and clearance phenomena, but variation within a group may be large.

Experimental Plan

Exposure to the aerosol should be to the nose or mouth to avoid deposition of large amounts on fur or skin. Deposition of material on fur of laboratory animals can interfere with subsequent assay of amounts in lung and can lead to ingestion of large quantities of particles. A determination of the initial deposited amount should be made immediately after exposure (within minutes), and the inhalation exposure should not last more than about 20-30 minutes. Long inhalation exposures are complicated by concurrent clearance occurring during deposition.

Amount in the lung should be determined with sufficient frequency to define the clearance curve. Ideally, the amount in the lung should be quantitated hourly for the first few hours and daily for several days. It is important that clearance be followed for long enough to properly define the clearance curve. The tracheobronchial tree is usually cleared by about 1-2 days but deep lung clearance can require several days, even months or years, for highly insoluble materials.

Data Handling

To determine the effect of toxic agents on clearance, statistical tests should be employed. This necessitates reducing clearance curves to numerical values; the fewer parameters used to define the curve, the simpler the tests will be. Figure 1 depicts three out of the many ways of quantitating clearance curves: (1) analysis into exponential components; (2) fitting with a polynomial function; and (3) calculation of moments. Each method yields numerical values that can be given mean values and standard deviations for a group of observations. Statistical tests for significance can then be performed on these values, permitting one to demonstrate significant changes in clearance patterns (Figure 2).

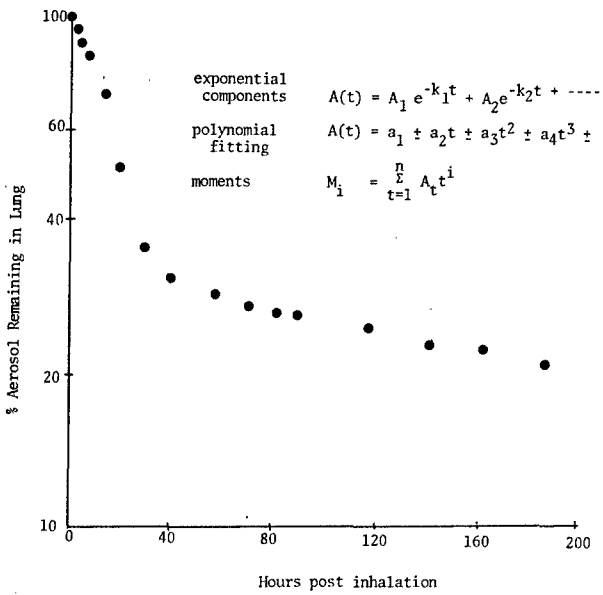

Figure 1. Three methods of reducing a clearance curve to quantitate parameters that can be used in statistical testing. The method that gives fewer parameters for testing would in general be more sensitive for detecting differences between groups.

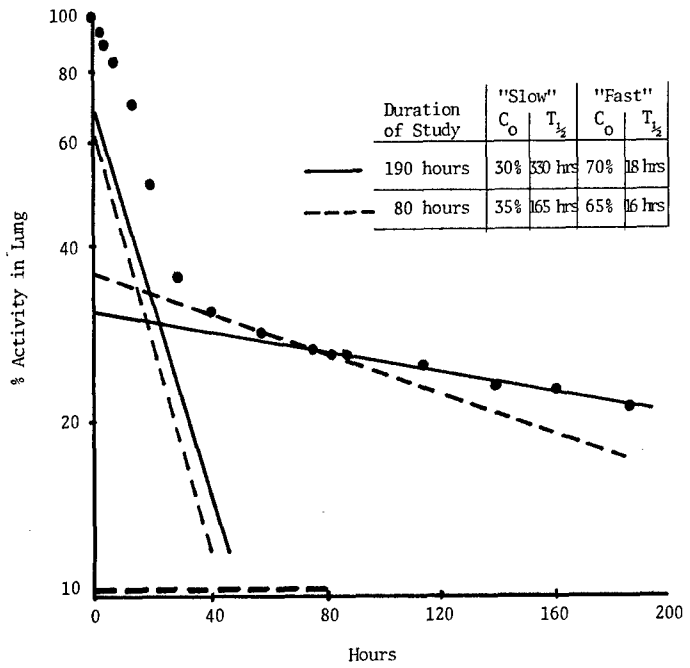

Figure 2. Demonstration that the duration of a clearance study has an effect on the exponential components of a clearance curve. Had this study been stopped at 80 hours different component curves would have resulted.

EFFECT OF CIGARETTE SMOKE EXPOSURE ON PARTICLE CLEARANCE IN THE RAT

Data resulting from a modest study will be presented to demonstrate the effect of a toxic agent on tracheobronchial clearance. Rats were used since they were inexpensive and easy to handle. Eight Sprague Dawley rats were briefly exposed, nose only, to a radioactive silver aerosol. The animals were then divided into two groups: one group was exposed to cigarette smoke for four hours, the other group breathed ordinary air and served as a clearance control. Clearance curves were determined for each animal by measuring the radioactivity in the thorax every 100 minutes for the first day, every 200 minutes the second day, and less frequently for about eight days. These measurements were made by using a collimated gamma-ray detector placed above an opening in a lead shield. The animals were placed beneath this collimator shield such that only gamma rays emitted from the thoracic region were measured. Feces were collected every time a thoracic count was made, and the radioactivity in each sample was determined.

Aerosol Exposure

An exploding wire aerosol generator (a 4 microfarad capacitor charged to 6.5 kilovolts) of the type described by Karioris and Fish (1962) was used to aerosolize 20 cm of 0.005 inch diameter silver wire. The wire had previously been neutron irradiated and had an induced activity of about 0.75 microcuries of ^{110m}Ag (beta and gamma, 250 day half-life) per cm of wire. The aerosol had spherical primary particles that were distributed approximately log-normally with a count median diameter of 0.07 microns and a geometric standard deviation of 1.6. Electron micrographs indicated that most of the aerosol was in the form of agglomerates of primary particles with an aerodynamic median diameter of about 1 μm when inhaled by the animals. The radioactive wire was exploded inside a 22 liter exposure chamber, five minutes were allowed for settling of large particulates, and the animals were exposed nose-only to the aerosol through ports in the chamber walls (Figure 3). During the actual explosion, rats were held in a separate room to isolate them from the loud noise. The exposure time was 15 minutes and the mean initial lung deposition was estimated (using wheat-filled phantoms) to be about 0.1 microcuries, or about 0.2 milligrams of silver. No anesthetics were used as the animals were docile and cooperative during both the aerosol exposure and the thoracic counting procedures.

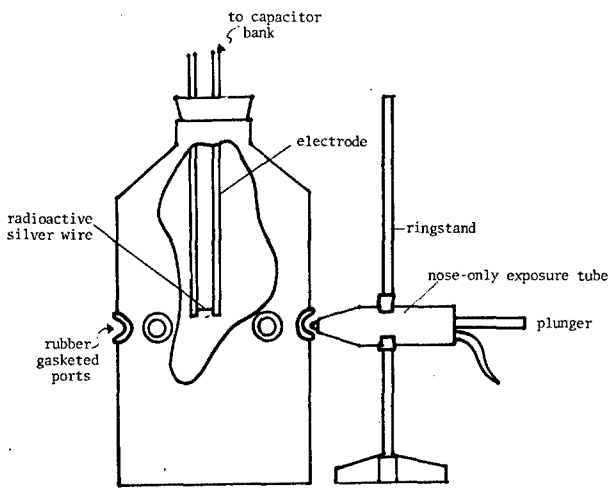

Figure 3. Aerosol exposure set up for simultaneous nose-only exposure of 8 rats to radioactive silver particles.

Smoke Exposure

The experimental group was exposed to fresh tobacco smoke generated by a machine which drew air continuously through several lit unfiltered cigarettes and gently blew the smoke into a large box (one cubic meter volume). The animals were placed inside cages within this box 30 minutes after exposure to the silver aerosol. They remained in the smoke for four consecutive hours, being removed only twice for two minutes each time, for thoracic activity measurements. The smoke concentration was maintained at a level such that taking a breath inside the smoke chamber (smoke exposure box) gave the experimenters the same subjective experience as inhaling during normal cigarette smoking.

Clearance Measurement

The animals were placed in plastic restrainers beneath a NaI(Tl) crystal (3" diameter) for gamma activity determinations of their thoracic regions. Two inches of lead were used to shield the head and gastrointestinal tract from the gamma detector. The shield, with a 2-1/2" wide opening above the thoracic area, had been designed using roentgenograms of all of the rats so that ^{110m}Ag in either the head, stomach or intestines was shielded from the detector.

Results

Longitudinal body scans of radioactivity indicated high initial activities in the head region, and it was feared that the fur on the head had been contaminated with significant amounts of ^{110m}Ag. However, this activity declined rapidly indicating a fairly clean nose-only exposure and that the initial activity was probably due to high deposition inside the nose and throat.

When compared to the air breathing group, the animals in the smoke-filled chamber were less active when in their cages, preferring to sit quietly, but they resisted handling by struggling considerably more than the control animals.

Clearance curves for control and smoke-exposed groups (Figures 4 and 5) were arrived at by averaging the values of all animals in each group. Cumulative activity excreted in the feces (Figure 6) is shown for both groups and is in terms of percent of total activity excreted during the data collection period.

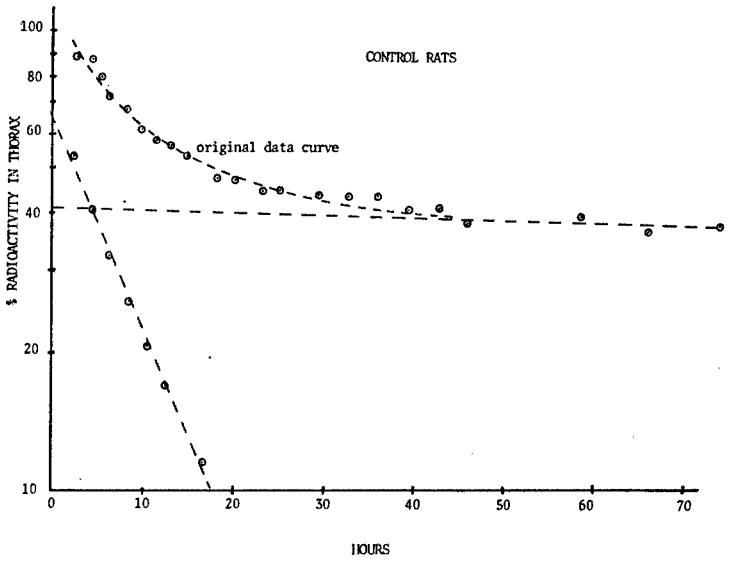

Figure 4. Thoracic clearance curves for control rats. Exponential components, "short" and "long" term, are shown.

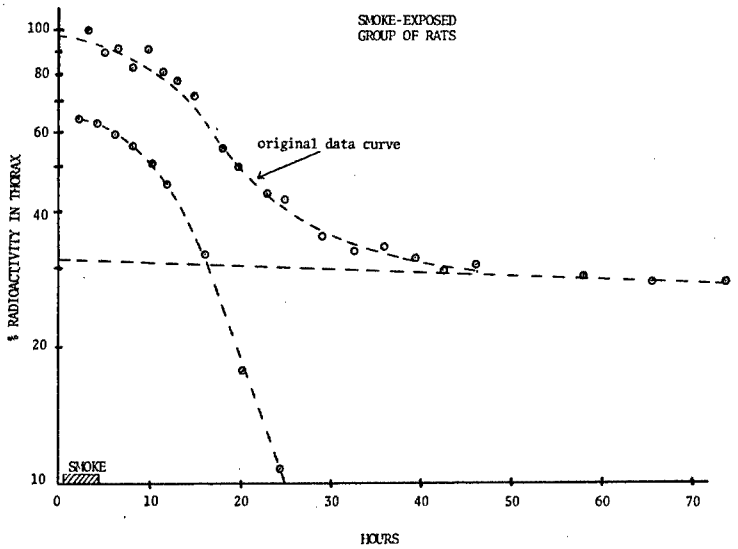

Figure 5. Thoracic clearance curves for rats exposed to cigarette smoke. Exponential components, "short" and "long" term, are shown.

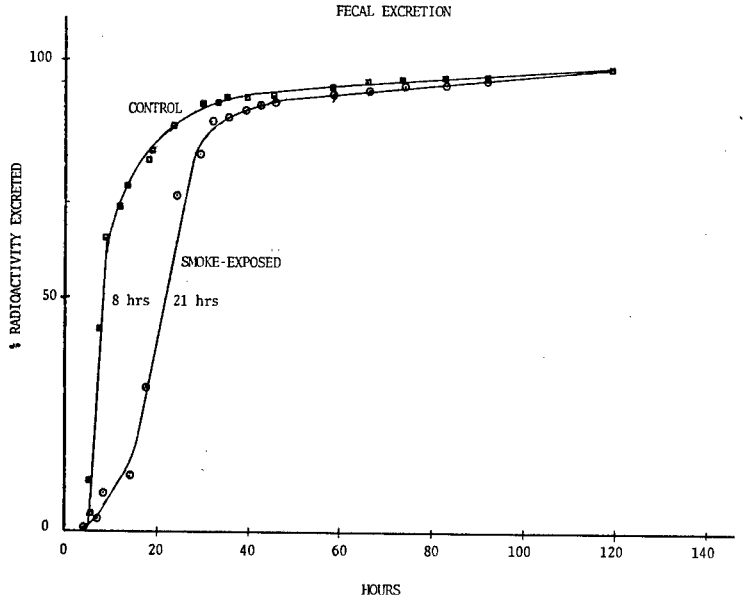

Figure 6. Cumulative fecal excretion of inhaled radioactivity for control and cigarette smoke-exposed rats. Half of the activity was excreted in the first 8 hours by the control group and in the first 21 hours by the smoke-exposed group.

The thoracic clearance curves appear linear (on a semi-log plot) after about 40 hours. This linear portion has a half-life of about 285 hours, with no significant difference between the two groups. Extrapolating this linear curve toward zero time and subtracting it from the original clearance curve produces a "short-term" clearance curve. For rats, this curve is usually also linear on a semi-log plot and is often assumed to represent mucociliary clearance of particles deposited on the ciliated portions of the respiratory tract. The control group's short-term curve is linear over its entire range and has a half-life of 6-1/2 hours. The smoke-exposed group's short-term curve does not appear linear until 12 hours after the animals were removed from the smoke chamber. The linear portion of this curve has a half-life of 6 hours which is not significantly different from the control value.

The time at which cumulative fecal excretion of ^{110m}Ag reached 50% of the total excreted was 8 hours for the control group and 21 hours for the smoke-exposed group. The difference, 12 hours, is almost identical to the period of time during which thoracic clearance was depressed

in the smoke group. This lag in excretion was not due to fecal retention by the smoke group since both groups produced fecal pellets at the same rate throughout the experimental period. It is, therefore, concluded that the brief exposure to cigarette smoke blocked movement of silver from the respiratory tract to the gastrointestinal tract, and that this block was effective for 12 hours after the smoke exposure terminated.

CONCLUSION

The status of deposition and clearance phenomena is an important consideration in inhalation toxicology. The techniques of aerosol challenge have been recently developed to a sufficient degree that routine testing of deposition and clearance of inhaled particles is now feasible in the toxicology laboratory.

ACKNOWLEDGEMENT

Dr. L. L. Skolil of the Physics Department, San Diego State University, encouraged and aided the original experimental work reported in the section titled "Effect of Cigarette Smoke Exposure on Particle Clearance in the Rat."

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AMRL-TR-75-125

PAPER NO. 4

MORPHOMETRIC EVALUATION OF LUNGS USING AUTOMATED IMAGE ANALYSIS

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INTRODUCTION

To further the understanding of the structural and functional relationships in pulmonary tissue, morphologists have used both quantitative and descriptive techniques. Morphometry is necessary for precise correlation of structure and function in both normal and diseased organs. It can provide accurate measurement of the severity of damage in diseased organs and it is the only means of confirming, by statistical methods, the existence of subtle lesions. The latter point is illustrated in this paper, in which the focus of attention is alteration in the airspaces of pulmonary parenchyma.

Our primary objectives in this study were to compare accepted manual methods to automated methods and to use automated methods in the quantitation of the distal airspaces of the lungs from 66 dogs distributed among control and 7 experimental groups exposed to a variety of air pollutants. These objectives were accomplished by a demonstration of similar stereologic parameters by both methods, by using a mathematical model based on the distribution of chord lengths to estimate stereologic parameters, and by the use of pattern recognition to classify the distal airspaces on the basis of the ratio between perimeter cube and area.

Accepted stereological methods (Weibel, 1963) were used to evaluate 14 young and aged dog lungs. Morphometric evaluation of histological sections by manual methods is time consuming and the probability of operator error increases with the number of slides processed and the number of operators. For comparison with manual methods we also used automated analysis in which optical images were analyzed by a television scanner coupled to a computer (Cole, 1966). Thurlbeck (1974) commented that automated measuring microscopes offer a reasonable compromise between the tedium and error of the human mode and the complexity and great expense of computer pattern recognition techniques which utilize binary image

digitization and analysis (Levine et al., 1970). Our study was designed to test in part the validity of this statement.

Automated measuring microscopes have been used to quantitate two divisions of the lung, the conducting airways and the distal (respiratory) airways. A comparatively accurate assessment of the mucous producing structures of the conducting airways was obtained in studies of experimental bronchitis induced by acute exposure to sulfur-dioxide vapor in rats (Mawdesley-Thomas and Healey, 1969a; 1969b) and in studies of experimental bronchitis induced by acute exposure to cigarette smoke in lambs (Mawdesley-Thomas and Healey, 1973). In the distal airways, automated analysis and manual methods were used to determine the internal surface area of 6 normal rat lungs from histological slides (de Bignon and Andre-Bougaran, 1969). In that study, the automated method demonstrated a lower standard error than the manual method. Normal and emphysematous human lungs were quantitated by automated analysis using macrosections (Anderson et al., 1971). The results of that study showed significant differences in the number of parenchymal airspaces, sectioned areas, and percentage of long intercept lengths (>500 mμ). An increase in the ratio of the number of lactate dehydrogenase (LDH) positive cells to

alveoli and the hypertrophy of LDH positive cells in the lungs of guinea pigs acutely exposed to nitrogen dioxide was shown using automated analysis (Sherwin et al., 1973b). A comparison of the ratio of the number of LDH positive cells to alveoli showed no significant difference between estimates obtained by manual or automated methods (Sherwin et al., 1973a).

METHODS

List of Symbols

Symbol	Definition	Dimension
$\hat{L}_c$	Estimated mean chord length calculated by linear integration	cm
$\hat{\mu}_x$	Estimated mean chord length calculated by numerical integration	cm
e	Base of the natural system of logarithms = 2.718	cm^0
V	Volume of structure	cm^3
V_{vi}	Volume density of component i	cm^0
v_i	Mean volume of component i	cm^3
A	Profile area of section	cm^2
A_i	Mean profile area of component i	cm^2
S	Surface of structure	cm^2
S_{vi}	Surface density of component i	cm^{-1}

List of Symbols (Continued)

Symbol	Definition	Dimension
N	Number of structures	cm^{0}
N_{vi}	Numerical density of component i	cm^{-3}
N_{ai}	Numerical profile density of component i	cm^{-2}
D	Linear dimension of structure ("caliper diameter")	cm
P	Profile perimeter on section	cm

Note: This symbolism corresponds to the notation used by the International Society for Stereology (Weibel and Elias, 1967).

Fourteen young and aged normal dogs were killed by electrocution subsequent to a series of physiological studies (Robinson et al., 1972; Robinson and Gillespie, 1973a; 1973b; 1975). These beagle dogs were from a closed colony and were housed in outdoor runs or individual cages since birth. The lungs were fixed via intratracheal perfusion of Karnovsky's fixative (Karnovsky, 1965) at 20 cm H_2O pressure and tissue sections were selected at random from 6 of the lobes to give a total of six slides per animal. Point count and linear integration methods were used to estimate manually the surface to volume ratio (S_{vA}) and the fractional volume of the distal airspaces (V_{vA}) and tissue (V_{vt}). Similarly automated analysis was

used to estimate the same stereologic parameters using fractional percentage of component areas and linear integration methods. The manual and automated estimates of the parameters S_{vA} and V_{vt} were regressed on age by the method of least squares. The slopes of the regression lines were compared using the difference between means of the Student's t distribution (Snedecor and Cochran, 1967).

Sixty-six beagle dogs, distributed among control and treatment groups were exposed to a variety of air pollutants for 61 months (Hinners et al., 1966). A wide variety of physiological parameters were determined before, during, and after exposure (Lewis et al., 1974; Bloch et al., 1972, 1973; Vaughan et al., 1969). About three years after cessation of exposures, the dogs were weighed, measured, and killed by an intravenous injection of pentobarbital. The lungs, trachea, and attached structures were removed from the thorax and trimmed of extraneous tissue. The lungs were weighed, and subsequently the left lung was removed at the left primary bronchus for biochemical analysis. The trachea was cannulated and the right lung was fixed in the normal dorsoventral orientation by intratracheal perfusion with dilute Karnovsky's fixative (Karnovsky, 1965) at 30 cm H_2O pressure. The volume of the right lung (V_{Lr}) was then measured by fixative

displacement. After samples were taken for scanning electron microscopy (SEM) and transmission electron microscopy (TEM), the lobes of the right lung were cut into 1 cm thick slices (Figure 1). The fraction of nonparenchyma (V_{vnp}) (bronchi and blood vessels down to 2 mm diameter) and parenchyma (V_{vp}) were determined using the point-count method of Dunnill (1962).

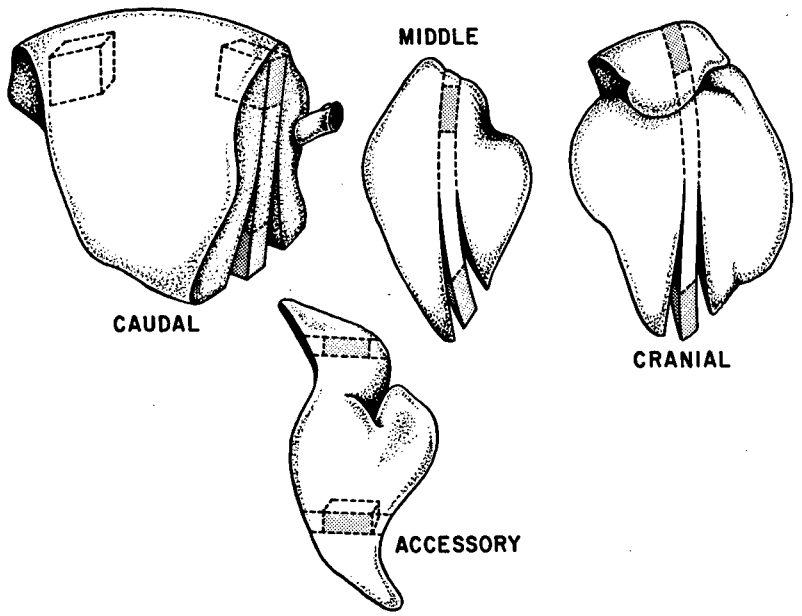

Figure 1. Blocks of tissue were selected from sites in the cranial and middle (transverse plane), caudal (sagittal plane), and accessory lobes (frontal plane). The cranial, middle, and caudal lobes are depicted from the lateral view, while the accessory lobe is depicted from the caudal view.

Nine 2 to 3 cm^2 blocks of tissue were cut from each right lung (Figure 1) and measured. Paraffin sections 7μm thick were cut, stained with hematoxylin and eosin, and measured microscopically using a calibrated eyepiece reticle. The linear correction factor (p) for shrinkage of the fixed tissue due to processing was calculated from these measurements.

The stained slides were examined at a magnification of 125X on a Quantimet 720 image analyzing computer (Cole, 1966). The instrument was set to detect the airspaces, and the detection level was standardized by use of a preset level of detection on a wire mesh grid. Measurements were made at 20 stratified random points on each slide (Thurlbeck, 1967). Increased sampling per slide did not reduce the variance significantly. Two programs were used for automated analysis. A chord-sizing program determined the volume fractions of V_{vA} and V_{vt} and the number of intra-alveolar chord lengths in 14 size classes from 12 to 684 μm in increments of 48 μm. A pattern recognition program (Gibbard et al., 1972) determined the number of airspaces and their sectioned areas (A) and perimeters (P) in 14 size classes. The measurements from both programs were printed on a teletype and punched on papertape. The punched papertape was read into a Burroughs 6700 computer for computation of the 266, 760 measurements. Fortran IV (level H) programs allowed data integrity checking,

editing, and reduction before computing and graphically displaying various stereologic parameters.

The volume fractions V_{vA} and V_{vt} were computed from the chord sizing program. The V_{vA} was further classified into the volume fractions of alveoli (V_{va}), alveolar sacs (V_{vas}), and alveolar ducts (V_{vad}) from their representative areas by the pattern recognition program.

The pattern recognition program was run on the NO_2 high group and 6 dogs of the control group closest to the mean of the entire control group. A P^3/A ratio was used to classify the distal airspaces by the following criteria: alveoli $<2 \times 10^4$ cm, alveolar sacs $2 \times 10^4 - 2 \times 10^5$ cm, and alveolar ducts $>2 \times 10^5$ cm. Visually these geometric ratios corresponded to the following criteria: alveoli = no interalveolar septa entering an airspace, alveolar sacs = 1-6 interalveolar septa entering an airspace, and alveolar ducts = 7 or more interalveolar septa entering an airspace. All volume fractions and sectioned areas were corrected for the Holmes, processing, and fixation effects (Weibel, 1963). The total length of scanning lines multiplied by the number of fields and by V_{vA}, divided by the number of intra-alveolar chords $>12\mu$ gave the mean chord length of the processed tissue

($\hat{L}_{C_1}$). Those chords $<12\mu$ were rejected because they represented mostly structures not normally air-filled, such as capillaries and post-capillary venules.

The estimated mean chord length ($\hat{L}_C$) of unfixed tissue was calculated from the processed to unfixed tissue by formula 1 (Thurlbeck, 1967):

$$L_C = L_{C_1}\,(p)\left(\frac{TLC_r}{V_{Lr}}\right)^{1/3} \qquad (1)$$

The total lung capacity of the right lung (TLC_r) was determined by multiplying TLC by 0.59 which is the fractional volume of the right lung (Cree et al., 1968). TLC was measured on these dog lungs by plethysmographic methods (Lewis et al., 1974). The S_{VA} was calculated by multiplying the reciprocal of $\hat{L}_C$ by 4 (Weibel, 1963). Significant differences between the S_{VA} of the control and exposure groups were tested using the Student's t test statistic (Snedecor and Cochran, 1967). The internal surface area of the unfixed right lung (S_{Ar}) was calculated as described by Weibel (1963) and Thurlbeck (1967). The internal surface area of the entire unfixed lung (S_A) was estimated using:

$$\frac{0.59}{S_{Lr}} = \frac{0.41}{S_{L1}} \tag{2}$$

to determine the volume of the unfixed left lung (S_{A1}) and adding it to S_{Ar}.

A distribution of percent cumulative frequency of airspace chords in 14 size groups was computed for each dog lung. A two parameter cumulative probability law of the form

$$F(x) = P(X \leq x) = (1 - e^{-\alpha x})^{\beta} \tag{3}$$

was found to provide the best fit to that distribution. The parameters α and β were estimated using a combination of minimum modified chi-square (Cramer, 1946) and the Newton-Raphson procedures (Froberg, 1965). The estimated mean chord length $\hat{\mu}_X$ was obtained by calculating the area above the curve $(1 - e^{-\alpha x})^{\beta}$ using the 6th Order Newton-Cote formula for numerical integration, formula 4 (Froberg, 1965):

$$\xi(X) = \int_0^{\infty} [1 - F(x)]dx \tag{4}$$

The variance of X was determined by formula 5 (Froberg, 1965):

$$\hat{\sigma}_X^2 = 2\int_0^{\infty} x[1 - F(x)]dx - [\int_0^{\infty} 1 - F(x)dx]^2 \tag{5}$$

$\hat{\mu}_x$ and $\hat{\sigma}^2_x$ were corrected for the Holmes, processing, and fixation effects. S_{vA} was calculated by multiplying the reciprocal of the $\hat{\mu}_x$ by 4 (Weibel, 1963). Since the average n per group was large (1347 fields), the differences between S_{vA} and S_{Ar} of the control group and exposure groups were tested using the standard normal test statistic (Snedecor and Cochran, 1967). From the sectioned areas and volume fractions of alveoli, alveolar ducts, the respective diameters were calculated. The estimated diameters of alveoli were calculated from their mean volumes by

$$v_a = \beta : A_a^{3/2} \tag{6}$$

where β is the dimensionless shape coefficient A_a/v_v. Since we were primarily concerned with comparisons between groups, we used a $\beta = 1.38$ and assumed that alveoli were roughly spherical in shape. The mean alveolar diameter was easily calculated from its spherical volume (Weibel, 1963). The diameters of alveolar sacs and ducts were determined using a shape coefficient from the length-to-diameter ratio = 2 (Weibel, 1963). They were assumed to be sectioned cylinders (Weibel, 1963). The number of alveoli (N_{va}), alveolar sacs (N_{vas}), or alveolar ducts (N_{vad}) per cm^3 were obtained by

$$N_{v(a,\ as,\ or\ ad)} = \frac{N_{A(a,\ as,\ or\ ad)}^{3/2}}{\beta\ V_{v(a,\ as,\ or\ ad)}^{1/2}} \tag{7}$$

where $N_{A(a,\ as,\ or\ ad)}$ are the number of a, as, or ad per cm^2 and $\beta = 1.55$, the true shape coefficient of an alveolus (Weibel, 1963). A $\beta = 2$ was used for alveolar sacs and ducts (Weibel, 1963). The total number of alveoli (N_{ar}), alveolar sacs (N_{asr}), and alveolar ducts (N_{adr}) of the processed right lung were obtained by

$$N_{(ar,\ asr,\ or\ adr)} = N_{V(a,\ as,\ or\ ad)} (V_{vp} \cdot V_{Lr}) \quad (8)$$

where $V_{vp} \cdot V_{Lr}$ is the parenchymal volume of the right lung. Significant differences between the means of morphometric parameters derived by pattern recognition methods were tested using the Student's t test statistic (Snedecor and Cochran, 1967).

RESULTS

In the 14 young and aged normal dogs, a comparison of manual and automated methods demonstrated no significant difference between the slopes of the two calculated regressions of S_{vA} with age. The automated method had a higher S_{vA} than the manual method. The mean S_{vA} for the control group plotted with the mean group age was about 10 cm^{-1} less than the value predicted for automated analysis (Figure 2). The standard error of S_{vA} for the automated analysis method was 2.65 cm^{-1}, while it was 5.67 cm^{-1} for the manual method. Likewise no significant difference was

observed between the two calculated regressions of the slopes of V_{vt} with age. The automated method had a higher V_{vt} than the manual method. The mean V_{vt} for the control group plotted with the mean group was about 2×10^{-3} cm° less than the value predicted for automated analysis (Figure 3). The standard error of V_{vt} for the automated analysis method was 1.72×10^{-3}, while it was 1.73×10^{-3} for the manual method. The standard error was based on 120 observations.

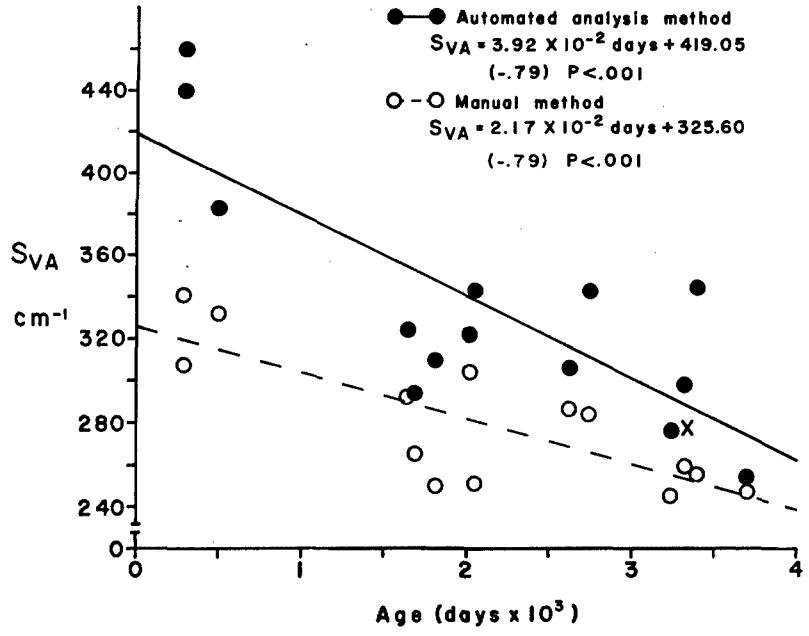

Figure 2. Decreases in surface to volume ratios (S_{VA}) in cm^{-1} of the distal airspaces with increasing age (days) as estimated by manual and automated methods. The regression equation, correlation coefficient in brackets, and significance are given for both methods. The mean S_{VA} for the control group is plotted with the mean group age and marked by an X.

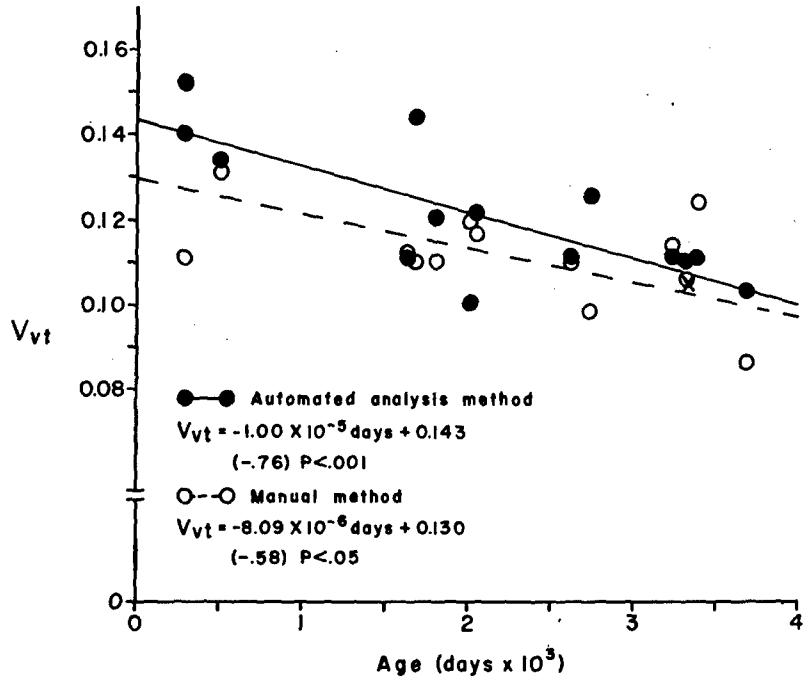

Figure 3. Decreases in the fractional volume (V_{vt}) of distal airspace tissue with increasing age (days) as estimated by manual and automated methods. The regression equation, correlation coefficient in brackets, and significance are given for both methods. The mean V_{vt} for the control group is plotted with the mean group age and marked by an X.

Automated methods were utilized exclusively in the evaluation of airspaces distal to the terminal bronchioles in the lungs of dogs exposed to selected automobile emissions. A comparison between S_{vA} calculated by a distribution of chord lengths (Formula 2) and S_{vA} calculated by linear intercept methods (Formula 1), did not demonstrate any significant differences in the estimation of group S_{vA} (Figure 4).

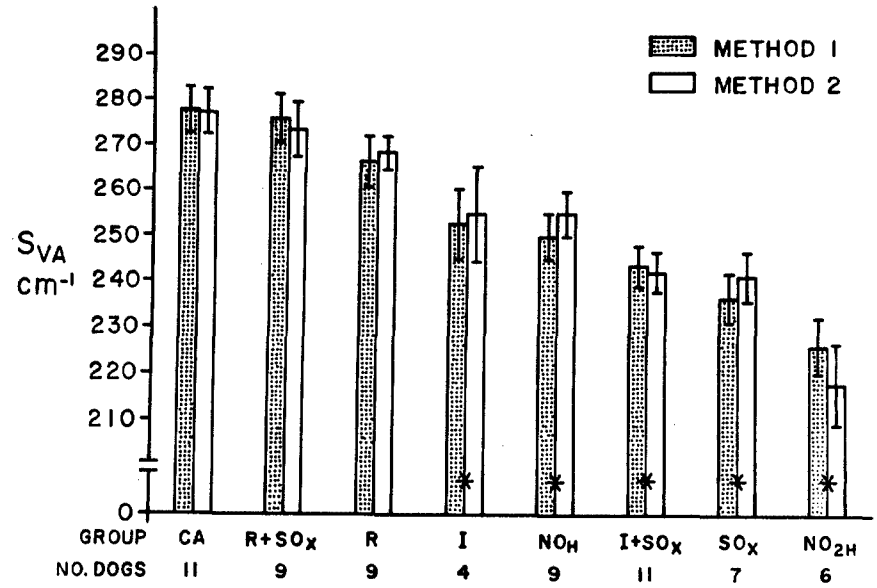

Figure 4. A comparison between the surface densities (S_{VA} cm^{-1}) of method 1, S_{VA} calculated by a distribution of chord lengths (formula 4), and of method 2, S_{VA} calculated by linear intercept methods (formula 1). The exposed groups are plotted with their standard errors from the greatest to least surface densities and significantly different groups are marked by an asterisk as compared to the control group.

Though both methods are acceptable estimators of stereologic parameters, the standard error (SE) of the distribution method was 5.58, while the SE of the linear integration method was 6.05. As a result of its lower SE and its estimation of variance from individual fields, the distribution method was the more sensitive indicator of group differences (Figure 4). Figure 4 showed that the most significant decreases in exposed group

S_{vA} as compared to the control group by the distribution method were nitrogen dioxide high/nitric oxide low (NO_2 high), oxides of sulfur (SO_x), irradiated automobile exhaust and oxides of sulfur ($I + SO_x$), nitric oxide high/nitrogen dioxide low (NO high), and irradiated automobile exhaust (I) respectively. The distribution method was used to estimate S_{Ar}. The S_{Ar}/TLC/BW ratio showed that the most significant decreases in exposed groups as compared to the control group were NO_2 high, SO_x, I, $I + SO_x$, and NO high respectively.

Using pattern recognition the volume fractions (V_{vi}), profile areas (A_i), diameters (D_i), number per cm^2 (N_{Ai}), number per cm^3 (N_{vi}), and number in the right lung (N_{ir}) of alveoli, alveolar sacs, and alveolar ducts-respiratory bronchioles for the control and NO_2 high groups were obtained (Table 1). The NO_2 high group showed significant decreases in V_{va} (35%), V_{vt} (17%), V_{vp} (3%), N_{Aa} (24%), N_{Aas} (23%), N_{va} (19%) and N_{vas} (29%), while V_{vad} (40%), A_{ad} (36%), D_{ad} (17%), and D_{ad}, with an assumed constant length of 964.56 μm (26%), were significantly increased.

TABLE 1. MORPHOMETRIC PARAMETERS OF DOG LUNGS DETERMINED BY PATTERN RECOGNITION

Parameter	Unit of Measurement	Control Group	NO_2 High Group
N	cm°	6	6
W	Kg	9.11 ± 0.39	9.16 ± 0.37
V_{Lr}	cm^3 (processed lung)	403.40 ± 36.49	535.86 ± 28.38
V_{vp}	cm°	0.8840 ± 0.0040	0.8567 ± 0.0021
V_{vt}	cm°	0.1147 ± 0.0029	0.0948 ± 0.0029
V_{va}	cm°	0.2808 ± 0.0573	0.1833 ± 0.0091
V_{vas}	cm°	0.2382 ± 0.0613	0.2096 ± 0.0132
V_{vad}	cm°	0.3663 ± 0.0258	0.5123 ± 0.0212
A_a	10^{-5} cm^2	5.15 ± 1.06	4.68 ± 1.60
A_{as}	10^{-4} cm^2	8.64 ± 0.21	8.39 ± 0.36
A_{ad}	10^{-3} cm^2	1.98 ± 0.10	2.70 ± 0.11
D_a	10^{-4} cm	99.13 ± 1.01	94.45 ± 1.61
D_{as}	10^{-4} cm	318.59 ± 3.82	313.94 ± 6.63
D_{ad}	10^{-4} cm	482.28 ± 12.56	563.19 ± 11.08
D_{ad}	10^{-4} cm constant length	964.56	608.60 ± 18.05
N_{Aa}	10^2 cm^{-2} processed tissue	73.56 ± 2.36	55.51 ± 3.14
N_{Aas}	10^2 cm^{-2}	7.73 ± 0.16	5.92 ± 0.33
N_{Aad}	10^2 cm^{-2}	2.80 ± 0.13	2.95 ± 0.88
N_{va}	10^5 cm^{-3}	7.68 ± 0.37	6.23 ± 0.54
N_{vas}	10^4 cm^{-3}	2.20 ± 0.07	1.57 ± 0.13
N_{vad}	10^3 cm^{-3}	4.15 ± 0.28	3.99 ± 0.24
N_{ar}	10^6 cm°	273.87 ± 13.19	286.00 ± 24.79
N_{asr}	10^6 cm°	7.85 ± 0.25	7.21 ± 0.60
N_{adr}	10^5 cm°	14.80 ± 1.00	18.32 ± 1.11

DISCUSSION

In this study we addressed our efforts to only one segment of the lung, the distal airways. The S_{vA} and V_{vt} estimates by both the automated analysis and manual methods for the 14 young and aged normal dogs were close. The S_{vA} (Figure 2) of automated analysis was higher than the S_{vA} of the manual method because the automated method included some small vessels as airspaces. This fact is further substantiated if one considers the approximate 1% difference between V_{vt} estimated by both methods (Figure 3). Since the volumetric portion of vessels (V_{vv}) is about 1%, then correction of V_{vv} gives almost identical values for V_{vt} by both methods. With the new module "Image Editor" on the Quantimet 720, the operator can exclude vessels from the screen. Under such conditions the S_{vA} and V_{vt} should be identical to the manual method.

The control group from the 66 dogs showed small decreases in S_{vA} and V_{vt} from that predicted from the calculated regression line of age and S_{vA} or V_{vt} (Figures 2 and 3). This is probably a result of differences in perfusion fixation pressures of 30 cm H_2O (66 dogs) and 20 cm H_2O (14 dogs). With a perfusion fixation pressure of 30 cm H_2O all areas of the

lung appeared distended to TLC, but at 20 cm H_2O pressure the lungs infrequently contained some areas that appeared not to be entirely inflated. Although these areas can be excluded from quantitation, as they were with the quantitation of the 14 young and aged dog lungs, 30 cm H_2O pressure fixation is believed to provide the more reproducible measurements.

Considering the increased S_{VA} estimate by automated analysis, we expected our calculated S_A to be slightly higher than S_A calculated by manual methods. We obtained a value of 36.26 10^4 cm^2 for the S_A of the control group. Using the prediction formula for S_A, $S_A = (3.9\ W) - 3.7$, from a study using manual methods (Siegwart et al., 1971), we obtained a S_A = 31.84 10^4 cm^2 for the control group. Since we are only concerned with detecting significant changes in groups, we feel our stereologic parameters, though not absolutely in agreement with manual methods, are very reliable indicators of change.

The two methods of calculating the mean chord length from automated analysis data were comparable, but the method that uses a distribution of chord lengths has some important advantages. The $\hat{\mu}_x$ calculated from a distribution of chord lengths is not as sensitive to extremely long chords as is $\hat{L}_c$. The shifts in a distribution of chord lengths were easily detected as an area shift and the variance per slide was much less than that calculated by linear integration methods. Thus $\hat{\mu}_x$ was a more sensitive statistical

indicator of group differences than $\hat{L}_c$. The information provided by the linear integration method is, however, useful for identifying groups of animals in which there are individuals with an increased number of large chords. These individuals are the ones that should receive closest scrutiny by the pathologist as being more likely to have focal changes which provide the basis for evaluation of pathogenesis.

The chord distribution program has the advantages of requiring 1/2 the time for analysis and computer processing as compared to the pattern recognition program. It requires only a few modules on the image analyzer and provides an acceptable estimator of surface density compared to the manual method. The architectural continuity of the distal airspaces is lost in severe pulmonary emphysema and we feel the pattern recognition program would not be able to accurately classify those abnormal airspaces. Architectural continuity is not a limitation for the chord distribution program. By scanning the computer print-out sheet of either program from a slide (20 fields) it is possible to determine if the lesion is focal or diffuse. If it is focal, i.e. only a few large values, it may not be significant enough to cause a quantitative difference for a slide or an animal in any of our programs, but the computer print-out sheet will key the pathologist to the presence of a lesion. The pathologist can use this information as a screening method to select animals and lobes of animals for detailed microscopic evaluation.

The control and one exposed group were quantitated using pattern recognition. The method proved very useful in classifying the distal airways (alveoli, alveolar sacs, and alveolar ducts-respiratory bronchiole). The method of classifying a distal airspace was dependent on its architectural pattern. Even though enlargement was significant in the alveolar ducts of the NO_2 high group, the architectural pattern was intact enough to allow classification by a P^3/A shape factor.

The significant decreases in V_{va} (35%), N_{va} (19%), N_{vas} (29%), and V_{vt} (17%) suggest a loss of alveoli and alveolar sacs coupled with destruction of the interalveolar septa. The concomitant increases in V_{vad} (40%), A_{ad} (36%), D_{ad} (17-26%) indicate enlargement of alveolar ducts and respiratory bronchioles. We assumed that the alveolar ducts and respiratory bronchioles did not significantly enlarge in length because we observed no significant change in V_{vas}, A_{as}, or D_{as}. Since no significant change was observed in the remaining alveoli and alveolar sacs as evidenced by A_a, A_{as}, D_a, and D_{as}, we believe the enlargement of alveolar ducts is best explained by a loss of alveoli and alveolar sacs within and adjacent to alveolar ducts. Our observations using the scanning electron microscope confirmed the centriacinar location of the lesion. Respiratory bronchioles showed dilatation and associated interalveolar septa an increase in the number of fenestrae and pores of Kohn. Second and third order respiratory bronchioles were not

excluded from quantitation because of their ubiquitous location in the parenchyma, thus their dilatation was recorded with the alveolar duct segment. It is not difficult to conceive of adjacent alveolar damage when one considers the extensive amount of collateral ventilation that results from the numerous pores of Kohn in the interalveolar septa of the dog lung. Our observations on the predominant centriacinar location of the lesion are in agreement with previous studies of lifetime exposures of rats to NO_2 (Freeman et al, 1968; Haydon et al., 1965).

Pattern recognition was useful in classifying airways, localizing lesions, and determining whether all elements of the distal airways are involved and to what extent they are altered. The absolute values estimated by pattern recognition closely approximated those values calculated for dogs by Siegwart et al. (1971). For example we calculated a $D_a = 99 \times 10^{-4}$ cm, while they graphically calculated a $D_a = 103 \times 10^{-4}$ cm for dogs of the same weight.

The shape factors for counting the number of alveoli and alveolar sacs plus ducts per unit volume introduced by Weibel (1963) have been questioned in a study that compared estimates of the number of alveoli and alveolar sacs and ducts from two-dimensional planimetric measurements and from measurements of three dimensional reconstructions of human lung parenchyma (Hanson and Ampaya, 1974). They found no valid method for estimating the

number of alveolar sacs plus ducts from a thin section, but they did not question the estimation of S_{VA} by the mean linear intercept method. We concluded from their evidence and from our evaluation of the methods tested in this study that all of our methods are useful for detecting comparative differences; however, the chord distribution method provides the most sensitive test for differences among large groups of animals with abnormally enlarged distal airspaces.

By all methods of evaluation the greatest enlargement was observed in the NO_2 high and SO_X groups and slightly less in I + SO_X, NO high, and I groups. Our SEM observations of these groups confirmed the centriacinar location of the lesion accompanied by tissue destruction.

Morphometry has not been used for the quantitation of the upper airways in this study. Such an application is required to explain the decrease in V_{VP} in the NO_2 high group.

It has been shown that the chord sizing and pattern recognition programs of automated analysis closely approximate sterological parameters derived by established manual methods. Those programs coupled with computer processing have proven to be useful morphometric tools for statistically confirming the presence of subtle lesions in the distal airspaces of a large number of lungs from dogs altered by experimental conditions.

ACKNOWLEDGEMENTS

This study was supported in part by Contract No. 68-02-0323 from the Environmental Protection Agency and Grant No. ES 00628 from the National Institutes of Health. We thank Dr. Jerry Gillespie for reviewing the manuscript and Mrs. Donna Consiglio for typing the manuscript.

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AMRL-TR-75-125

PAPER NO. 5

METHODS TO EVALUATE THE TOXICITY OF NOXIOUS GASES AND AIRBORNE PARTICULATES ON PULMONARY BACTERIAL DEFENSE SYSTEMS

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INTRODUCTION

Methods to evaluate the toxicity of the airborne chemicals (gases and particles) are of the utmost importance to society. Increasing numbers of potentially toxic contaminants are at present entering the atmosphere as a consequence of otherwise beneficial industrial processes. Accordingly, information is essential to assess the need for control strategies to protect occupational workers and the public from contaminant induced injuries. Traditionally, toxicological studies have utilized physiological, pathological, and biochemical measurements to assess toxicity. Recognition of the association between exposure to noxious gases [ozone (Goldsmith, 1968; Bates, 1972), nitrogen dioxide (Lillington, 1974; Goldstein, 1975; Ramirez and Dowell, 1971), sulfur dioxide (Goldsmith, 1968; French et al., 1973), or airborne particulate sulfates (French et al., 1973), silica (Tepper and Redford, 1970), asbestos (Selikoff et al., 1972)] and the development of respiratory infection has resulted in the use of microbiological or immunological parameters to evaluate toxicity. This article reviews methods used to assess these defense parameters, their sensitivity in detecting abnormality, and the extent to which these tests have been applied in toxicology.

MUCOCILIARY FUNCTION

The mechanical removal of bacteria from the lung is a primary means of pulmonary protection against bacterial infection (Kilburn, 1967; Green, 1970). Three mechanisms of varying capabilities participate in this process - the mucociliary system which removes bacteria from the tracheobronchial

tree, an alveolar system which primarily removes particulates but may also remove bacteria from the alveoli, and cough reflexes which extricate inspissated secretions from bacteria from all pulmonary regions (Green, 1970). The available evidence indicates that of the three, mucociliary transport is the most important. The mucociliary system extends from the nares to the terminal bronchioles (Kilburn, 1967). Ninety percent of bacteria that deposit along its surfaces are transported from the lung within one hour (Green, 1968). In contrast, the alveolar system requires days to remove intraalveolar materials. Because of the rapidity of bacterial proliferation, such slow rates are ineffective in preventing bacterial infection (Green, 1968). The significance of cough mechanisms for maintaining pulmonary sterility is not known. Cough is of undoubted importance when the lung is already diseased. Whether cough also compensates for ineffective mucociliary removal in the normal lung has not been determined. Depression of the cough reflex may ultimately be shown to be a factor in reducing pulmonary resistance to infection. However, until such data appear, the significance of alterations in cough frequency and intensity as regards infection are unevaluatable. In accordance with these considerations, this section will confine itself to a discussion of the normal physiological relationships which govern mucociliary removal of intrapulmonary bacteria, the effects of toxic substances on these relationships, and experimental systems that measure toxin induced mucociliary dysfunction.

TRACHEOBRONCHIAL ARCHITECTURE

The anatomy of the nasopharynx and the tracheobronchial tree serves as the first line of defense against bacterial entrance into the lung. Architecturally, the system is a series of bifurcating tubes of ever-diminishing caliber. This configuration allows certain aerodynamic generalizations concerning sites of bacterial deposition. Since many factors in addition to anatomic considerations interact to determine particle movement (inertia, velocity, diffusion, gravity, respiratory frequency, tidal volume), these generalizations apply only for the majority of particles of a given size. Bacterial particles of 0.5 to 2.0 μ in diameter usually traverse the bronchi via Brownian movement and settle in alveolar regions. Larger particles of 2.0 to 10.0 μ in diameter are influenced more by gravity than by Brownian movement. These bacterial particles often settle within the tracheobronchial tree (Green, 1970). Bacterial particles above 10 μ in diameter tend

to sediment almost immediately in the nasal or pharyngeal cavity. Because the longest diameter of most bacteria is 1.0 to 2.0 μ, single or paired bacteria are likely to reach the alveoli where they are relatively safe from mechanical removal; small groups of bacteria tend to settle along tracheobronchial surfaces where they are amenable to rapid removal and bacterial clumps are usually too large to enter the lung.

TRACHEOBRONCHIAL MUCOSA, MUCUS, AND MUCOCILIARY MOVEMENT

The cellular components of the mucociliary system are goblet, clara, and ciliated epithelial cells and apocrine glands (Kilburn, 1967). Thegoblet and clara cells and the apocrine glands continuously produce large amounts (0.1-0.3 ml/kg body weight/24 hr) (Toremalm, 1960) of a sticky, viscous mucus which serves as the backbone of the mucociliary system. This complex fluid has a surface layer with gel-like properties and underlying layer which behaves like a sol (Kilburn, 1967). Bacteria that come into contact with mucus are trapped by its adhesiveness. Because of these physico-chemical properties, mucus and attached bacteria move as a single layer upward against gravity. Ciliated epithelial cells provide the force for this purposeful movement. The cilia that line the free surface of these cells beat synchronously to propel the overlying mucus at rates of 10 to 20 mm/min (Toremalm, 1960).

MECHANISMS OF INJURY

Even though the mucociliary system is continuously exposed to the atmosphere, environmental injury is uncommon in the absence of technology. A few natural perturbations (extremely dry or cold air, allergens, viruses) can inhibit mucociliary function by hardening the mucus layer, constricting bronchioles or destroying mucosal cells (Dalhamn, 1956; Kra Jina, 1964; Walsh et al., 1961; Vaughan et al., 1973). The numerous man-made insults (cigarette smoke, industrial particulates, gaseous pollutants, etc.) are much more important causes of mucociliary dysfunction. Exposure to these toxins damages cilia, ciliary cells, and mucus producing cells (Kensler and Battista, 1966; Dalhamn and Sjoholm, 1963; Ballenger, 1960). Such insults result in dyssynchronous ciliary movements, the production of excessive quantities of mucus, and retarded rates of bacterial clearance with an increased susceptibility to pulmonary infection.

MEASUREMENTS OF MUCOCILIARY FUNCTION

Mucus

Methods exist for determining the amount of mucus produced, its physico-chemical characteristics and its biochemical consistency. Mucus formation can be measured by collecting respiratory secretions from an anesthetized animal via an intratracheal or endotracheal cannula (Boyd and Ronan, 1942; Perry and Boyd, 1941; Boyd, 1970). The quantity of secretions collected over defined time periods allows calculation of hourly or daily rates of formation. The rate in normal animals is then compared with corresponding rates for animals that have received test treatments. It is also possible for the animal receiving the treatment to be its own control. Important sources of error exist in these techniques. The need to anesthetize and to intubate the animals introduces artifacts of mucus formation (anesthesia) and extraneous substances (intubation). A more physiological method for obtaining respiratory secretions has been described by Wardell and associates (1970). A 5 to 6 cm segment of the cervical trachea of a dog is separated and formed in situ into a subcutaneous pouch. The isolated system functions for months in a normal fashion. Milliliter quantities of tracheal mucus can be collected at intervals throughout this period. Studies with this experimental model have shown that exposure to sulfur dioxide alters the rate of mucus production (Litt, 1974).

The rheologic or flow properties of mucus can be measured with various types of viscometers (Davis and Dippy, 1969; Lieberman, 1968). It is worth emphasizing that considerable technical expertise is required to perform these delicate measurements. Mucus is subject to sheer degradation and only the gentlest of handling will prevent its breakdown. Furthermore, the test stresses must be similar in magnitude to intrapulmonary ones, or the results will not be biologically significant. When properly performed, viscoelastic measurements provide valuable information, as increases in viscosity or elasticity are associated with reductions in mucociliary function (Litt, 1970).

The concentrations of individual constituents of mucus (acid polysaccharides, neutral mucopolysaccharides, sialic acid, and sulfonated compounds) can be measured by various biochemical techniques (Jakowska, 1963; Lamb and Reid, 1969). At present, however, the value of these measurements is uncertain since relationships between chemical concentrations and mucociliary function have not been established (Reid, 1970).

Ciliary Activity

Much more is known about ciliary activity than about mucus. Ciliostasis has been used as a bioassay for the detection and assessment of hazardous agents for years (Dalhamn, 1956; Hilding, 1957; Dalhamn, 1964; Dalhamn and Rosengren, 1968). These bioassays are performed in vitro by removing a section of trachea, visually measuring changes in ciliary rate, and then comparing either the rate change or the time to cessation of ciliary movement with similar values in specimens that have received the test treatment. Numerous technical variables are inherent in the methodology (Donnelly et al., 1974). Observer error occurs in judging ciliary rate or ciliary cessation. Ciliary rates differ among animals of the same species and even in the same animal at different time periods. Lastly, difficulties can occur in reproducing treatment procedures. Because of these variables, statistical evaluations using blind protocols and randomized testing are a necessary part of these experiments (Donnelly et al., 1974).

Errors in visualization can be minimized by using nonobserver methods for measuring ciliary rates. Ciliary activity can be continuously filmed (Dalhamn, 1956; Proetz, 1932; Dalhamn, 1970), or the rate of flickering can be counted photoelectrically (Dalhamn and Rylander, 1962) or stroboscopically (Rivera, 1962; Gray, 1930; Jennison and Bunker, 1934; Brokaw, 1966). Filming appears to be the most reliable of these methods. Very accurate measurements of ciliary rates can be made by means of high speed photography. Preliminary observations with photoelectric methods indicate its probable future utility (Dalhamn and Rylander, 1962). Stroboscopy has been successful in measuring slow ciliary frequencies in non-mammalian systems (Rivera, 1962; Gray, 1930; Jennison and Bunker, 1934; Brokaw, 1966). However, attempts to use this technique to record more rapid mammalian rates of ciliary movement have not had the same success (Dalhamn, 1970).

Methods are also available for observing in vivo the rate of ciliary movement (Dalhamn, 1970). A microscope with a vertical light is attached to the trachea via a tracheostomy. This system allows measurement of ciliary function while the animal is exposed to noxious gases or particulates.

Mucociliary Transport

Mucociliary transport rates are determined by visually monitoring the rate of movement of particles placed within an excised tracheobronchial system (Barclay and Franklin, 1937), or an incised and externalized in situ system (Lightowler and Williams, 1969; Laurenzi et al., 1969; Albert et al., 1967; Morrow et al., 1967). More physiological measurements can be obtained by externally monitoring the movement of inhaled radioactive particles in intact animals and in humans (Lourenco et al., 1971; Thomson et al., 1973; Anderson et al., 1974). The visual methods which require less expertise and costly equipment have been used in most studies. In these experiments the progress of carbon particles, India ink or graphite has been measured microscopically within murine, feline, or canine tracheas (Lightowler and Williams, 1969; Laurenzi et al., 1969; Albert et al., 1967; Morrow et al., 1967). Since all particles do not move at the same rate, arbitrary end points such as the fastest rate of transport are recorded (Albert et al., 1967). Prolonged exposure to levels of air pollutants much above ambient impair these rates of particle movement (Kra Jina, 1964; Lightowler and Williams, 1969; Morrow et al., 1967).

The intrapulmonary movement of radioactive particles (^{198}Au-Fe_2O_3, ^{51}Cr-Fe_2O_3, $^{54}Mn\,O_2$) can be determined by externally monitoring radioactive events (Lourenco et al., 1971; Thomson et al., 1973; Anderson et al., 1974). These elegant techniques require a source of uniformly sized gamma emitting particles and an Anger Camera or similar type of measuring device. The human subject or the experimental animal inhales the radioactive particles from an aerosol generator. Sequential measurements of particle location are made with sodium iodide crystals. Since the particles distribute throughout the lungs, alveolar as well as tracheal clearance rates can be determined from the radioisotopic scans. These techniques have a number of potential advantages for studying air pollution. The methods are physiological, nonhuman primates can be studied, the animal serves as his own control, and clearance rates are measured precisely and sequentially over prolonged periods of time.

ALVEOLAR MACROPHAGE SYSTEM

The distal alveolar regions of the lung are protected against bacterial infection by the alveolar macrophage system (Green, 1970; Bowden, 1971). These ubiquitous pulmonary phagocytes are dispersed throughout

the alveoli in a manner which allows them to intercept invading bacteria within minutes after their entry into the lung (Goldstein et al., 1974). The bactericidal armamentarium of the macrophage inactivates and degrades the ingested bacteria in the ensuing two to four hours (Cohn, 1963; Gill and Cole, 1965; Elsbach et al., 1973). The efficiency of this phagocytic system maintains the sterility of the alveolar region despite the constant introduction of microorganisms (Green, 1970; Green, 1968; Kass et al., 1966).

The extraordinary ability of the macrophage to seek out, ingest and inactivate invading bacteria results from the integration of a number of complex biophysical reactions (Stossel, 1974). Phagocytes are attracted to bacteria by chemotactic factors that are elaborated by the bacteria themselves, or are formed from the interaction of bacteria and host tissues (Stossel, 1974). Simultaneously, serum opsonins attach to the bacterial cell wall rendering the microorganism susceptible to phagocyte ingestion. This opsonizing process is extremely important as phagocytes that are surrounded by equally palatable particles will selectively ingest ones that have been opsonized (Cohn, 1968). Once ingested, bacteria are internally isolated within phagosomes (Weissman et al., 1972). Microbiocidal enzymes that were in inactive states (secondary lysosomes) are activated and then fuse with the phagosome, to form the phagolysosome. This process allows the delivery of highly toxic enzymes to the operational site without subjecting the cell's cytoplasm to potentially injurious effects (Stossel, 1974; Weissman et al., 1972). Although the exact role of each of the many enzymes and toxic substances which participate in intracellular bacterial inactivation is not known, the available evidence suggests that lysozyme, catalase, hydrogen peroxide, and malonyldialdehyde, a catabolite of lipid peroxide with antibacterial activity, are among the more important bactericidal substances (Stossel, 1974; Weissman et al., 1972; Gee, 1970).

The complexity of the above sequence of phagocytic events provides numerous potential sites for an environmental toxin to interfere with phagocytic function. The toxin can impair chemotactic attraction by destroying chemotactic substances or by reducing phagocytic mobility secondary to edema formation. The toxin can inhibit the ingestive process by damaging the phagocytic membrane. Lastly, the toxin can damage the cell itself or the enzymatic systems involved in bacterial inactivation and degradation.

Experimental Systems

Methods for the evaluation in vivo of the intact pulmonary antibacterial system of rodents (mice, rats, guinea pigs) have been developed (Goldstein et al., 1974; Laurenzi et al., 1964; Green and Goldstein, 1966). Briefly, rodents are infected with aerosols of test bacteria. The infected animals are sacrificed immediately after infection and at four hours thereafter. The lungs are excised to determine the numbers of viable bacteria by pour plate techniques. The rate of bacterial clearance can be determined by comparing the numbers of viable bacteria at each time period. If the bacteria are radiolabelled, comparisons of radioactivity for the two time periods can be used to assess the rate of mucociliary removal of the inhaled bacteria (Green and Goldstein, 1966). Histologic determinations of the intra or extracellular locations of the intrapulmonary bacteria at the two time periods reveals the rate of bacterial ingestion by pulmonary phagocytes (Goldstein et al., 1974). The effects of exposure to ozone, nitrogen dioxide and sulfur dioxide on pulmonary antibacterial systems have been studied with these experimental methods, (Goldstein et al., 1974; Goldstein et al., 1971; Goldstein et al., 1973; Ehrlich, 1966). According to the data obtained, exposures to relatively low levels of ozone and nitrogen dioxide depress intrapulmonary bactericidal function (Goldstein et al., 1971; Goldstein et al., 1973; Ehrlich, 1966). Tests with ozone also show that the depression in bactericidal function is due to severe impairments of intrapulmonary killing and lesser impairments of bacterial ingestion by the alveolar macrophage (Goldstein et al., 1974). It should be noted that when a pathogenic organism (Klebsiella pneumonia) is used in these systems, death occurs as a consequence of the diminished intrapulmonary bactericidal activity (Ehrlich, 1966).

Chemotaxis

The above cited rodent system allows the measurement of chemotaxis and ingestion in combination (Goldstein et al., 1974). Experimental systems which measure in vivo rates of chemotaxis alone, have not been developed. Rates of chemotaxis can be measured in vitro by removing pulmonary macrophages and then testing them in a Boyden chamber (Boyden, 1962). The macrophages can be obtained in a high state of purity by lavage techniques (Ward, 1972). The cells are then placed in the chamber and allowed to migrate through the micropore filter toward an attracting media (Boyden, 1962). These assay systems are sufficiently standardized to quantitatively assess chemotactic function (Ward, 1972; Snyderman et al., 1972). The effect of pollutants can be determined either by exposing the intact animal and then removing the phagocytes, or by exposing phagocytes that have already been removed.

Bacterial Ingestion

A number of in vitro tests have been developed to measure the rate of bacterial ingestion by alveolar macrophages (Coffin et al., 1968; Gee et al., 1970). Bacteria can be injected intratracheally into animals that have been exposed to a pollutant and alveolar macrophages lavaged and the number of intracellular bacteria counted (Coffin et al., 1968). Comparison of the number and distribution of intracellular bacteria in macrophages from treated animals with the values for control animals is a measurement of the effect of the pollutant on phagocytic ingestion (Coffin et al., 1968). Alternatively, macrophage monolayers can be prepared and tested with bacterial suspensions. At varying intervals, the bacteria are washed from the monolayer, the macrophages are stained with a Wright-Giemsa stain, and the number of ingested bacteria are determined by counting intracellular bacteria (Reynolds and Thompson, 1973). Staphylococcus epidermidis is often used as the test organism in these systems because of its unique susceptibility to lysostaphin (Tan et al., 1971). The addition of lysostaphin to a culture dish containing macrophages and staphylococcus epidermidis results in rapid lysis of extracellularly located staphylococci without injury to the macrophages or already ingested bacteria.

Bacterial Inactivation

The above in vitro systems for determining bacterial ingestion rates can also be used to measure rates of bacterial inactivation. Instead of histologically counting the number of ingested bacteria, the cell suspensions or monolayers are lysed and the numbers of viable bacteria are determined by pour-plate techniques (Reynolds and Thompson, 1973; Tan et al., 1971).

Enzymatic Measurements of Phagocytic Function

The concentrations of various enzymes and bactericidal substances within alveolar macrophages can be accurately measured with various biochemical techniques. In these in vitro systems, macrophages are lavaged from the lung following exposure to an air pollutant (Hurst et al., 1970). If, as is believed, these enzymes are important participants in intracellular bacterial inactivation, their inhibition is an adverse consequence of the pollutant exposure.

Histochemical techniques are also available for determining the concentrations of acid phosphatase, beta glucuronidase, and glucose-6-phosphate dehydrogenase (Barka and Anderson, 1962; Hayashi et al., 1964; Pearse, 1972). These methods can be used to determine in vivo the effect of pollutant exposure on concentrations of phagocytic enzymes in macrophages that contain ingested bacteria. Reductions in these enzyme concentrations may correlate with the inability of phagocytes to kill ingested bacteria following exposure to pollutants.

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AMRL-TR-75-125

PAPER NO. 6

DYNAMIC LUNG VOLUMES IN ANESTHETIZED ANIMALS

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INTRODUCTION

The dynamic lung volumes are the most popular and useful tests of human respiratory physiology. These volumes comprise all the volume and rate parameters commonly recorded during maximum inspiration and maximum expiration. Lung volumes of this type are basic to the measurement of numerous other pulmonary function tests and provide essential diagnostic information concerning ventilatory performance. The accuracy and validity of all test parameters depend on the subject's ability to perform certain breathing maneuvers.

The inability of animals to perform the breathing maneuvers required for many pulmonary function tests has greatly limited applied animal research in terms of sensitivity and comparability, relative to human testing. Such common human clinical tests as the forced vital capacity (FVC), forced expiratory volume/sec (FEV_1), and the maximum expiratory flow-volume parameters ($\dot{V}max$) have had no relatable counterparts in animal studies. Of significant importance here is that numerous investigators involved in human studies are determining that ventilatory functions, or more specifically ventilatory functions of the small airways, are early indicators of disease (Hyatt and Black, 1973). In addition, the most prevalent human respiratory disease is chronic obstructive pulmonary disease (Macklem, 1971). Certainly animal models for obstructive lung disease are needed (Macklem, 1971), so three are presented here for consideration: the cynomolgus monkey, guinea pig, and the rat.

Rationale of Tests Selected

Because of the flow limiting conditions which develop only during expiration, it is the forced vital capacity maneuver that is most essential to evaluate in determining the status of the conducting airways and surrounding lung tissue. In order to evaluate obstructive disease we have selected the $FEV_{0.5,\ 1.0}$ and $\dot{V}_{max}$ at various lung volumes during the maximum expiratory flow volume test. Limitations in peak flow relate to the resistance, primarily in the trachea and main bronchi. These airways are self-supported by cartilage rings and are not greatly influenced by volume change. The small airways, however, lack self-supportive structures and depend on radial traction provided by surrounding lung tissue; therefore, as the lungs deflate during expiration, they tend to collapse. This is the reason flow has effort independent limitations during expiration.

Restrictive patterns of disease can be studied by the use of the lung volumes and their ratios. The volumes directly obtainable are the inspiratory capacity (IC), vital capacity (VC) and expiratory reserve volume (ERV).

METHODS

Animal Preparation

Prior to testing, the animals were anesthetized with sodium pentobarbital at a dose of 35 mg/kg. Each species was fitted with an endotracheal tube as large as possible. The monkeys in the 3-7 kg range can handle an 18 to 22 F cuffed endotracheal tube which is commercially available. The guinea pigs and rats were fitted with polyethylene tubing which was slightly tapered by pulling the ends of the tubing while passing it through a flame. The tapered ends were trimmed and an appropriate length was gently wedged into the trachea. The void of the pharynx and mouth was then filled with fresh plastic dental impression cream to insure sealing. After one minute the cream hardens but is easily removed after the testing. Essentially, all rats and guinea pigs tolerate this procedure and can be re-employed.

Pulmonary Function Testing Apparatus

A combination plethysmograph-respirator provided the direct motivating force required to obtain the breathing maneuvers necessary for this type of pulmonary function testing in the anesthetized animal. The basic method employed is similar to that used in an external tank respirator; however, a hydraulic control system enables the operator to completely control inspiration, expiration, breath-holding, and breathing rate within the anatomical and physiological limits of the animal (Moorman, 1975).

Figure 1 is a diagram of the plethysmograph-respirator. The animal is situated in a stainless steel airtight chamber ventilating through a pneumotachograph housed in the side. The top of the chamber is fitted with a rubber diaphragm which is operated by a small displacement hydraulic cylinder. By raising the diaphragm, the pressure surrounding the animal decreases, causing inspiration. By lowering the diaphragm, the pressure increases, resulting in expiration. Plus and minus 70 cm H_2O pressure is used to achieve maximum inspiration and expiration. To insure that we achieve maximum volumes and flows, we have monitored intrathoracic pressures during the imposed maneuvers. During a forced expiration, the intrathoracic pressures are instantaneously raised to 35 cm H_2O which has been shown to produce flow maxima by investigators studying IVPF curves. The anesthetized animals do not resist these breathing maneuvers because of the apnea produced on inflation as a result of the Hering Breuer reflex.

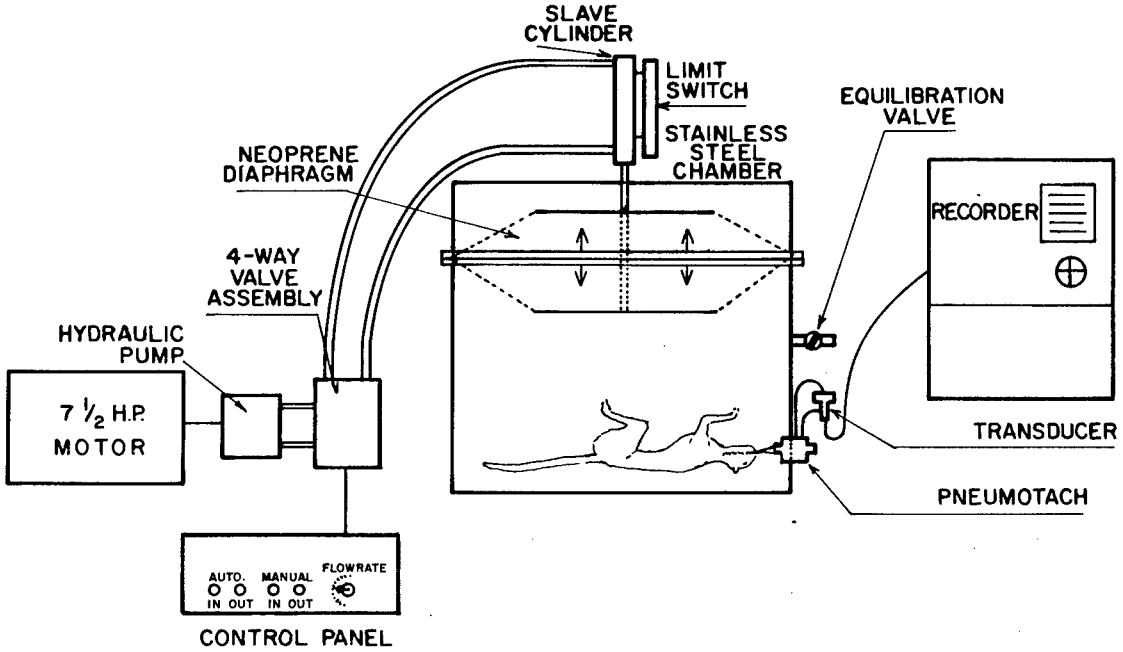

Figure 1. Diagram of Plethysmograph-respirator.

NORMAL VALUES - SPECIES COMPARISON

Representative normal values for the parameters studied are presented in Tables 1, 2, and 3 for the monkey, guinea pig, and rat, respectively. As one might predict, the larger animals demonstrate larger volumes and flow rates; however, by expressing the flow rates in terms of VC's per second (which adjusts for differences in animal size), the smaller species outperform the larger ones. This species difference increases as flow rates at smaller lung volumes are compared. This demonstrates that guinea pigs and rats do not reach effort independent flow limitation until the last 20% of their FVC, whereas man and monkey reach flow limitation at much higher lung volumes (1/2 to 1/3 FVC).

TABLE 1. DYNAMIC LUNG VOLUMES OBTAINED IN 12 CYNOMOLGUS MONKEYS (NORMAL VALUES)

	MEAN	S.D.±	RANGE
WEIGHT (KG)	5.06	1.81	2.80- 7.60
INSPIRATORY CAPACITY (cc)	259.00	92.00	140.00-363.00
VITAL CAPACITY (cc)	412.00	105.00	285.00-520.00
EXPIRATORY RESERVE VOLUME (cc)	153.00	38.00	112.00-223.00
PEAK EXPIRATORY FLOW (cc/SEC)	720.00	212.00	473.00-1056.0
PEAK EXPIRATORY FLOW (VC/SEC)	1.77	0.34	1.24- 2.36
FORCED EXPIRATORY VOLUME (0.5 SEC %)	88.20	4.90	80.00- 95.00
FORCED EXPIRATORY VOLUME (1.0 SEC %)	94.20	3.67	89.86-100.00
MAXIMUM EXPIRATORY FLOW-VOLUME CURVE			
V MAX 25% VC (cc/SEC)	482.00	229.00	260.00-843.00
V MAX 25% VC (VC/SEC)	1.18	0.43	0.58- 1.77
V MAX 10% VC (cc/SEC)	101.00	52.00	47.00-181.00
V MAX 10% VC (VC/SEC)	0.28	0.21	0.13- 0.76

TABLE 2. DYNAMIC LUNG VOLUMES OBTAINED IN 12 GUINEA PIGS (NORMAL VALUES)

	MEAN	S.D.±	RANGE
WEIGHT (KG)	1.30	0.13	1.10- 1.60
INSPIRATORY CAPACITY (cc)	29.25	4.87	20.40- 38.80
VITAL CAPACITY (cc)	33.05	4.76	26.09- 41.80
EXPIRATORY RESERVE VOLUME (cc)	2.07	1.20	0.27- 3.68
PEAK EXPIRATORY FLOW (cc/SEC)	137.71	30.18	90.44-198.00
PEAK EXPIRATORY FLOW (VC/SEC)	4.55	0.80	3.58- 6.43
FORCED EXPIRATORY VOLUME (0.5 SEC %)	95.27	2.15	90.70- 97.85
FORCED EXPIRATORY VOLUME (2.0 SEC %)	99.30	0.72	97.90-100.00
MAXIMUM EXPIRATORY FLOW-VOLUME CURVE			
V MAX 25% VC (cc/SEC)	53.52	16.18	35.67- 84.00
V MAX 25% VC (VC/SEC)	1.83	0.70	1.20- 3.23
V MAX 10% VC (cc/SEC)	21.62	7.88	10.45- 33.25
V MAX 10% VC (VC/SEC)	0.70	0.29	0.39- 1.24

TABLE 3. DYNAMIC LUNG VOLUMES OBTAINED IN 16 RATS (NORMAL VALUES)

	MEAN	S.D.±	RANGE
WEIGHT (KG)	0.252	0.032	0.196- 0.305
INSPIRATORY CAPACITY (cc)	9.100	1.110	7.080- 0.730
VITAL CAPACITY (cc)	10.810	1.460	7.500- 12.810
EXPIRATORY RESERVE VOLUME (cc)	1.760	0.540	0.420- 2.350
PEAK EXPIRATORY FLOW (cc/SEC)	62.360	8.570	48.550- 77.210
PEAK EXPIRATORY FLOW (VC/SEC)	5.800	0.740	4.830- 7.430
FORCED EXPIRATORY VOLUME (0.5 SEC %)	96.020	1.900	92.100- 99.100
FORCED EXPIRATORY VOLUME (1.0 SEC %)	98.560	1.650	93.400-100.000
MAXIMUM EXPIRATORY FLOW-VOLUME CURVE			
V MAX 25% VC (cc/SEC)	19.270	7.220	9.480- 35.620
V MAX 25% VC (VC/SEC)	4.620	1.110	2.160- 6.010
V MAX 10% VC (cc/SEC)	5.170	0.970	3.760- 6.780
V MAX 10% VC (VC/SEC)	1.660	0.670	0.440- 3.080

A comparison of the dynamic lung volumes of the three species presented along with representative values for man demonstrate the monkey's similarity to man. Table 4 presents the normal values for man and the three species presented. A comparison of the relative magnitude of VC to body weights (cc of VC/gms body weight) reveals that monkeys have larger VC's than rats or guinea pigs. The ratios for monkeys, guinea pigs and rats are 0.081, 0.025, 0.043 respectively. It was also noted that guinea pigs and rats have such smaller ERV's than monkeys. The ERV/VC ratios for monkeys, guinea pigs and rats are 37%, 6%, and 16%, respectively.

TABLE 4. COMPARISON OF NORMAL MEAN VALUES FOR MAN* AND THE 3 SPECIES STUDIED

	MAN	MONKEY	GUINEA PIG	RAT
WEIGHT (KG)	70.000	5.000	1.300	0.252
INSPIRATORY CAPACITY (cc)	3600.000	259.000	29.250	9.100
VITAL CAPACITY (cc)	4800.000	412.000	33.050	10.810
FORCED EXPIRATORY VOLUME (0.5 SEC %)	65.000	88.000	95.270	96.020
FORCED EXPIRATORY VOLUME (1.0 SEC %)	83.000	94.200	99.300	98.560
PEAK EXPIRATORY FLOW (VC/SEC)	1.390	1.770	4.550	5.800
V MAX 25% VC (VC/SEC)	0.330	1.180	1.830	4.620
V MAX 10% VC (VC/SEC)	0.070	0.280	0.700	1.660
VC/BODY WEIGHT	0.068	0.081	0.025	0.043
ERV/VC %	25.000	37.000	6.000	16.000

* NORMAL VALUES FOR MAN TAKEN FROM "THE LUNG" AND "HANDBOOK OF PHYSIOL. (RESP. II)"

APPLICATIONS

The application of the various animal species and test procedures described are useful in evaluating responses to experimental exposures in at least four general ways. First, such applications are essential in detecting similar early ventilatory changes characterizing many of the human exposure related diseases. This applies particularly to many irritant gases and respirable dusts which produce early obstructive changes even at very low concentrations. Second, they provide a useful tool for segregating the individuals within a treatment group which are responding to a toxic substance for further characterization by the biochemist, immunologist, and pathologist. This situation, characterized by some individuals responding within a treatment group while others do not, is very common in long-term low level exposures, yet frequently data treatment obscures this type of response by the use of statistical means. Third, these techniques can reveal the mechanisms of response such as large or small airways obstruction resulting from smooth muscle changes or restrictive dysfunction, resulting from fibrosis, tumors, or pulmonary vascular congestion. Fourth, the dynamic lung volumes are useful in monitoring serial changes which occur during the progression of disease because they do not require animal sacrifice. This may be extended to monitoring recovery or reversibility of toxic responses.

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OPEN FORUM

DR. TYLER (University of California, Davis): This morning the speakers presented a variety of ways of evaluating the effects of inhalation toxicology. We also heard earlier in the morning the necessity to get at basic mechanisms of injury if we are to extrapolate this information to man, so I think I'd like to ask which of these methods provide the most information which can be extrapolated to man.

DR. WILSON (University of California, Irvine): All the techniques presented have the potential of being applicable to man. Your question is which will be the most sensitive or which will be the most applicable.

DR. TYLER: My question was, "Which method determines the mechanisms of injury so that the mechanisms then can be assumed to be the same in man as in the animal so that one can extrapolate from animal experimentation to man much as we heard earlier this morning?" The mechanisms are extrapolatable, perhaps the effects are not.

DR. WILSON: I think the anatomic approach would have to be the first approach which would have to be used because there one can see where the lesion is and compare it to a similar lesion in man. The problem with pulmonary physiology is that it's not always directly applicable from animal to man because the number of generations of airways, the relative tapering of the airways, the defense mechanisms, and a number of other factors are different. On the other hand, I think once the anatomic lesions are identified then the physiologic techniques can be utilized to identify anatomic lesions without actually opening the chest. So I think I would like to start with an anatomic approach and then go on to the physiologic.

DR. PHALEN (University of California, Irvine): I think maybe the way I would have put your question is not what technique extrapolates best to man but what animal extrapolates best to man, and that is endpoint dependent. But I think that the answer is those that have been shown to extrapolate well to man. Where people have shown a correspondence between man and animal using a given technique, that method is probably good for extrapolation. I would like to see anatomical work done on more than one species as you are doing in your lab and much more actual direct relating of animal results to man by the people who do animal studies or the people who do human studies.

MR. MOORMAN (National Institute for Occupational Safety and Health): I think there are a lot of other answers to this question. First of all, it's not which technique. I think to be comprehensive, all of the techniques have their place. Certainly, to start off with an anatomical approach, I don't know that there are any anatomic techniques to demonstrate certain functional abnormalities. People look for techniques which correlate the two, and in some cases, they do; in other cases, I think it's a mistake to look for correlation. I doubt very much whether early obstructive changes can be attributed to an anatomical condition but there are changes in physiologic functions. Likewise, certain specific areas of the lung that have dust deposited in them may develop a specific lesion that isn't detectable by physiologic means. I think that question has to relate to what is the exposure. I think you have to direct the methodology to the type of exposure and the type of response you might expect. In the general sense, they are all necessary.

DR. DUNGWORTH (University of California, Davis): I think that sensitivity of the techniques is one of the points that Dr. Tyler was questioning. If we are looking for the most sensitive method of detecting something because of the heterogenous response of the lung, I have no doubt that it is a cellular biology method, that is ultrastructural examination and cellular physiology. One point here I think is important to be made wasn't made here by anybody this morning. There are, of course, a number of noninvasive techniques which rely on isolation of cells, and Elliot Goldstein did go into most of these. It's become increasingly apparent that we have to be sure what cells are doing in their normal environment wherein the phenotypic expression is modulated as usual and not as a genotypic expression represented by what the cell is doing in cell culture which can be greatly different. It is important to have the normal phenotypic expression by the local microenvironment. So I'd say cell biology methods introduce more than the standard morphologic techniques that most of us think about. I think that the pulmonary function parameters are particularly useful for some acute studies wherein there is a bronchoconstrictor agent. In these studies, there can be direct comparison of monkeys and of man at low exposure levels. There the pulmonary function parameter is sensitive. I think when we talk about chronic low level exposure studies that pulmonary function measurements are less sensitive than the morphologic and cell biologic measurements.

DR. WILSON: I stayed away from the whole idea of sensitivity. My own thought is that once you've established an anatomic lesion at some high level of pollution to prove that it's there and then reduce the level of pollution down to the point where no longer can an anatomical lesion be shown, then other techniques must be used to see whether an effect is present. Now, my own prejudice, as I say, is that either pulmonary function or biochemical techniques will show the lesions to be there even after clear-cut anatomic lesions are not there.

DR. BACK (6570 Aerospace Medical Research Laboratory): This question is for Dr. Phalen, and I think it may have something to do with the whole question we're talking about. This has to do with anatomical juxtaposition of different organelles within the lung and has to do with the differences in the biochemistry at each of the levels found throughout the lung. I would like to hear from Dr. Phalen to find out whether we're really looking at the correct animal model. Which animal most closely resembles man from an anatomical point of view and how does Reynolds number come into effect on the animal model we use?

DR. PHALEN: The Reynolds number is a dimensionless parameter that describes the ratio of two types of forces acting on particles: inertial forces and viscous forces. The idea is that if you're in a high Reynolds number area, the behavior of the fluid is turbulent, or the behavior of the particles is best described by the word "turbulent." If you're in a low Reynolds number region, you can think of your particle, for example, as being in molasses where the viscous forces are large and it has a tendency to follow air streams. With respect to Reynolds numbers, there is some fairly recent evidence that maybe that's not as an important parameter in the lung as was thought. Even in the region of the lung where Reynolds numbers are low and one would expect smooth laminar flow, air seems to have what is called secondary flow patterns that are produced by bifurcations. Before you really get fully developed laminar flow in a pipe, you have to go several pipe diameters. This doesn't really happen in the lung. Our morphometric studies showed, for example, that the length of the diameter ratio in tracheal bronchial tubes varies quite a bit between species. The shape of the flow divider probably becomes the important anatomical feature if you are worried about the detailed nature of air flow deep in the lung. A blunt flow divider disturbs air flow a lot more than a sharp flow divider.

There are species differences here. With respect to anatomical similarity, it depends on what you are interested in. If you are a trachea man, you are going to want an animal with a trachea similar to the human. If you are a small airway disease person, you are going to want an animal that either responds physiologically in the small airways like man or anatomically looks similar. I'm willing to say that the dog and the monkey have respiratory bronchial structures that, to me, look like human. The rat and the hamster and the mouse do not. Therefore, I wouldn't recommend using animals that don't have a respiratory bronchiole if you're studying a disease that affects the respiratory bronchiole in the human. So I think that it's not clear cut. The question of what is the best animal, I think has a simple answer. I think there isn't one. I think it's been amply demonstrated that man is also a poor model for man. The way I respond in a chamber may not have much to do with the way I'll respond out on the street. The way my wife responds to an inhaled material may have very little to do with what my son does. There are times when it can be argued that I'm not even a good model for myself because now I'm 35 years old and some day I'm going to be 45 years old. I don't now have small airway disease and someday I may. I think that question is the easiest one to answer.

DR. DUNGWORTH: I support what Dr. Phalen said. The only amplification is that when you said it depends on whether you're a trachea man or a small airway man, I think that most people looking at early chronic obstructive pulmonary disease are small airway people, you might say almost by definition. Just about every study which has demonstrated the experimental effects of an inhaled irritant has shown that the vulnerable or critical site is at the junction of the conducting airways and the acinous, that is, the region of the respiratory bronchiole. So I think it is important to have an animal in which the topography of this region, not only in the development of the respiratory bronchial and the terminal bronchial are similar to man but also in which the cell population types are as close to man as can be obtained. So I would support him on the comment that the dog and the monkey are better than the rat and other smaller rodents. I think we might say that the monkey is better than the dog but I would also agree that nothing is perfect.

DR. BACK: That's the point I wanted to make. We don't begin with a preconceived notion that a compound is causing an effect anywhere. We've got to find out where, indeed, that compound is acting; and if we're using the

wrong model to begin with, we don't become alveolar men per se or bronchial men per se. We have to follow our nose in terms of toxicology because we don't know where the compound is working.

DR. PHALEN: May I suggest that you use 8 or 10 animal species, all different, rather than 10 animals of the same kind.

DR. BACK: Well, I'm suggesting that depending upon the animal you use, you might find a completely different milieu of end organ changes. If a material is impinging on the bronchi, then it never gets to the alveoli.

DR. DUNGWORTH: I think we said first of all that we should examine morphology. We said you stand back and look at everything. We don't have any preset notion of where the damage is going to be. You sample widely. Nevertheless, in just about all species that have been exposed to irritant gases such as ozone, NO_2, and phosgene, the most critical damage is produced at the junction of the conducting airways and the acinous. This varies anatomically from species to species but repeatedly in rats, mice, hamsters, guinea pigs, dogs, monkeys and man, the damage tends to occur here. But you have to stand back and take the whole survey before you assume that. In fact you don't, you prove it.

DR. CROCKER (University of California, Irvine): When you set out to look at a compound and want to know if there is an effect anywhere in the lung, what method would you use? This is part of the question. I'd like to go even a step further back. What if you expose a whole animal to an atmosphere in which you do not know whether there is a lung effect at all? By what indication do you undertake any one of the more specialized studies that we have discussed this morning? Do you, for example, watch to see whether at a high concentration the animal shows unwillingness to breathe, a tendency to hold its breath or breathe fast or cough and only then say this may be a pulmonary irritant? Or if you have put it into a concentration where none of these occur, but the animal has other effects, CNS effects, for example, would indicate that there is pharmacologic activity, do you make a decision to go with or without additional lung studies? Now, all of us know that we need to do pathology on that animal. Therefore, there will be a morphologic start at some point. Would anyone care to describe the next step after the animal has been exposed and has shown a response which is not necessarily

pulmonary? When do you then decide which of the more special function studies to apply in analysis of a potential lung target response? Do I make my question clear? Do you wait until you can see something gross happening in the intact animal's response to the atmosphere, an irritant of the lung evidently being demonstrated in the way the animal acts and then you begin to look? Or do you make a judgment on the basis of the compound itself and the probability that it acts on the lung? Mr. Moorman, you're doing this sort of practical thing at NIEHS. When do you begin to do any of your studies on the lung and after what indications?

MR. MOORMAN: Well, I have a thought to preface this. Consider an exposure to 75 ppm of carbon monoxide. Where would you start to look for a response with this exposure? Compare that to 10 mg/m^3 of coal dust. There isn't any answer. I couldn't say that one technique should be employed without knowing more about what specifically you are talking about. Every study evolves and should evolve correctly into more definitive values.

DR. WILSON: As a physician, I can answer that one way. If the patient comes to you and he has no complaints at all, it's difficult to know what to do with him. You do a routine history and physical and get some lab work. That's sort of like an FDA protocol, isn't it. You do things then maybe something comes out of it, maybe something doesn't. But without any hints, it's very difficult to know what to do. If you did every possible test that you can do to a patient, and ask every question you could ask, you and he could be there for one year steadily and still may not find anything. So I think without any hints, it's very difficult to know where to start. Now if the question was asked, what about a possible pulmonary irritant in industry, I think I could answer that question. As I indicated earlier, I think there are 4 or 5 single breath techniques which are highly sensitive and which cover a large enough breadth of pulmonary functions, with which one could with fair sensitivity answer the question.

DR. CAVENDER (Becton, Dickinson and Company): It seems to me that the question which should be asked here and everyone is afraid to ask is this: Is there a suitable screening test that we can set up and absolutely say that this is the criteria, this is the NCI test or what have you for screening possible pulmonary irritants or toxins? As Dr. Dungworth has already said, if you look at irritant gases, the response that occurs in just about any

species is in the respiratory bronchioles. With things like sulfuric acid mist, different answers are obtained depending on which species you look at. So it seems to me that what we're trying to say is that with mutagenesis, for instance, a microbial test as a screening test is a possible way of getting rid of some of these 14,000 compounds of interest and center on a few that may be true mutagens in man. If that's the question we are asking, what protocol would you select? It's true, and I would agree with Dr. Wilson, that it's best in looking at a specific toxic compound to give it at a high enough dose to find where it affects morphologically or anatomically and then go back and look at it with some of these other tests later on. But if you are trying to scan the whole spectrum of possible compounds, it's a more difficult situation and you wouldn't get through with it in your lifetime or in all of our lifetimes put together. And so as a screening method, what would you suggest as an approach to looking for pulmonary lesions?

DR. DUNGWORTH: Obviously being a morphologist, I'm going to answer from a morphological point of view and not bother anymore with what I think is a self-defeating argument of who's more sensitive than the other. As far as what is usually practical, this means using small animals where a wide variety of such tests as we've been talking about this morning are either impractical or too costly. I suggest that if I were to set up a screening type of test, it would be to choose various dose levels. In classical toxicology and if we are talking about inhalation toxicology, to expose a couple of species of animals to those serial dose levels for 7 days, to kill them at that stage, and look at them at that stage by the methods we commonly use and I described this morning. To me that's the one central feature. We prefer the 7-day stage because a lot of things happen rapidly in the lung and at the end of 7 days, you've reached a fairly stable sequence of events. So it makes a nice time at which to compare the relative effect of different substances. You would have to compare the effects at different levels of the respiratory tract, not just assume. The 7 day is a very useful staging point because after that you either begin to get into adaptation in which case you allow the lesions to disappear or you get a very slow change into a chronic lesion.

DR. MUDD (University of California, Riverside): I'd like to change the subject a little bit. I'd like to make a comment on Dr. Goldstein's paper and then ask a question. Dr. Goldstein showed us pictures of macrophage

cells which after exposure to ozone had ingested bacteria but the macrophage cells were not capable of digesting them which indicates perhaps that ozone has passed this particular biological membrane without causing lysis at any rate and yet affected the enzymes inside. I think this is a little different than some of the presupposed ideas of some people who feel that ozone must affect the biological membrane directly because it usually contains a lot of unsaturated fatty acids. The question that I would like then to ask is this. Can the morphology methods such as scanning and transmission electron microscopy show us those changes in the macrophage cells in the absence of the added bacteria?

DR. DUNGWORTH: In our studies, even with very high levels of ozone, we found that there was a population of macrophages that remained unaltered, that is the same as the controls. There was a second population of equal number that appeared to be undergoing changes which probably meant that their kinetics were altered. Somewhat akin to your question, not in the macrophage, is that with EM cytochemistry on acid phosphatase, Bill Casselman showed that there was what is referred to as labelization. What do you mean by labelization? Let's say that the enzyme products were scattered in the cytoplasm of cells and there was no ultrastructural evidence of damage. That is, the mitochondria, the ribosomes and endoplasmic reticulum appeared normal even though the reaction product was diffused throughout the cell sap. So there you have a cytochemical method that showed an effect that was not detectable by morphologic ultrastructural changes.

DR. TYLER: I think the only thing I would like to add relative to histo- and cyto-chemistry is that we really have to be cautious with these procedures. I think they can provide a lot of information as Dr. Goldstein has shown. I think one possibility is that indeed the membranes are altered by the ozone in this case. And this alters the permeability relative to the substrate and the capture agent. I think this may explain some of the things which you are seeing. I'm not sure they are the explanation, but I think it's one possibility which one should consider. If, indeed, a membrane has changed so that it is less permeable rather than more permeable, that's one possibility.

DR. CROCKER: I don't know whether if Dr. Goldstein were here he would make a point about the possible degree of sensitivity of the bacterial digestion model, but it might be to Dr. Cavender's point to ask if anyone would consider an in vitro method that would follow from what Dr. Goldstein was saying about the macrophage function and its depression by exposures to atmospheric pollutant materials. Now, for example, if one wanted a very simple way at this point to recognize certain effects of ozone, once having established these through a series of observations that have made it possible to see a macrophage function effect, it would now be possible to make some of those studies with a macrophage alone at a level of sensitivity that would be equivalent to the intact animal. Would anyone suppose that this is an in vitro method for scanning other gases in the fashion that Dr. Cavender refers to methods of an in vitro sort for screening mutagens?

DR. DUNGWORTH: I would imagine that it's possible to set up a relative potency test using the alveolar macrophage as the test subject, and on that basis to get a relative grading of amounts. This would be for what it would be worth in that in vitro system. The correction factor from in vitro to in vivo brings in all sorts of unknown imponderables, like if you have 0.02 ppm ozone in the air above the macrophages in the culture system, what does this mean relative to breathing 0.1 ppm ozone through the nose? This type of concentration gauge, as far as I know, just isn't available. But that would have to go into a possible correction factor. It would probably be a horrendously complex thing to try to derive. But if you wanted to relate a substance to one other, then the macrophage system would be one quick screening.

DR. S. D. LEE (Environmental Protection Agency): I'd like to ask Dr. Dixon a question. If I heard you correctly, you seem to feel that effects of sulfuric acid mist have been documented well enough. If so, based on your information, what level would you recommend as the environmental standard and based on what kind of information?

DR. DIXON (National Institute of Environmental Health Sciences): The references I made were merely to a large number of research projects going on the the Institute. This particular work is not mine, but perhaps Dr. Cavender can comment on it. NIEHS is not in the regulatory business, and we're not setting standards. I'm obviously ducking your question, but I have no information on the setting of such standards as you're talking about.

DR. CAVENDER: Perhaps we're preempting Trent Lewis because he's going to be talking about TLV's and so forth very shortly, and sulfuric acid mist is one in which the TLV has been decreasing each year. I presume the rationale for the question is some of the new information that is available on sulfuric acid mist that refers to the finding that smaller particles are more toxic than larger particles. This, again, goes to a particular model that's been used and, perhaps abused, one to which I was alluding earlier. The fact that the guinea pig and the irritant response model has been abused to a large extent because in one sense the irritant response that you see in the guinea pig is due to particle number. And so if you drop your particle from one micron down to a tenth of a micron you are going to see a greater irritant response. But if you keep your mass loading the same and have it so that you will see a morphological change in the lung, you will find that there is very little difference between a tenth micron particle and a one micron particle. So some of these complications that we get in trying to pick an ideal model that will do everything is one that has not been answered yet and we still don't have an answer.

DR. CROCKER: Dr. Lee, did your question have background to it that might lead to your mentioning some information of your own?

DR. LEE: Well, I feel that there isn't enough information available at this point and what is available is too controversial to set any standards. Maybe I overinterpreted what Dr. Dixon mentioned earlier but it came over to me that there is enough information to set such standards.

DR. CROCKER: Yes, I think we all agree that the setting of sulfuric acid and sulfate standards is still in the very active stage of investigation.

MR. HYDE (University of California, Davis): Just to bring the discussion back to the original question about which animals or anatomical techniques are most useful in evaluating lesions in the lung, I found in my studies that the morphometric methods allow one to statistically test lesions in various lobes and regions within a lobe where they cannot be visualized with the scanning electron microscope or the light microscope or pulmonary function data do not show that there are significant changes there. So I think that quantitative morphometry methods have been overlooked simply because of the time consuming nature that has been associated with them. I think that we should look a little more closely at morphometric methods for setting air pollution standards.

DR. CROCKER: Would you care to take that a step further and say that would be a desirable or even acceptable fashion for routine examination of lungs? You did a very extensive study that was by no means a routine one. It was on a series of very valuable animals at the end of a long program of study. Had that not been done you might not have found in the detail that you did those valuable findings. But if you had to use your method for a large number of rats in a screening system, just how applicable is it? Is it something that you can use at that level of operation?

MR. HYDE: Yes, it is. That's what we're attempting to do now, to go back and look at many of our animals that we've worked up morphologically and run them through this screening process. I think that's the application that I'm trying to encourage. I'd like to elaborate a little more on the time frame. It takes approximately an hour and a half to run one animal through the procedure and this is with nine slides, or nine samples, out of one lung. That's quite specific sampling. The other thing that one can do then is feed it into a computer and that's relatively short in time. Once the programs are written, it's fairly inexpensive. I think that one thing that is overlooked a lot by morphologists is that it's very difficult to set up a gradation of lesions from one lobe to another when it's not possible to actually give a size variance, just through subjective methods. This is what I think is such a key feature of morphometric methods. It gives a statistical base to comparisons.

MR. MOORMAN: For discussion purposes, I'd like to consider a request by industry in which you have a number of people who have been exposed to a certain toxic substance. A question being asked about the toxicity of a specific compound, what toxicologic tool do you use to determine this? Of course, in this case, you would go back to functional studies. But let me point out that the Blakeston Medical Dictionary defines disease as dysfunction and altered structure resulting from anything. So it seems to me that many people feel that disease is strictly identified by the production of an irreversible lesion which is in part correct. I thought it was very interesting that you noted in your study that the NO_2 high exposure group had the most severe effect. I performed pulmonary function tests on the same dogs for about 3 years on this study, and we also found similar results. We found that the single breath nitrogen washout was significantly lower in that group. I think that the question of whether or not a morphologic

technique is better than a function technique is not really a question to ask. One provides 50% of the answer and the other one, the other 50%. Disease does occur functionally and without morphometric or structural change. Likewise, structure change often occurs without functional change.

DR. DUNGWORTH: Just to reinforce the point that Mr. Hyde made. Looking purely from the morphology and morphologic assessment only, it depends on the endpoint being sought. If it's a tumor in the mouse model system, that's one thing. But the endpoint in these extremely expensive chronic studies often turns out to be a chronic obstructive pulmonary disease in which one is looking at changes in the size of air spaces distal to the terminal bronchiole by definition of emphysema, and changes in the bronchiole and bronchi which would denote a degree of bronchitis. The place that the Quantamet comes into its own, as Mr. Hyde has shown, is where there are changes but they are changes that are to a lesser degree in the control animal population. It's inevitable in an aging population of whatever species and I don't need to go into that anymore. I'm sure it's plagued everybody in this room. A Quantamet enables specific measurements to be made such that we can prove with the usual statistical methods to apply to other quantitative data in which up to now have been lacking in morphology. And that's where the pulmonary function people have been much more able to arrive at specific conclusions. The Quantamet enables this to happen. The Quantamet was a reasonable investment to get the specific answer to support the pulmonary function data.

MR. J. DAVIS (University of California, Irvine): I have a question for Mr. Moorman. I'd like to know if you have information on the relationship of pulmonary function tests between anesthetized and unanesthetized animals. You were making a lot of statements about pulmonary function tests on anesthetized animals so can you give us some information on that?

MR. MOORMAN: I don't think that there is any information. In some respects, certainly there is a vital capacity change whether the animal is anesthetized or unanesthetized. There isn't any information. I'm sure you could make some speculation. I think the tests by definition are valid. With respect to flow rates, the anesthesia does have some effect. It could be discussed for hours. It seems to me in pulmonary function testing with an anesthetized subject, you want to avoid parameters that are most related to the effects of anesthesia. For instance, the rate parameters:

respiratory rate, minute volume. Certainly it would be invalid to make comparisons using these parameters in the anesthetized animal. However, it's my experience that most of these rate parameters are nondiagnostic. They don't lead you to the definition or the mechanism of disease. I know of no functional test that will demonstrate small airway obstruction specifically. It seems as though you are relegated to taking whatever parameters you can at whatever respiratory rate the animal is breathing at in the awake animal and often times, there's more variance as a result of the animal's response to the exposure than there is to the pharmacologic action of the drug. I think you have to be aware of the possibilities that the drug may effect. All of these tests are done on a comparative basis. The controls and the exposed animals are anesthetized so you have a controlled condition. Specifically, it would be very difficult to say whether some of the flow rates like small lung volumes which are significant of obstructive disease would be changed in the nonanesthetized subject. We have found that animals undergoing chronic exposure to a number of dusts produce the same physiologic dysfunction that humans have demonstrated in screening or prevalent-type studies. I wanted to comment also that recently I said I was presenting three animal models for obstructive lung disease. I didn't mean that the rat and the guinea pig were excellent models. I said they were usable. You can't detect small airway obstruction in these two species by comparing flow rates at small lung volumes. There is quite a bit of difference in the maximum expiratory flow volume curve in comparing the man and the monkey which are very similar to the rat and the guinea pig. But after the flow limitation characteristics exist, the comparison is useful.

DR. WILSON: I'd like to compliment Mr. Moorman on what he's done. I think that the type of test that he offers is a tremendous advantage over what we've had with intact animals and also Mr. Hyde for what he's done. He's taken the time consuming problems with looking at lungs in detail and made it very quantitative and much better. At least from my standpoint, this trip was worthwhile to learn about these things because, I think, they are probably what is going to occur in the future, i.e., an automated morphometric measurement approach to lungs that have been exposed to various toxins and a forcing function on dynamic lung volumes, on animals that have been exposed over a period of time. I think this is probably where the immediate future lies.

DR. CROCKER: I think that was a very nice summary statement.

DR. ELLIOTT (University of California, Irvine): I'd just like to raise one point of caution about the statement that anesthesia doesn't affect things because the anesthesia literature is full of studies of pulmonary function in anesthetized versus unanesthetized man. Of course, ether is well known as a bronchodilator and cyclopropane is well known as a bronchoconstrictor. So you have to know the pharmacologic parameters of your anesthetic before you can study any pulmonary function meaningfully in the anesthetized animal.

MR. MOORMAN: Limitation in flow during small lung volume as I understand relates primarily to the elastic recoil properties of the lung which provides patency to the small airways. Considering this, I would find it very difficult to consider what the artifact may be as a result of anesthesia. Do you feel that anesthesia effects recoil properties at the lung? I have looked at compliance in anesthetized and nonanesthetized animals and don't find significant difference. In the Handbook of Applied Physiology, there's a section on anesthesia and I don't think there's any clear evidence. Certainly some aspects of lung function are altered during anesthesia but I think you have to sacrifice this effect if you want to do diagnostic type tests and if you want your tests to relate to the industrial health physician. It's difficult enough to extrapolate to man when you're using the animal. It's even more so when the tests used in the animal model are quite a bit different than the common single breath type tests and the dynamic lung volumes that are used in man. I think it is our experience that we can use these tools and very significantly detect differences.

DR. ELLIOTT: You may be correct but if you neglect the specific pharmacologic action of the anesthetic, you may mask some functional changes.

MR. MOORMAN: I agree.

SESSION II

ANIMAL TOXICOLOGY MEASUREMENTS -- APPLICATIONS AND ASSOCIATED TECHNIQUES

Chairman

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AMRL-TR-75-125

PAPER NO. 7

RELATIONSHIP OF INHALATION TOXICOLOGY DATA TO ESTABLISHMENT OF TLVS

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It is a difficult task for a speaker to present the subject of the relationship of inhalation toxicology data to establishment of TLVs in a conference such as the present one in the fifteen minutes which have been allotted to this presentation. The speaker must decide in his own mind how he can present the subject so as to give the participants a maximum of useful information. Because of this limitation, a decision was made to present the basic underlying principles of the subject with very little detail concerning the establishment of any single Threshold Limit Value in the hope that these basic principles will serve as a framework for your individual knowledge accumulated from formal education, published literature and other presentations on similar or associated topics.

It is essential that one understands the concepts of Threshold Limit Values (TLVs) before one can relate toxicologic data to their establishment. The TLVs represent, with certain exceptions, time-weighted average concentrations of airborne substances associated with industrial operations and manufacture, designed to protect the health and well-being of nearly all workers repeatedly exposed during a 7- or 8-hour workday and 40-hour workweek, not only for their working lifetime, but after retirement (Stokinger, 1972). The concept of a time-weighted average carries with it two important considerations. First, time weighted averages permit excursions above the limit provided they are compensated by equivalent deviations below the value during the workday. Secondly, the time-weighted average method and its excursion limits merely represent guides in control of occupational health hazards and are not to be considered as absolute figures differentiating between

safe and dangerous concentrations (Stokinger, 1969). Exceptions to the time-weighted averaging procedure are the approximately 6% of the nearly 500 substances for which limits have been established based on a ceiling value, i.e., a maximum value that should not be exceeded (Stokinger, 1972). Ceiling values are imposed on substances essentially fast acting in nature and encompass one or more of the following properties: (a) intolerable irritation, (b) chronic or irreversible damage and (c) narcosis of sufficient degree to increase accident proneness, impair self-rescue or materially reduce work efficiency (Stokinger, 1964).

The most obvious place to begin assessing the toxic actions of airborne industrial chemicals is the point of contact between man and chemical. Toxic chemicals can enter the body by various routes. The most important route of exposure in industry is inhalation. Despite its tremendous importance in occupational medicine, cutaneous effects will not be considered. Furthermore, local effects on the mouth or alimentary tract play a minor role in occupational toxicology and, like the eyes, they seem to be sensitive areas where unpleasant contact is readily noticed.

Inhalation toxicology data are most relevant for other anatomical sites. The upper respiratory tract, nasal passages, pharnyx and larnyx are sensitive to irritants, e.g., acid and caustic aerosols, and specific materials removed by filtration, e.g., nasal septum perforations and carcinomas arising from exposure to chromates. Pathological changes and functional impairment of the lower respiratory tract and systemic toxicity arising from absorption of toxic chemicals are uniquely different and at times particularly difficult to assess under long-term, low-level exposure.

Since Threshold Limit Values are based upon repeated daily exposures of workers to chemicals, chronic animal inhalation studies, one to two years in duration, usually provide the only acceptable data, exclusive of human data, to establish a limit. Acute and subchronic exposures may fail to provide information on the accumulation of the compound in the body, or progressive development of the effect. These two factors in turn may dictate that the only response noted is chronic in nature or that the chronic effects differ markedly from the acute effects. There are, however, instances in which the acute and not the chronic response is the basis for the TLV. This is due to the cardinal toxicologic principle that the intensity of toxic action is a function of the

concentration of the toxic substance which reaches the site of action (Amdur, 1973). Hence, if the detoxification and excretion rates of the body exceed or equal the intake, it is possible that no toxic response will occur. Conversely, if the intake rate exceeds the body's excretory mechanisms, toxic responses will occur. For example, the TLVs for hydrogen cyanide and hydrogen sulfide are based upon data from acute effects from a single exposure.

Another requisite of inhalation toxicology and its adequacy for utilization in establishing threshold limit values is employing multilevel exposures (Stokinger, 1965). A minimum of two exposure levels should be tested; three exposure levels are preferable, however. One level should be such that frank effects develop, so that the chronic animal response can be precisely delineated. The second exposure concentration is one in which minimal effects occur in a small percentage of the animals exposed. The third and most important exposure concentration is one termed as a "no effect" level. It is obvious that the term "no effect" is relative to the criteria employed for its determination. Since the prime objective of experimental studies directed toward threshold limit evaluation is to reveal the subtle as well as the gross changes following exposure, comprehensive, sophisticated and sensitive procedures should be employed to substantiate as much as possible the airborne concentration having no effect on the health and welfare of the experimental subjects. Preferably, these procedures should be designed in a manner which would permit the ultimate application of the results to the worker. The day is past when routine, unimaginative and short-cut procedures are acceptable as evidence of toxic potential. Thus, it is essential that the procedures akin to inhalation toxicology are tailored so that they delineate the specific toxicologic characteristics of the chemical under study.

It is encouraging that recently the trend in inhalation toxicology is to expansion of criteria of toxic response to include biotransformation, neurophysiological and behavioral effects, pharmacodynamics, assessment of physiological impairment, scanning electron microscopy, etc. There is a continuing need for the development of more sensitive tests for injury due to exposure to chemicals. Early emphasis in the United States was placed on physical, chemical and morphological criteria of injury. The electron microscope, the cytogenetic, histochemical and histopathological techniques, enzymology and metabolic studies, however,

may not detect abnormalities of various organ systems, e.g. nervous, pulmonary, etc., which are manifest only as aberrations in function. Significant advances in functional toxicology have been achieved over the last decade but in many such areas the surface has merely been scratched or the first furrow is yet to be turned.

Each of you is familiar with the three major technical advantages of animal research - (1) strict control of exposure concentrations, (2) strict control of duration of exposure and (3) versatility for more detailed study. One aspect of significance to the latter is the desirability of performing serial testing and sacrifices. Significant toxic responses may be missed if data at 1 week, 1 month and/or later months are omitted, due to the activation of the body's adaptation mechanisms. Adaptation to changing environmental conditions is a normal body function; the effects of low-toxicity exposures to chemicals are no exception.

The use of animals to simulate the toxic hazards faced by workers has several shortcomings which must be addressed by inhalation toxicologists. There is no one animal species which can serve as a model for all agents toxic to man. Therefore, inhalation studies should be conducted with a minimum of two species selected as to: (1) a demonstrated response in subchronic studies, (2) anatomic and/or physiologic relationships to the research objectives, (3) particular sensitivity of response to classes of test substances and (4) statistical and/or logistical limitations. The exposure of multiple species of animals will increase the probability of finding one that will mimic the human response. One must be cognizant of the close parallelism of toxic responses repeatedly seen in animal and man. Hence, species similarities are the rule and species differences represent the exception.

A few cogent comments relative to factors associated with the inhalation route are appropriate. The dose received and retained by the animal (or man) is not known with the same accuracy as when a chemical is given by other routes, e.g. oral, intravenous, or intraperitoneal. The airborne concentration and duration of exposure can, however, be measured accurately and provide an estimate of the dose. Such variables as the respiration rate and volume, the deposition, retention and clearance from the respiratory tract, and the systemic distribution and excretion are all variables defining the dose the animal

receives. It is important to bear in mind that absorption by the pulmonary route can be more important than absorption through the gastrointestinal tract. When a toxic substance is absorbed through the gastrointestinal tract, the blood which carries this material must first pass through the liver before going to the body tissues. In the liver, the material can be detoxified prior to systemic distribution. Conversely, the blood which absorbs toxic materials from the lungs does not pass through the liver initially but passes directly to the body tissues by means of the arterial circulation.

A second fact of great importance is the residence time of the toxic material in the target organ. A good example of this is the metallic dusts. Most of the lead and cadmium which is ingested is excreted rather directly through the intestinal tract whereas a significant portion of inhaled lead and cadmium particulates remain in situ until dissolved or translocated into the circulatory system. Lead and cadmium are toxic both by ingestion and by inhalation. An equivalent dose, however, is more readily absorbed from the respiratory tract than from the gastrointestinal tract and hence produces a greater response (Nordberg, 1974).

Incorporated into the TLVs are safety factors determined essentially on the gravity of the toxic potential; the more serious the potential, the larger the factor. These safety factors are in the form of several-fold decrements from (1) borderline effects, if the limit is based on human experience or (2) the "no effect" level, if based on animal data. The determination of the safety factor is the responsibility of the Committee on Threshold Limit Values and commonly is 2 to 5 fold but in extreme cases where death might be the end-point such factors are approximately 10 fold.

In situations where there are available volunteers, medical supervision and assistance, and substances with appropriate toxicologic properties, human exposures of brief duration should be performed at the proposed TLV to elucidate the following factors: (1) total body burden, (2) biologic indicators of exposure, (3) behavioral or other physiological impairment, (4) narcosis, (5) effect on vision, (6) odor threshold and offensiveness, (7) nausea and (8) subjective factors. The exposures should be repeated to determine whether a reduction or increase in a toxic response occurs.

A second form of human experimentation involves human volunteers with terminal disease, or those defined as legally dead, without involvement of those organs and tissues known to be affected by the test substance. In this situation such humans may be exposed at or around the level at which the most sensitive response test was positive in animals.

Industry contributes directly to the evaluation of the suitability of a limit and hence the adequacy of its safety factor, through industrial plant experience (Stokinger, 1965). Medical data obtained by close worker surveillance combined with simultaneously obtained environmental data permit epidemiological studies to be performed which are directed at the worker whose health and welfare the TLVs are designed to protect.

In assessing toxicologic data for the establishment of a TLV, there are times when certain knowledge is inadequate and/or questionable. It is then that the TLV Committee must make the most judicious decision from the facts at hand. This philosophy was appropriately expressed by Sir A. B. Hill (1965) when he wrote: "All scientific work is incomplete - whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have, or to postpone the action that it appears to demand at a given time."

Thus in relating inhalation toxicology data to the establishment of TLVs, limits must be based upon the best knowledge at hand and be guided by the principle of the enhancement of the quality of human life. Such action is based on a philosophy of preventive medicine. Necessary research should proceed concomitantly to fill the existing gaps in our state of knowledge.

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AMRL-TR-75-125

PAPER NO. 8

MODELING OF CHRONIC TOXICITY

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Mathematical modeling thoroughly verified by experiments is a reliable tool to obtain information on the role of individual factors (physical and chemical properties of vapors and their metabolites, physiological parameters of species and environmental conditions) in uptake, accumulation and washout of vapors. Once the significance of these factors is understood, they can be analyzed in order to predict threshold limit values from the acute toxicity.

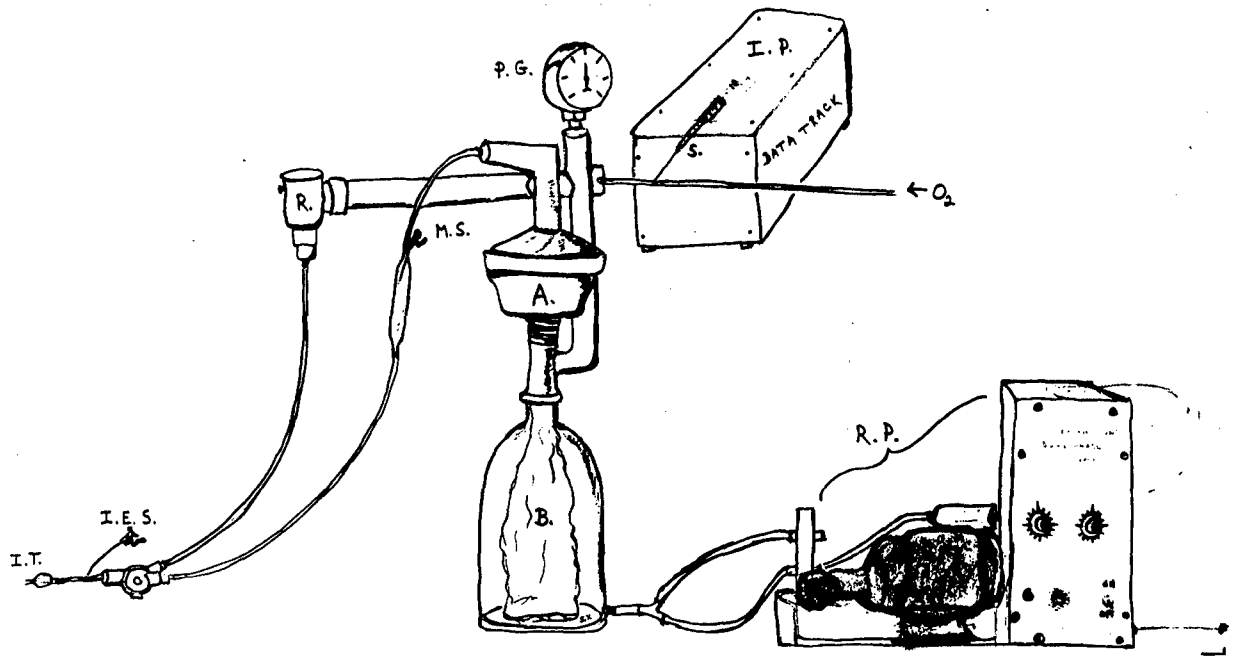
In the formulation of the mathematical model, the following assumptions are made: uptake, distribution and excretion are described by exponential functions, in which the rate constants are determined by tissue volumes, blood perfusion and by the solubility of vapors (partition coefficients). According to these parameters, tissues are grouped into four pharmacokinetic compartments. The first compartment includes alveolar air (FRC), the volume of lung tissue and arterial blood. It is a rapid gas exchanging group "REG". The second compartment - vessel rich groups "VRG" - includes well perfused tissues (mainly viscera). Muscles and skin form the third compartment, "MG". Its blood perfusion is variable and usually much lower than in the VRG. Because of the high solubility of solvent vapors in fat, adipose tissues and fatty marrow form the fourth compartment, "FG". The half times (time interval necessary for saturation of the compartment to fifty percent) vary significantly for each compartment. They are of the magnitude of seconds for REG, minutes for VRG, and hours for FG.

Vapors of water insoluble compounds undergoing metabolism have two excretory pathways: the lung and metabolism. The rate of overall metabolism of compound "v_m" may be determined from uptake rate when an apparent steady state is reached. The rate of metabolism, determined

from concentration differences in inhaled and exhaled air and minute ventilation "$\dot{V}$". $v_m = (C_{inh} - C_{exh})\dot{V}$ is used for the calculation of the concentration independent metabolic coefficient "K_M" = v_m/C_{inh}. The metabolic coefficient determines the rate constant for metabolism in the mathematical model (Fiserova-Bergerova et al., 1974). The model assumes that metabolism occurs in tissues carrying microsomal enzymes, which are included in the VRG compartment. It also assumes metabolism to be an irreversible reaction of the first order, not inhibited by substrate or by product, and that it does not accommodate for enzyme induction.

Suitable physiological parameters required for mathematical formulation of pharmacokinetics in man have been published by Eger (1963) and tested in our laboratory (Fiserova-Bergerova, 1972 and 1975; Fiserova-Bergerova et al., 1974; Holaday et al., 1975). However, a suitable pharmacokinetic animal model was not available. Mongrel dogs, used by Cowles et al. (1972) were found unsuitable in our laboratory because of variability in their physiological parameters. We found beagle dogs and rhesus monkeys to be satisfactory subjects for pharmacokinetic study. The physiological parameters collected from the literature for rhesus monkeys and beagle dogs were tested in inhalation experiments with Forane. Forane (1 chloro-2,2,2-trifluoroethyl difluoromethyl ether) is a lipid-soluble compound (vapor pressure 239 torr at 20 C), which does not undergo biotransformation and, therefore, its uptake and distribution depends only on physiological parameters and solubility.

In order to administer an inhalation drug and measure its concentrations in arterial blood and in inhaled, mixed-exhaled and end-exhaled air, animals were anesthetized with Brevital or Sernylane and a polyethylene catheter was inserted into the femoral artery. The animals were intubated with a cuffed endotracheal tube which was connected to a closed CO_2 absorbing total rebreathing circuit. The scheme of the circuit is in Figure 1. The circuit was made of metal, glass, nylon and tygon tubing in order to minimize absorption of vapors. The volume of the circuit was approximately one and one-half liter for dogs and one liter for monkeys.



A. = CO_2 - absorbent
B. = Rebreathing reservoir
I.P. = Dose regulated anesthesia pump
S. = Hamilton gas tight syringe
P.G. = Pressure gauge
R. = Wright respirometer
R.P. = Respiration pump
V. = Two way breathing valve
I.T. = Endotracheal tube
I.E.S. = Sampling port for inhaled and end exhaled air
M.S. = Sampling port for mixed exhaled air

Figure 1. Equipment used for administration of Forane and methylene chloride to monkey and dog.

During exposure, liquid drug was injected into the circuit from a Hamilton gas-tight syringe via a stainless steel needle by an infusion pump (Dose Regulated Anesthesia Pump, Mark II, Quan, Inc.). The infusion pump was programmed to maintain a constant alveolar concentration. The infusion rate of "v_t" of liquid Forane was calculated according to the equation:

$$v_t = C_{alv}\left(F_{VRG}\,\lambda_{bl/air}\,e^{-\frac{F_{VRG}}{V_{VRG}\lambda_{VRG/bl}}t} + F_{MG}\,\lambda_{bl/air}\,e^{-\frac{F_{MG}}{V_{MG}\lambda_{MG/bl}}t} + F_{FG}\,\lambda_{bl/air}\,e^{-\frac{F_{FG}}{V_{FG}\lambda_{FG/bl}}t}\right) \quad (1)$$

The values of constants were derived from data in Tables 1 and 2.

TABLE 1. WEIGHT (W) AND PERFUSION (F) OF TISSUES

SPECIES	MAN		MONKEY		DOG		RAT	
Body Weight kg	70		3		10		0.25	
Cardiac Output l/min	6.7		1.0		2		0.052	
Total Ventilation l/min	8.0		1.0		3.5		0.074	
Alveolar Ventilation "V_{alv}"	5.3		0.6		1.0			
Respiratory rate/min	12		38		23		85	
Parameter	Wg=V	Fml/min	Wg=V	Fml/min	Wg=V	Fml/min	Wg=V	Fml/min
Liver	2600	1500	90	194	370	300	8.2*	10
Kidneys	300	1188	16	140	50	175	2.0*	10
Brain	1500	795	87	60	75	45	2.0*	0.8
Heart	300	243	14	50	100	50	1.0*	1.3
Spleen	142		3.5	22	27	13	0.6*	
Pancreas			6.0	20	22	27		
Thymus	160	80	3.3		10		0.5	
Adrenals	20	102	0.9	2	1.5			
Thyroid	28	100	0.5	2	1.0	3.5	0.04	
GIT	1340	500	143	130	480	112	8.0	
VRG	6390	4508	364	620	1137	725	23	
Muscles	31000	1440	1505	254	4730	946	120	
Integuments	3400	310	300	66	1700	100	45	
MG	34400	1750	1805	320	6430	1046	165	
Fat	8000	270						
Fatty Marrow	2200	70						
FG	10200	340	100	20	500	54	5	
Blood	5400		210		300		12	
Lungs	600		28		80		1.3*	

* Taken from MacEwen et al., 1972 (page 24).

TABLE 2. PARTITION COEFFICIENTS "λ" TISSUE/AIR OF FORANE AND METHYLENE CHLORIDE AT 37 C

Compartment	Man	Monkey	Dog	Rat
		FORANE		
Blood	1.4*	1.0	1.3	1.6
VRG	2.5	2.9	3.6	2.9
MG	2.4	1.4	3.4	1.6
FG	69.0	66.0	75.0	63.0
		METHYLENE CHLORIDE		
Blood	6.5	8.2	11.2	12.8
VRG	6.6	8.0	8.9	8.6
MG	4.8	4.0	8.0	5.0
FG	82.0	86.0	105.0	92.0

*Taken from data by Lowe (1968), means of 3 individuals.

During exhalation period, the samples of mixed exhaled air were drawn from a mixing chamber in the expiratory limb of the circuit. During inspiration or at the end of exhalation, inhaled or end-exhaled air was sampled through a teflon catheter which was inserted in the endotracheal tube. The measurements of inhaled air, mixed exhaled air and minute ventilation were used to calculate uptake. A similar experiment with methylene chloride was performed one month after the experiment with Forane. The uptake rates from experiments with both drugs in one dog and one monkey are presented in Figure 2.

Two striking differences appear in the distribution of these two drugs: after one hour of exposure, the concentration ratio of Forane in inhaled air/alveolar air is equal to 1 for both species; for methylene chloride it equals 1.8 for the dog and 1.9 for the monkey. In our study in man the concentration ratio for Forane is 1.2 (Holaday et al., 1975), while for methylene chloride, estimated from data of DiVincenzo et al. (1972) it equals 4. Secondly, the uptake curves for Forane predicted by equation 1 match the experimental data, but the uptake curves for methylene chloride do not match the measured values (Figure 2). Similar observations were made in man (Figure 3).

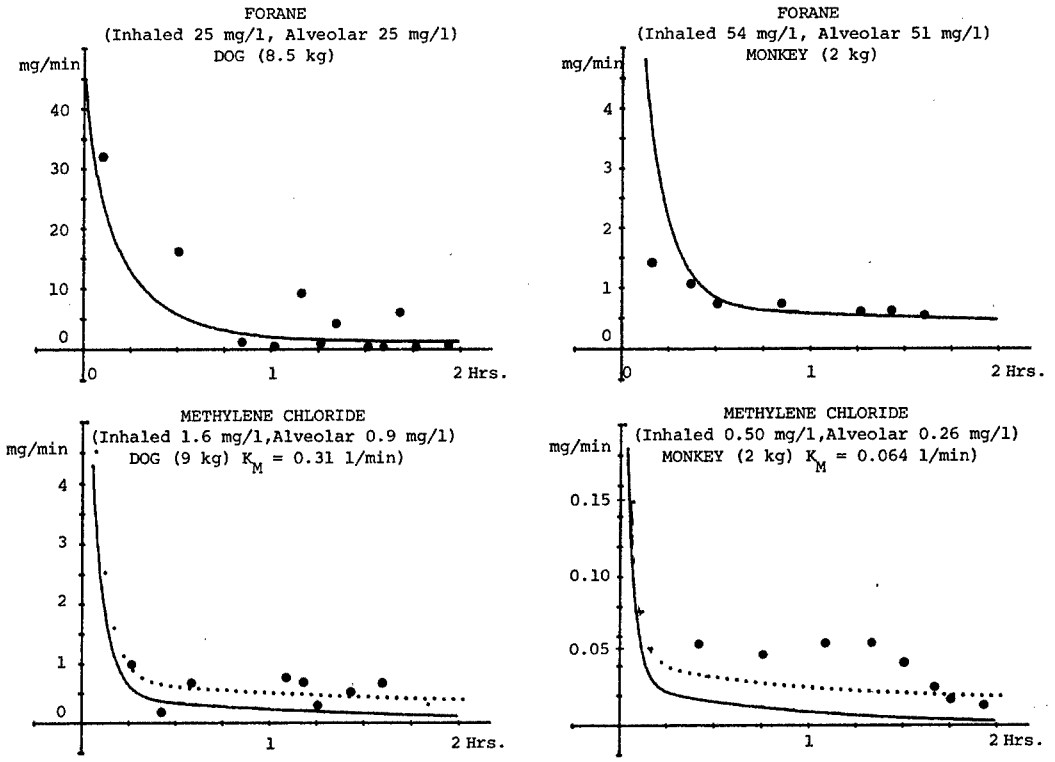
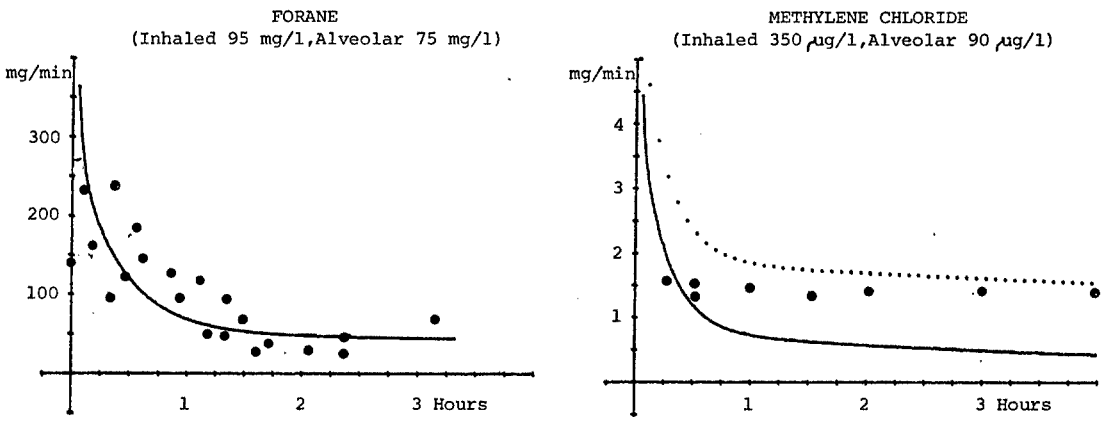


Figure 2. Uptake of Forane and methylene chloride.



Body Weight: 99 kg
Body Height: 187 cm
Ventilation: 7 l/min

Experimental data from Holaday, Fiserova-Bergerova, Latto, Zumbiel 1975

Body Weight: 70 kg
Body Height: 170 cm
Ventilation: 7.5 l/min

K_M = 3.2 l/min
Experimental data from Di Vincenzo, Yanno, Astill 1972

Figure 3. Uptake of Forane and methylene chloride by man.

Since the pulmonary uptake rate is equal to the retention rate of unchanged drug in tissues plus the rate of overall metabolism of the drug, the difference between experimental data and retention rate calculated from equation (1) for given alveolar concentration was used to calculate the rate of metabolism and the metabolic coefficient "K_M". The metabolic coefficient was used to predict the uptake curve by equation (2). (Dotted lines in Figures 2 and 3.)

$$v_t = C_{alv}\left[\frac{(F_{VRG}\lambda_{bl/air})^2}{F_{VRG}\lambda_{bl/air}+F_X}e^{-\frac{F_{VRG}+F_X}{V_{VRG}\lambda_{VRG/bl}}t} + \frac{F_{VRG}\lambda_{bl/air}F_X}{F_{VRG}\lambda_{bl/air}+F_X} + F_{MG}\lambda_{bl/air}\,e^{-\frac{F_{MG}}{V_{MG}\lambda_{MG/bl}}t} + F_{FG}\lambda_{bl/air}\,e^{-\frac{F_{FG}}{V_{FG}\lambda_{FG/bl}}t}\right] \quad (2)$$

The symbols in equation 2 are the same as indicated in Tables 1 and 2 and in equation (1), and F_X is calculated from equation (3)

$$\frac{1}{F_X} = \frac{1}{K_M} - \frac{1}{\dot{V}} - \frac{1}{F_{VRG}\lambda_{bl/air}} \quad (3)$$

in which $\dot{V}$ is alveolar ventilation.

Differences in physiological parameters determining uptake, distribution and excretion of inhaled vapors are demonstrated for 3 species in Table 1 and in Figures 4 and 5.

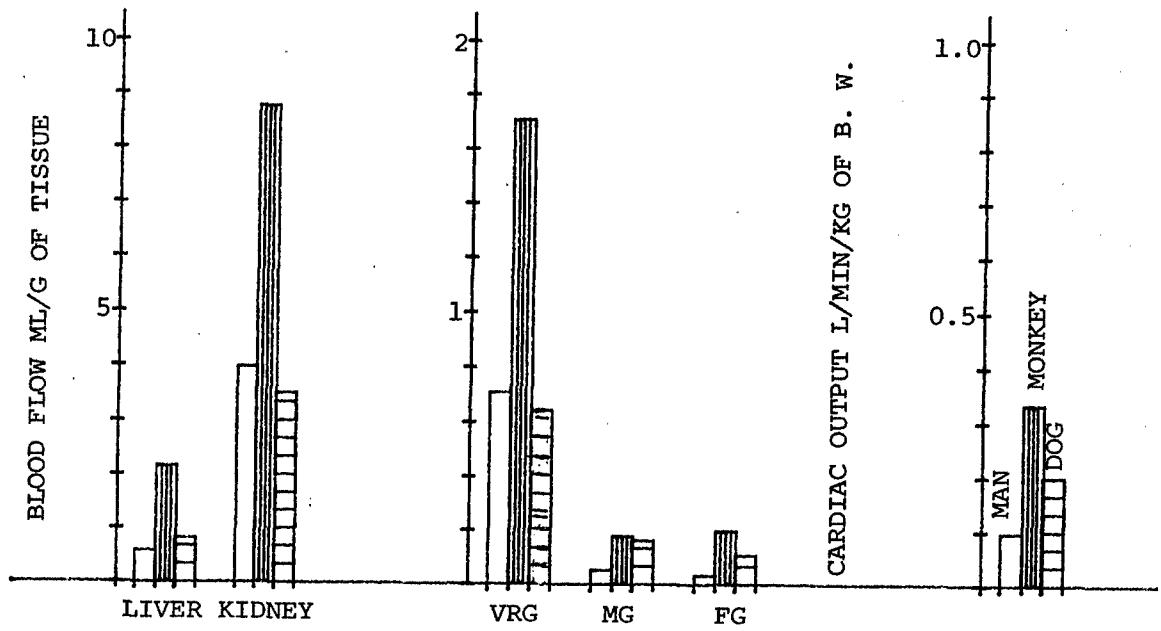


Figure 4. Species differences in cardiac output and blood distribution.

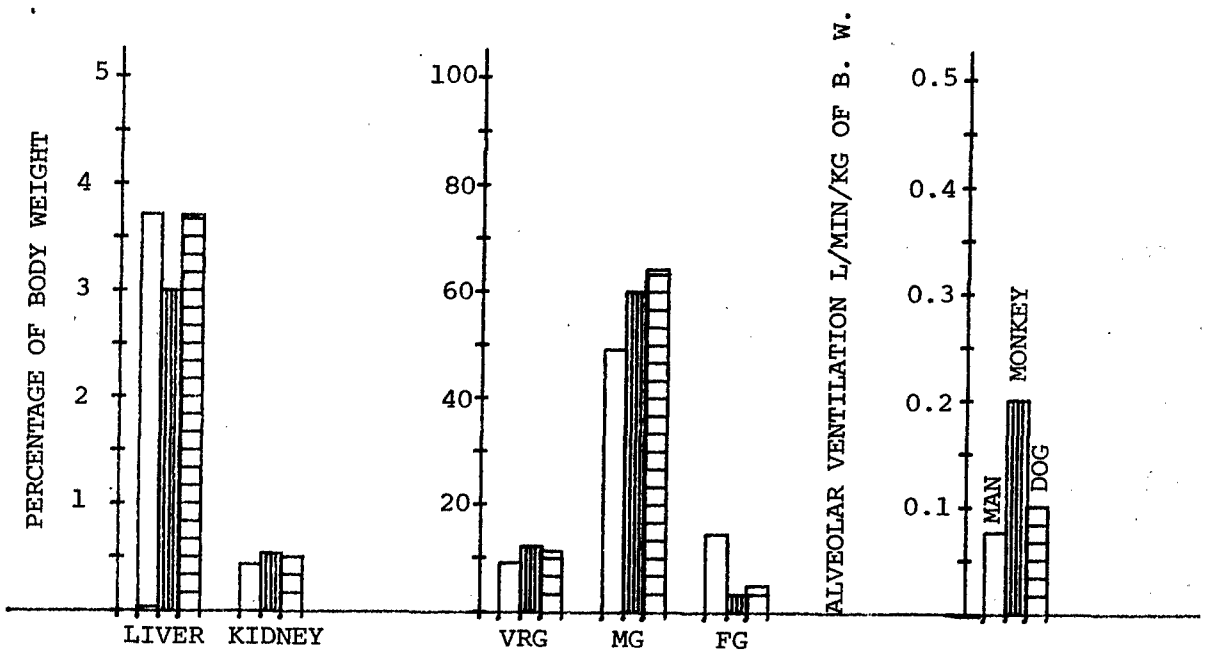


Figure 5. Species differences in organ weight and alveolar ventilation related to body weight.

The species variations in pulmonary ventilation and cardiac output result in significant species variations in the transportation rate of vapor from lung to tissues and vice versa. There is a positive relationship between body surface areas of species and their cardiac output and pulmonary ventilation. Because of relatively large minute ventilation and tissue perfusion of small animals, the availability of inhaled vapors for metabolism is greater in small animals than in large species. For the same reason the partial pressures of vapors in tissues of small animals are equilibrated faster with the environment than of man. Of special concern is the large species variation in blood flow to the excretory organs (liver and kidney) because of the important role of these organs in metabolic and excretory clearance.

Also, the volumes of pharmacokinetic compartments vary for different species. A special consideration must be given to the volume of FG compartment when dealing with lipid soluble vapors. The percentage of fat in man is approximately four times the percentage of young rhesus monkeys, which is another reason why vapor partial pressures equilibrate faster in small species than in man.

The solubility of methylene chloride and Forane (Figures 6 and 7) among four species used in this study varies in the range reported for other vapors (Steward et al., 1973). Species differences in the tissue/air partition coefficients affect the vapor concentrations in tissues at the steady state. The transportation rate of vapors from lung to the tissues and vice versa is a product of blood flow and the blood/air partition coefficient. The differences in blood/air partition coefficients (Table 2 and Figures 6 and 7) substantially affect the equilibration rate for different species. Rate of transportation of the vapor from environment to the site of metabolism "v" during steady state depends on alveolar ventilation, blood flow and solubility in blood.

$$v = \frac{\dot{V}_{alv}\, F\, \lambda_{bl/air}}{\dot{V}_{alv} + F\, \lambda_{bl/air}} C_{inh} \qquad (4)$$

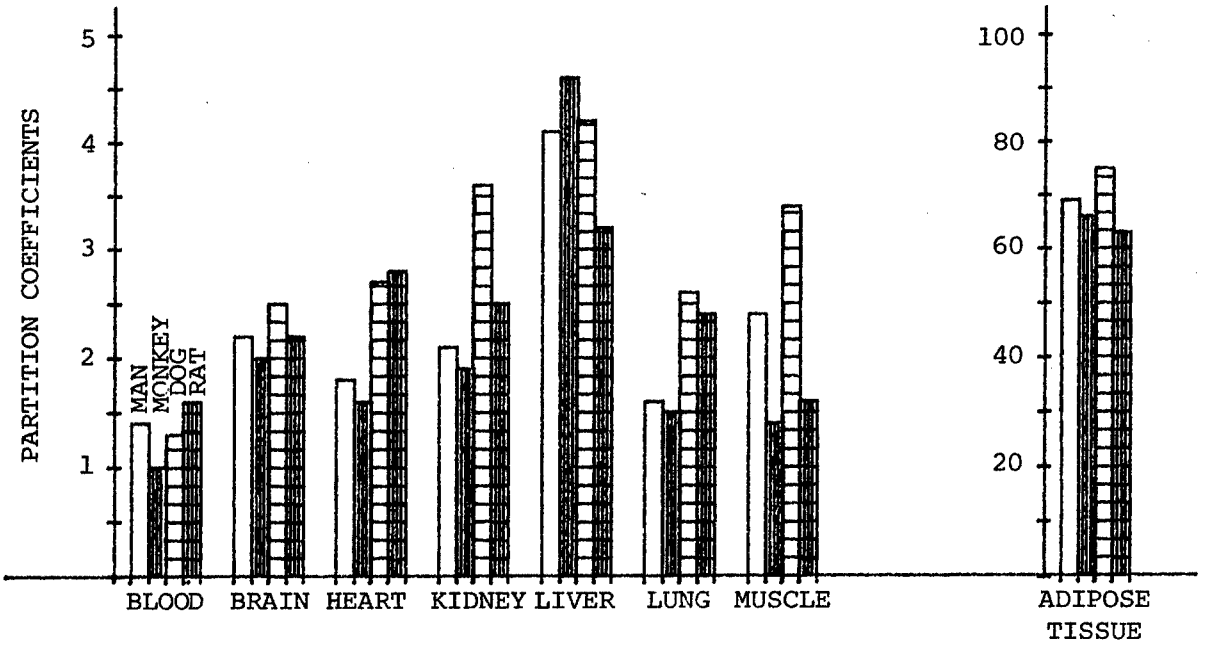


Figure 6. Species differences in partition coefficients of Forane (at 37 C).

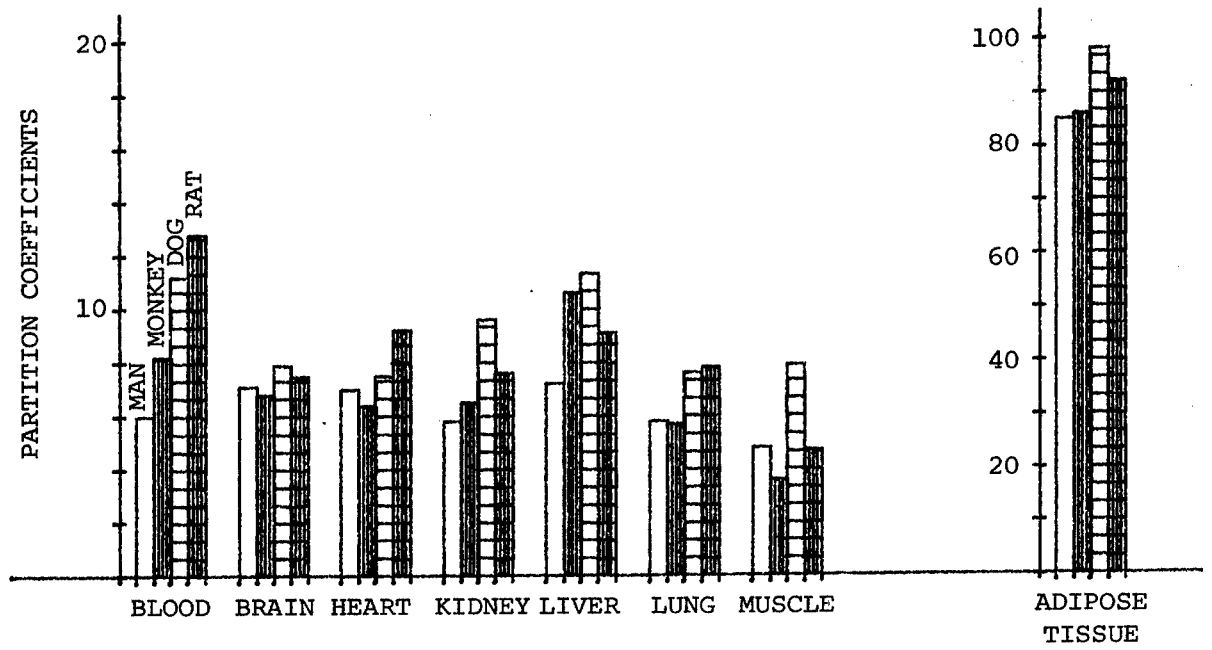


Figure 7. Species differences in partition coefficients of methylene chloride (at 37 C).

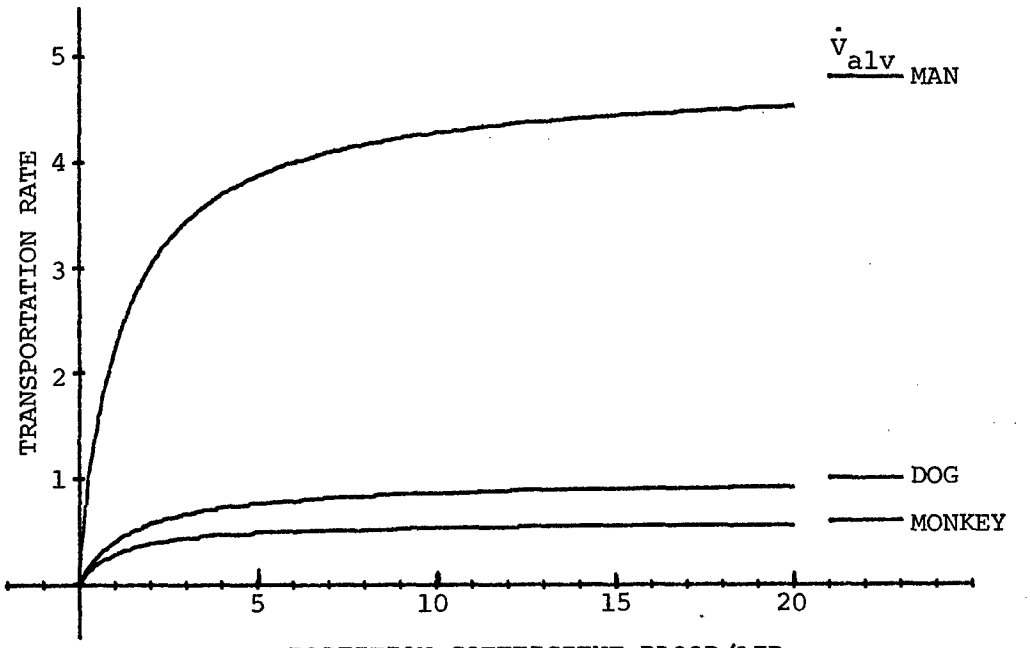
For highly soluble drugs λ bl/air $\ggg$ 1, and equation (4) may be simplified to

$$v = \dot{V}_{alv} C_{inh} \tag{5}$$

which means that the rate of metabolism of highly soluble vapors is determined mainly by alveolar ventilation. For slightly soluble vapors, λ bl/air $\lll$ 1, the simplified equation (4) is

$$v = F \lambda \text{bl/air } C_{inh} \tag{6}$$

It means that the rate of metabolism of slightly soluble vapors is determined mainly by transportation rate of vapors by the blood. The relationship between transportation rate and blood/air partition coefficient is demonstrated in Figure 8.



$$\text{Transportation Rate} = \frac{\dot{V}_{alv} F \lambda \text{ bl/air}}{\dot{V}_{alv} + F \lambda \text{ bl/air}}$$

F = Blood flow to site of metabolism;
in this figure = $F_{VRG} - F_{brain}$

Figure 8. Transportation rate as a function of partition coefficient blood/air.

Continuous or interrupted chronic exposures were studied by mathematical models. Occupational exposure is an interrupted exposure - eight hours a day, five days a week.

The following conclusions were made from the data:

1. The equilibration of partial pressure of slightly soluble vapor in the body with the environment is rapid. Therefore, the accumulation of vapor in the body during interrupted exposure is negligible and the daily variations in the concentrations of vapor in tissues are large.

2. The equilibration of partial pressures of soluble vapors in the body with the environment is a long-lasting process. Therefore, the accumulation of vapor in the body takes place during interrupted exposure and the daily variation of vapor concentrations in tissues is small.

3. Because of the positive relationship between body surface area and cardiac output and ventilation, the equilibration of partial pressures is faster and the accumulation is smaller in small species than in large species.

4. During continuous exposure the vapor concentrations in tissues at the steady state are higher than the peak concentrations reached during the interrupted exposure if the same vapor concentration is inhaled. The time weighted averages of vapor concentration in tissues during a week are the approximation of one quarter of concentrations reached during continuous exposure. The difference between concentration reached at steady state during continuous exposure and peak concentrations in interrupted exposure is larger for vapors easily soluble in biological media than for slightly soluble vapors.

5. The amount of vapor metabolized weekly is approximately four times larger during continuous exposure than during interrupted exposure if the same vapor concentration is inhaled.

6. During interrupted exposure, the partial pressure of vapor in tissues is never completely equilibrated with partial pressure in the environment. The sketch in Figure 9 demonstrates the vapor concentration in a tissue following different types of exposure. Curve 1 refers to continuous exposure.

Curve 2 shows the interrupted exposure with peaks lower than curve 1. The peak partial pressure in tissues reached during interrupted exposure when the steady state is established is indicated by "$C_{int.}$" "C_t" is the partial pressure equilibrated with environment during continuous exposure. In order to establish for continuous exposure equivalent to peak concentration in tissues "$C_{int,}$" the inhaled concentration must be reduced by the ratio C_{int}/C_t. The vapor concentration in tissues during continuous exposure to such a reduced concentration is indicated by curve 3.

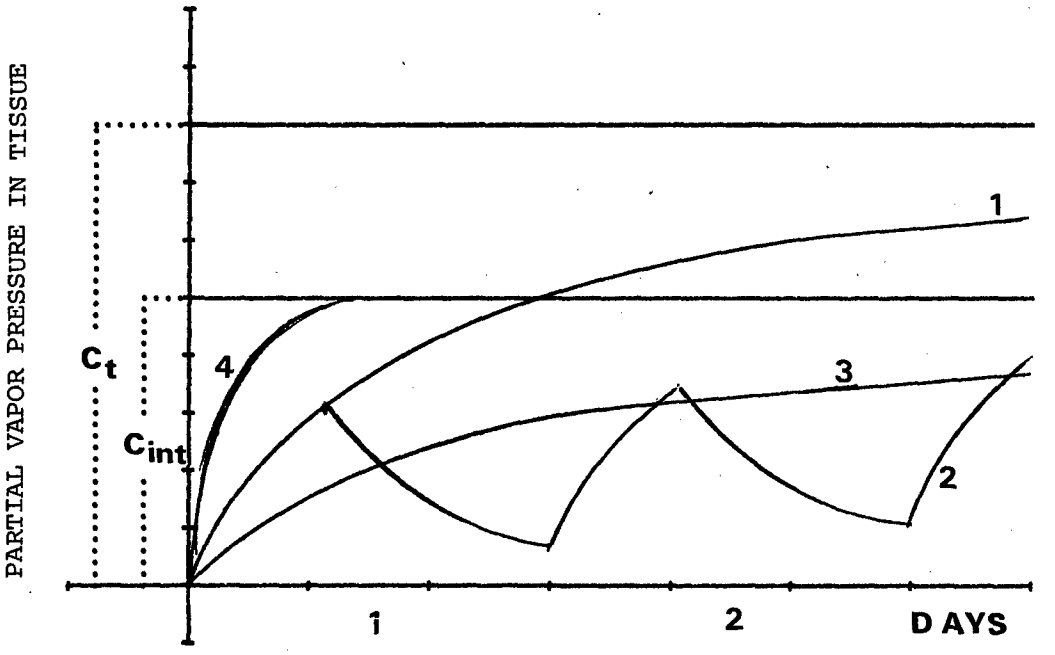


Figure 9. Scheme of equilibration of partial pressures in tissue during continuous and interrupted exposure.

7. The establishment of steady state is a slow process because, at the beginning of exposure, the venous blood returns to the lung almost free of vapor, the vapor having been retained by tissues. The amount of inhaled vapor is smaller than would be needed to equilibrate the partial pressures of vapor in blood with the inspired air without decreasing extensively the vapor concentration in the air specially if the vapor is well soluble in blood. The partial equilibration of blood with inspired vapor in a single passage through the lung at the beginning of the exposure may be calculated from the ratio of outflow and inflow to the lung:

Inflow = amount inhaled = $C_{inh} \cdot \dot{V}_{alv}$

Outflow = amount exhaled + amount removed by blood

$$= C_{alv} \cdot \dot{V}_{alv} + C_{art} \cdot Q$$

where "Q" is the cardiac output. Since the concentration in arterial blood is equilibrated with alveolar air:

$C_{art} = C_{alv} \cdot \lambda$ bl/air the outflow = C_{alv} ($\dot{V}_{alv} + Q \lambda$ bl/air)

Making inflow equal outflow the following equation results:

$$C_{inh} \cdot \dot{V}_{alv} = C_{alv} (\dot{V}_{alv} + Q \lambda bl/air) \quad (7)$$

Equation (7) may be used to calculate the partial equilibration:

$$\frac{C_{alv}}{C_{inh}} = \frac{\dot{V}_{alv}}{\dot{V}alv + Q \lambda bl/air} = \frac{1}{1 + \frac{Q}{\dot{V}_{alv}} \lambda bl/air} \quad (8)$$

The ratio of cardiac output to alveolar ventilation is 1.25 in man, 1.7 in monkey and 2 in dog. It follows from equation (8) that a larger blood/air partition coefficient results in a smaller partial equilibration of blood with inspired air. For methylene chloride, it would account for 11% in the man, 6.7% in the monkey, and 4.3% in the dog. As the exposure continues, the tissues become saturated with vapor, and the venous blood carries vapor back to the lung and the ratio C_{alv}/C_{inh} approaches 1 for inert vapors or 1 - $K_M/\dot{V}_{alv}$ for metabolized vapors (Fiserova-Bergerova, 1975). The establishment of the steady state may be accelerated by using the higher vapor concentration at the beginning of exposure, which compensates for the low outflow/inflow ratio. The steady state would be established most rapidly if the concentration in inhaled air "C_t" is programmed for the concentration "C" to which the body should be equilibrated by equation:

$$C_t = \frac{C}{V_{alv}} \left[V_{alv} + \frac{(F_{VRG}\,\lambda_{bl/air})^2}{F_{VRG}\,\lambda_{bl/air} + F_X} e^{-\frac{F_{VRG}+F_X}{V_{VRG}\,\lambda_{VRG/bl}}t} + \frac{F_{VRG}\,\lambda_{bl/air}\,F_X}{F_{VRG}\,\lambda_{bl/air} + F_X} + F_{MG}\,\lambda_{bl/air}\, e^{-\frac{F_{MG}}{V_{MG}\,\lambda_{MG/bl}}t} + F_{FG}\,\lambda_{bl/air}\, e^{-\frac{F_{FG}}{V_{FG}\,\lambda_{FG/bl}}t} \right] \quad (9)$$

The meaning of constants is the same as in equation (2). The inhaled concentration "C_t", programmed according to equation (9) would never allow a concentration in arterial blood or in any tissues to be higher than the concentration to be reached at steady state (curve 4, Figure 9). For highly soluble compounds, the programmed initial inhaled concentrations could be so high that they would irritate the lung. Therefore, certain adjustments must be made. As an example, equation (9) was used to calculate the required concentration of methylene chloride for a dog (Figure 10). The calculated initial concentration is about 20 times higher than the concentration to be studied. Let us assume that such concentration is four times higher than the short-term safe concentration (ceiling). In order to equilibrate the partial pressure of vapor fast and safe, the subject should be exposed to the ceiling concentration for a time interval "T" after which the concentration must be reduced to the level given by the theoretical curve. The time interval "T" for which the initial concentration should be kept constant is estimated graphically from Figure 10 by making areas I and II approximately equal.

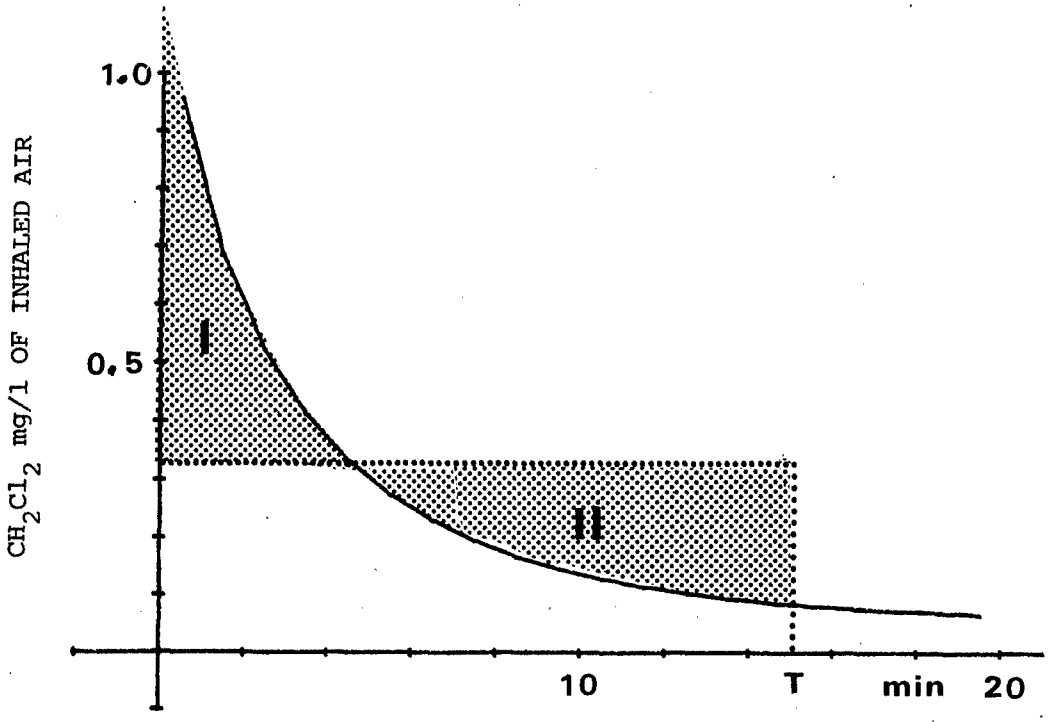


Figure 10. Speed up exposure of dog to 0.07 mg/l of methylene chloride.

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AMRL-TR-75-125

PAPER NO. 9

ANALYTICAL METHODOLOGY FOR INHALATION EXPOSURE CHAMBERS

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Batch sampling methods were once very popular because these methods are easy to set up, versatile, and use standard instrumentation. Batch sampling requires representative air collection in a syringe or by bubbling through a solvent. If the sample is syringe collected in the vapor phase, it is commonly analyzed by infrared or gas chromatographic injection. If the sample has been collected in a solvent, it can be analyzed by liquid chromatography, chemical analysis, fluorometry, visible or ultraviolet spectroscopy. The major problems with batch sample methods are the need for constant personal attention and the delay between sampling and analysis. For these reasons, continuous automatic types of analysis are now used whenever possible.

The most simple and useful continuous automatic type of analyzer for organic contaminants has been the hydrocarbon analyzer. It is necessary only to keep the material volatilized and flowing at a constant rate through the analyzer. The only interferences are other volatile organic materials either pulled in the chamber air intake or given off by the animals. If the background organic volatiles are relatively constant, the chambers can be analyzed by comparison to the chamber baseline response. Because of the background, the hydrocarbon analyzer minimum sensitivity is about 1 ppm.

By the use of a loop in the sample air stream, a gas chromatographic analysis can be made automatic. The major sampling problems are keeping the contaminant volatilized and the sample loop switch sealed. The choice of column packing and detector depends on the contaminant and the interfering materials. The packing is chosen to give a reliable, fast separation of the

contaminant from the other materials and a rapid elution of all materials to permit frequent sampling. Porous polymers such as Poropaks or Chromosorbs provide good column packing materials because one of them will generally do the job, and since they are not coated, they will bleed only very slowly and not change the sample retention time as coated columns do. The first choice in detectors is the flame which would have a minimum sensitivity for a 1 ml sample of about 100 ppb. If the material is not detectable by the flame, a thermal conductivity detector is probably best but water, which is generally fairly abundant in the chamber air, is also detected and the minimum sensitivity is about 10 ppm. For halogenated or nitrated contaminants, the electron capture detector has a minimum sensitivity of 10 ppb but is tricky. Water, for instance, can alter a signal, thereby changing the peak height of a specific concentration with humidity. Work done by Mike Holdren and presented by Dr. Farwell from Air Pollution Research, Washington State University, at this year's ASTM meeting held in October, shows an example. Peroxyacetyl nitrate injected in air of varying humidity shows the increase in signal with increasing water (Figure 1).

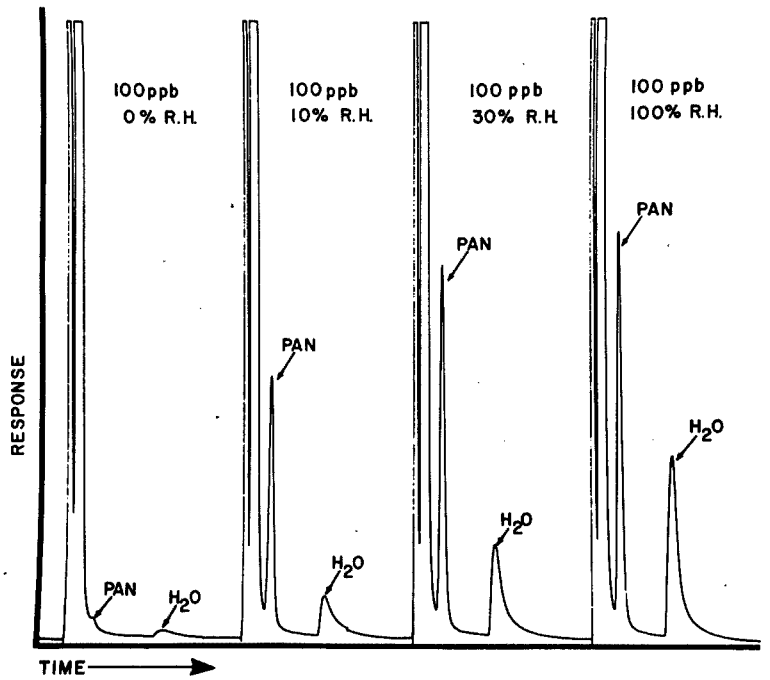

Figure 1. Humidity influence upon electron capture detection of P.A.N.

Infrared is another very useful continuous analysis method. There are commercial infrared analyzers specifically for a single compound such as the Beckman Model 215A which is for carbon monoxide or carbon dioxide. Adjustable infrared analyzers are available from Miran which can monitor wavelengths from 2.5 to 14.5 microns. Water, however, can interfere at shorter wavelengths, 5-7 microns, if the humidity is high. The infrared sensitivity is dependent upon the compound's absorption and the sample cell length. The minimum sensitivity is about 100 ppb using a 20 meter cell. The infrared analyzers are relatively compound specific, so much so that it is generally easy to run more than one compound and analyze each separately. For instance, we ran a dual 100 ppm methylene chloride, 1000 ppm trichloroethane exposure and monitored with two Mirans. The first Miran measured methylene chloride at 7.8 microns, the second measured trichloroethane at 13.8 microns with only about a two percent cross interference at these concentrations. The detector response was very fast and the presence of the animals caused no baseline interference.

The only ultraviolet continuous analyzer we have used is the Dasibi ozone analyzer. The analyzer appears to be relatively insensitive to water, dust or materials given off by the animals. It is sensitive to 10 ppb ozone and can detect atmospheric ozone changes.

There are a number of other available instruments highly sensitive to specific compounds. However, if sensitivity and specificity are not required, it is better to have a more generally useful instrument.

Atmospheric contaminants can also be dissolved and concentrated in liquid solution before analysis. The contaminant must be soluble in some solution preferably an aqueous reactant solution. The contaminant is concentrated, if possible, by scrubbing liters of air into milliliters of solution (Figure 2). Air containing the contaminant is mixed with the reactant solution in a scrubber tower and then separated below. The air is pumped through filters and a flowmeter. The pump is located after the tower so it does not affect contaminant concentration. The liquid is pumped from the separator through a colorimeter when used with a Technicon AutoAnalyzer apparatus. This method is currently being used by us to analyze hydrazine. The hydrazine is absorbed in a colored iodine solution, reacts to form nitrogen gas and a colorless solution of hydrogen iodide. The minimum detection level is about 40 ppb because of other reducers emitted by the animals affecting the baseline. We have analyzed ozone and nitrogen dioxide colorimetrically after scrubbing and there are a number of other potential uses for this analysis.

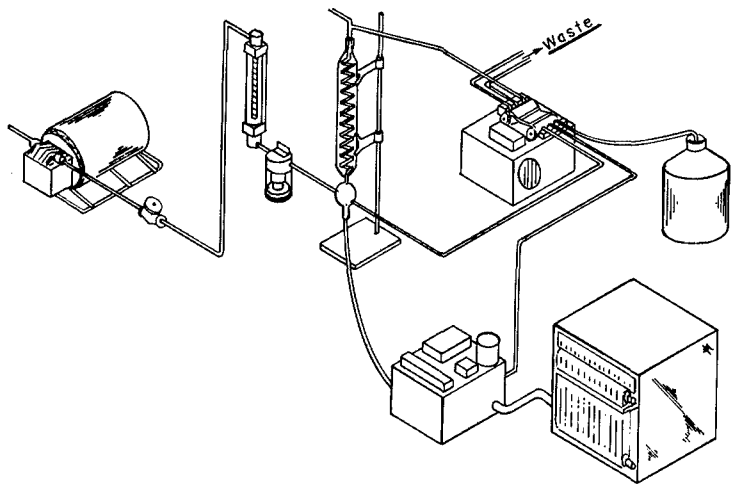

Figure 2. AutoAnalysis apparatus.

The reactant solution can also be analyzed with specific ion electrodes (Figure 3). The electrodes are generally not affected by anything found in the dome but the contaminant. For continuous analysis, a flow-through electrode cell was designed with low volume for rapid concentration changes. The specific ion electrodes available for use with this analysis include fluoride, chloride, bromide, iodide, cyanide, sulfide, sodium, thiocyanate, and possibly others. Due to the low interference of materials in the atmosphere and the high sensitivity of the electrode analysis, the minimum sensitivity would be about 100 ppb or lower in the atmosphere depending upon the contaminant solubility.

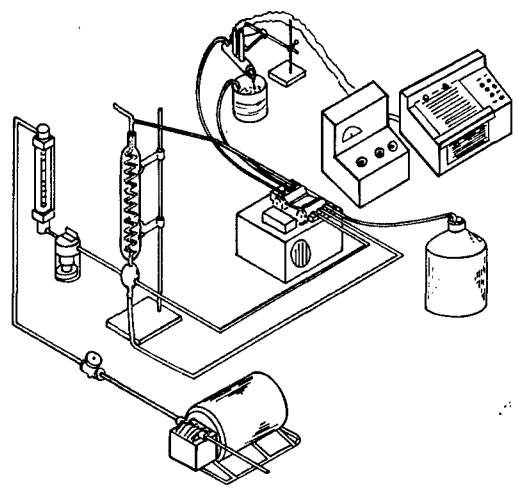

Figure 3. Automatic specific ion electrode apparatus.

We ran a dual HF, HCl exposure monitoring both materials with electrode analysis methods. Both contaminants were about 1000 ppm. There was no cross interference and nothing detectable was given off by the animals.

The electrode method was also used to measure 500 ppm cyanide in the presence of 14,000 ppm carbon monoxide. The carbon monoxide was measured by a Beckman 215A. No cross interference was found between contaminants.

A third analysis method for dissolved atmospheric contaminants could be the use of a liquid chromatograph (Figure 4). Periodic samplings and separations could be made by use of an automatic liquid loop sampler after the contaminant scrubber tower. We have not yet done this but have the necessary instrumentation. This method should be sensitive to 1 ppm in air depending upon the sample solubility in the scrubber liquid and sensitivity of the ultraviolet or refractive index detectors to the compound. This method should not be complicated by water or other materials in the dome.

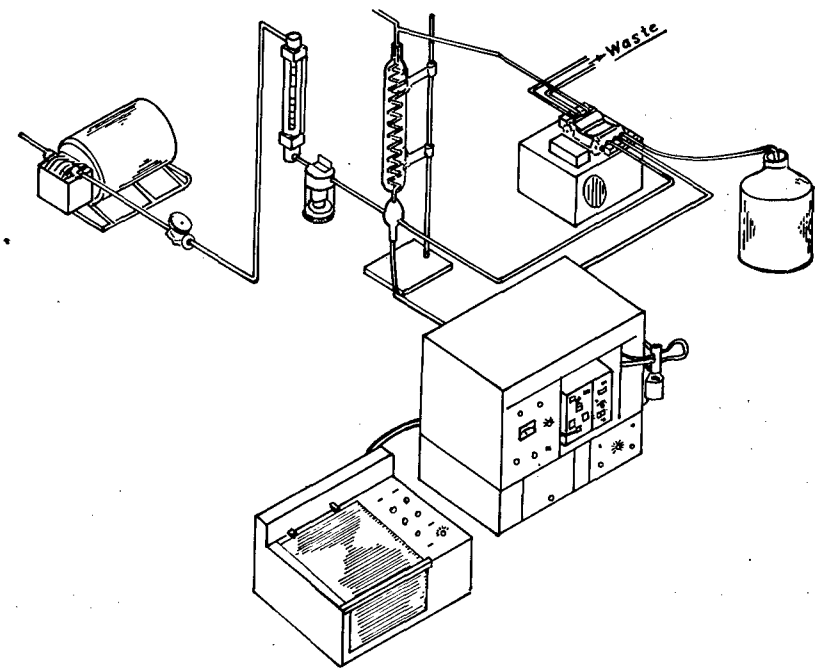

Figure 4. Automatic liquid chromatographic analysis.

Table 1 and 2 summarize the methods and limits of detection.

TABLE 1. DIRECT ATMOSPHERIC ANALYSIS METHODS

Instrument	Detects	Lowest Level
Dasibi - UV	Ozone	10 ppb
Electron Capture - GC	Halogenated Hydrocarbons	10 ppb
Miran - IR	Organics	100 ppb
Flame - GC	Organics	100 ppb
Flame - Hydrocarbon Analyzer	Organics	1 ppm
Thermal Conductivity - GC	Universal	10 ppm

TABLE 2. ATMOSPHERIC ANALYSIS METHODS AFTER SCRUBBING

AutoAnalyzer Colorimeter	A large variety of organic and inorganic contaminants	10 ppb
Specific Ion Electrode	F^-, Cl^-, Br^-, I^-, $S^=$, CN^-, Na^+, and SCN^-	100 ppb
Liquid Chromatograph	Universal	1 ppm

In conclusion, an atmospheric contaminant analysis should be designed around the physical and chemical properties and the concentration. The simplest, most reliable, and most specific analysis should then be chosen from the possibilities.

Modern instrumentation has drastically decreased the difficulty of measuring a single contaminant. The area of difficulty is now in monitoring dual exposures. The Mirans and specific ion electrodes have solved many of these problems for us.

AMRL-TR-75-125

PAPER NO. 10

TOXIC HAZARD EVALUATION BY MASS SPECTROMETRIC THERMAL ANALYSIS

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INTRODUCTION

Late one Friday afternoon, eleven days ago, we received a telephone call from the Wright-Patterson Medical Center for quick advice concerning an emergency case admitted to the hospital's intensive care unit. A laboratory technician became severely ill after he inhaled toxic fumes of thermal decomposition and pyrolysis products from a synthetic polymeric material which was incorporated as a structural component in a jet engine inlet guide vein that he had mistakenly heated to a temperature of up to about 500 C. The synthetic organic product involved was a polymer blend of buna-N, nylon, and a phenolic resin. The attending physician's question was whether we could give him any information concerning the chemical identity of the products his patient might have been exposed to that could aid him in the proper treatment of this emergency case. Based on prior experience in the analysis of thermal decomposition products of synthetic nonmetallic materials, we were in a position to offer some suggestions as to the general class of compounds that might be formed as decomposition products from the polymeric substances in question. But in order to be precise we requested a piece of the authentic polymer for examination. A sample was rushed to our laboratory and immediately analyzed by mass spectrometric thermal analysis for a more detailed toxic hazard evaluation. After little more than a three hours time the analysis was complete with twenty-two major decomposition products identified.

The compounds found were: unsaturated, saturated, and cyclic aliphatic hydrocarbons as well as aromatic hydrocarbons; also alcohols, ketones, and heterocyclic compounds, but most importantly large quantities of a series of aliphatic cyanides such as methylcyanide, ethylcyanide, vinylcyanide, etc., including smaller amounts of hydrogen cyanide, were identified. Without delay these results were reported to the concerned physician and after forty-eight hours the patient was released from the hospital. This is the lucky ending of an emergency case where we tried to help prevent a disaster. Normally, however, we do not deal with emergencies; rather the objective of our research activities is to prevent similar accidents from occurring by identifying natural or synthetic polymers intended for use in habitable enclosed spaces that might produce toxic thermal decomposition products either under normal use conditions or when exposed to heat under catastrophic circumstances such as fire. It was three years ago that I first reported at a previous conference of this series on the method that we developed for automated mass spectrometric thermal analysis (MTA) by combining thermogravimetry and mass spectrometry (Kleineberg and Geiger, 1972). Since that time we have widened our instrumental approach to incorporate a gas chromatograph and a pyrolyzer unit. We also replaced the Cahn thermobalance with a DuPont thermogravimetric analyzer system because it offered the advantage of substantially reduced dead volume resulting in improved sample transfer characteristics. In addition, we expanded the computer software of the library search routine from the original 6,000 to about 23,000 reference mass spectra.

DISCUSSION

Today I would like to talk to you about what we can accomplish with our various MTA techniques and present chemical analytical data that formed the basis for toxic hazard evaluation of thermal decomposition and pyrolysis products from some few selected polymeric materials of recent interest. Figure 1 shows a simplified block diagram of the instrumentation arrangement as presently employed in our laboratory. The sample under investigation is placed into the thermogravimetric analyzer (TGA). This instrument consists of a highly sensitive recording electrobalance in which the balance load inside a quartz tube is positioned in a combustion furnace. The furnace can be heated at various programmed rates and, depending on the kind of purge gas used, the sample is either under inert or oxidative

environment. For instance, a dynamic atmosphere of room air is the sample environment of choice if the test objective is the simulation of a real fire situation. Three parameters - weight change, the rate of the weight change, and temperature increase - are recorded simultaneously by the TGA. Volatile products evolving from the heated material are introduced into the electron bombardment ion source of the mass spectrometer (MS) in one of two ways: (a) continuously and directly via a capillary interface, or (b) at discrete times of degradation through a sample loop into a gas chromatography column. The mixture of decomposition products is then separated and the individual components are subsequently introduced into the mass spectrometer in the order of elution from the gas chromatograph (GC) with most of the carrier gas removed by a jet separator. In both cases, the mass spectrometer scans automatically in the repetitive mode, typically between 600 and 12 atomic mass units (amu) at appropriate scan speeds, for instance at 2 sec per decade for the combined GC-MS approach. Signals generated at the detector/multiplier end of the mass spectrometer are channeled directly into the memory of a minicomputer through the MS/computer interface which counts and thresholds the data. Intermittently, the raw data are transferred and stored on magnetic tape for subsequent data processing. A computer software package, consisting of several subroutines including a library search of about 23,000 reference mass spectra is available for data interpretation. When necessary, a pyrolyzer capable of very rapid heat rise can be coupled to the injection port of the GC to obtain additional analytical data. (This option is not included in the diagram.)

An experimental copolymer of acrylonitrile and styrene in the ratio of 6:4 was heated in an air atmosphere at a rate of 20 C per minute. The evolving thermal decomposition products were continuously and directly introduced into the mass spectrometer which repetitively scanned at a speed of 10 sec per decade. A total of 153 complete mass spectra were obtained for this analysis.

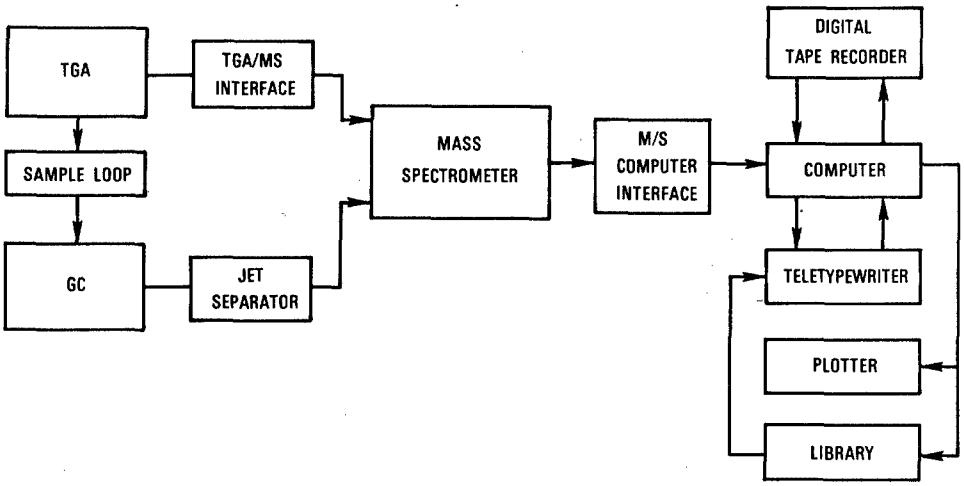


Figure 1. Block diagram of thermal analyzer mass spectrometer system.

Figure 2 represents a bar graph of the 110th mass spectrum of this series and it was recorded when the TGA trace indicated a rapid weight loss at a sample temperature of about 400 C. This spectrum is a composite mass spectrum of all decomposition products evolving from the sample at this point. From the mass spectrum alone it is very difficult to identify all of the individual components that are presented in it. However, with the aid of a computer subroutine, called plot mass chromatogram (PLOTMC), rate curves for the formation of single compounds are readily obtainable. This program provides the plot of a selected specific ion (mass) chromatogram. It plots the number of ions of a selected specific mass in each scan, or the summation of up to four specific masses to include fragment ions versus the scan number.

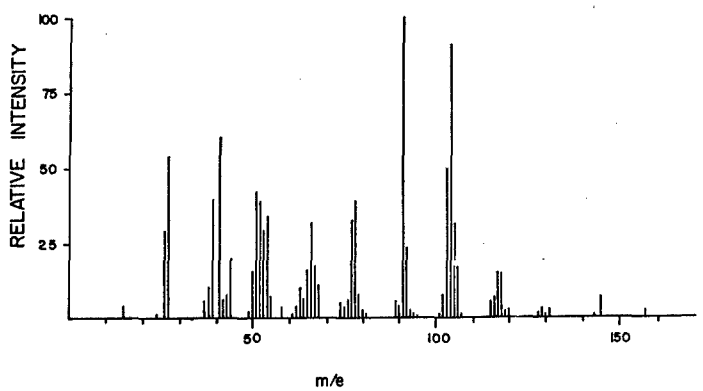


Figure 2. Composite mass spectrum of thermal decomposition products from acrylonitrile-styrene copolymer.

From the presence of peaks at masses 53 and 52 one can assume that monomeric acrylonitrile is one of the products formed. The signals at masses 104 and 103 could result from the styrene monomer, indicating that depolymerization might be a major decomposition reaction, while the mass peaks at 41 and 39 point to the presence of acetonitrile as a decomposition product.

Figure 3 shows the curves for the formation of the monomers acrylonitrile and styrene and the decomposition product acetonitrile. These curves were obtained utilizing the PLOTMC program when the sums of the specified ions were plotted against the scan numbers. From this presentation it can be seen that the three compounds evolve from the decomposing polymer at only slightly different temperatures. [Concerning the additional information contained in this figure: the order n of the decomposition reaction and the activation energies E for the formation of the respective compounds were calculated from the shape and the area under the rate curves for a different study, (Kleineberg et al., 1974)]. Additional investigations substantiated that depolymerization forming acrylonitrile is a major thermal decomposition reaction of this polymer, making it undesirable for applications where elevated temperatures might be encountered.

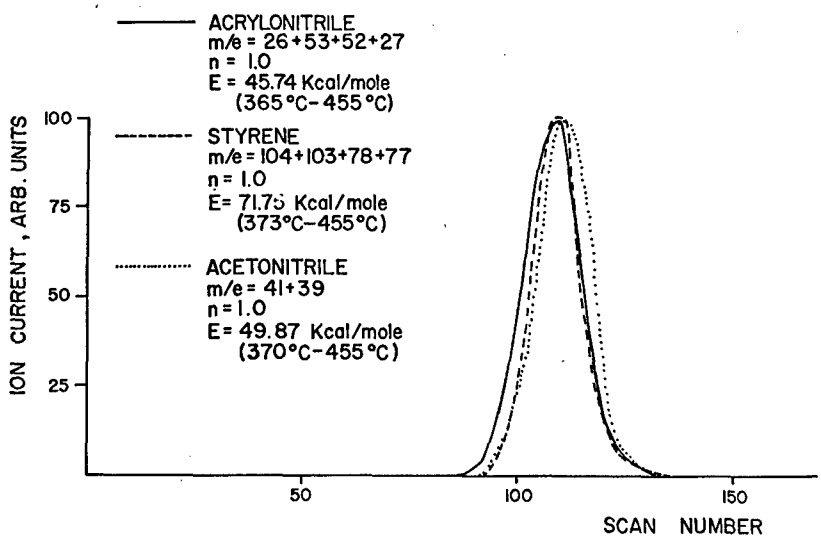


Figure 3. Rate curves for the formation of acrylonitrile, styrene, and acetonitrile from thermal degradation/depolymerization of acrylonitrile-styrene copolymer.

This was an example where the method of introducing the decomposition products directly into the mass spectrometer yielded sufficient information to arrive at a valid toxic hazard evaluation.

The next example I am going to present is a comparative study that was conducted to aid in the decision based on toxicological consideration to recommend either natural wool or synthetic Nomex for a specific application.

Figure 4 shows the TGA traces of Nomex and wool samples that were analyzed under identical conditions. It can readily be seen that the Nomex material is the thermally more stable species. The wool begins to degrade seriously as low as 250 C. The Nomex fabric approaches the same rate of decomposition at a temperature of as much as 300 C higher than that of wool. At those points for the respective degradation processes where both materials had lost equivalent amounts of weight, which was at 330 C for wool and at 575 C for Nomex, a volume of 2.0 ml of air passing over the degrading sample and containing the volatile decomposition products was injected through the sample loop of the GC-MS system for separation and subsequent identification of the complex mixture of compounds.

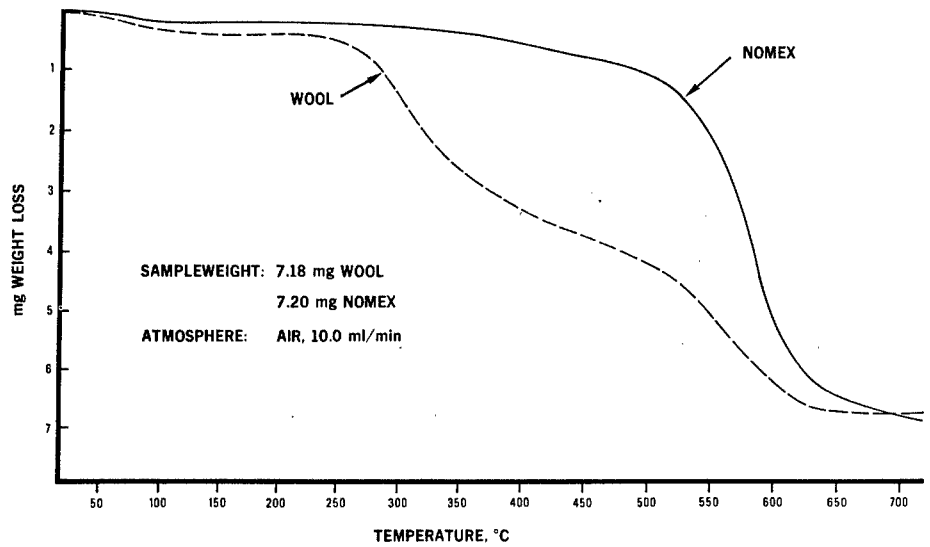


Figure 4. TGA traces of Nomex and wool.

Figure 5 represents the total ion plot recorded by the mass spectrometer for the degradation products of the wool sample formed at 330 C. This plot can be considered as a reconstructed gas chromatogram where each individual peak represents at least one separate decomposition product released at the specific time or temperature when the sample was injected.

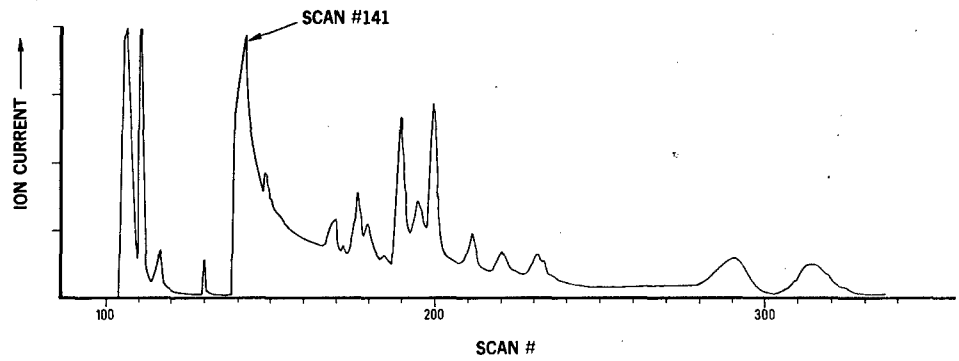


Figure 5. Total ion plot from GC sample of wool at 330 C.

The x-axis in this plot represents the number of each complete mass spectrum obtained for this single GC sample, i.e. a total of 336. To accomplish the identification of all compounds represented by the individual peaks, the corresponding mass spectra are recalled from the data system for display and interpretation. As an example, the spectrum 141 was recorded on top of a major peak as indicated in this presentation. This mass spectrum is shown in Figure 6. Unlike the composite mass spectrum of the unresolved mixture of compounds presented earlier, this spectrum offers no difficulty in interpretation. Outstanding features are the two peak doublets at masses 60-62 and again at 32-34. These masses, together with the observed relative abundance in these doublets, point to the presence of sulfur in the molecule. Accounting also for the additional peaks at masses 12, 16, and 28 produces the complete answer. The spectrum displayed here is that of carbonylsulfide (COS).

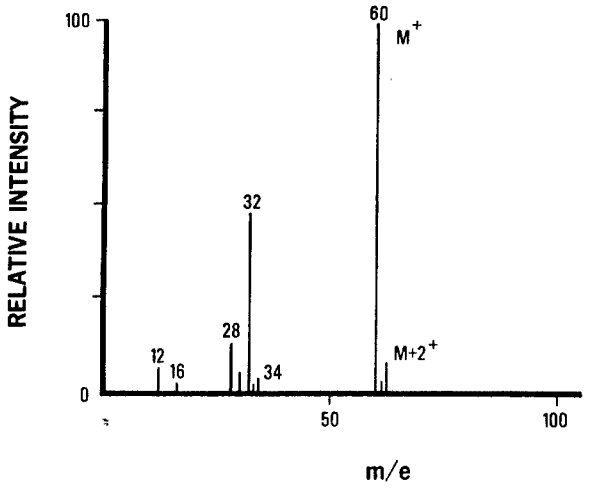


Figure 6. Mass spectrum recorded as scan #141.

The library search program offers a convenient aid for spectrum identification. In applying this computer routine, the "unknown" spectrum, in this case scan 141, was compared to all 23,000 reference spectra on file.

Table 1 shows an abbreviated form of the computer printout that was received as the answer. The list consists of the five spectra that best fit literature spectra including a quantitative measure of the similarity and identification codes. Indeed, two reference spectra of carbonylsulfide compared with a high similarity index to the "unknown" spectrum (#141), confirming our previous tentative interpretation.

TABLE 1. COMPUTER PRINTOUT FOR LIBRARY SEARCH

TGA Wool in Air Run #9

Ignore 18, 17, 0, 0

Scan #141 Goodness	Base 60 ID. No.	Page
844	API-0166	0000 Carbonyl Sulfide
762	APIH9066	0001 Carbonyl Sulfide
403	MSCH9004	0001
400	KOD-0163	0000
375	DJEH9003	0001

When at all possible, an additional confirmation for positive product identification is made by comparing the observed gas chromatographic relative retention times of the unknown to those of authentic compounds.

From determinations of relative retention times we know that water is eluting from the gas chromatograph, which uses a 2m, 1/4 inch Chromosorb 102 column, at the same time as carbonylsulfide. For this reason we excluded the mass peaks at 18 and 17 as interference from the spectrum of carbonylsulfide for the search routine. We have the option of ignoring a maximum of up to four peaks from the spectrum under investigation. This routine together with the capability to subtract one spectrum from another is frequently utilized to clean up the spectra from instrument background or interfering substances in unresolved chromatographic peaks.

The procedure for identifying all the individual peaks that we see in the total ion plot is essentially the same as I described for the identification of carbonylsulfide.

Table 2 shows the result of this effort for the analyzed wool sample. Seventeen identified compounds evolving from the degrading wool at 330 C are grouped in this list. The numerical values represent the rates of evolution expressed in mg of product released from one gram of wool during the period of one minute. Besides large amounts of CO_2 and water, there are the sulfur containing compounds of hydrogen sulfide, carbonylsulfide, and carbon disulfide. Saturated and unsaturated hydrocarbons are represented by the group listed from ethane through toluene. We also find the oxygenated compounds acetaldehyde and acetone, besides the heterocyclic pyridine, but most important is the presence of several organic cyanides. In contrast, Table 3 lists the decomposition products from a Nomex sample which was treated and analyzed under identical conditions. Aside from the fact that the thermal decomposition of Nomex is shifted to a considerably higher temperature, the degradation products are far less in number. In addition to the major components of carbon dioxide and water, we find smaller amounts of only acetaldehyde, hydrogen cyanide, and methylcyanide. Based on these chemical analytical data, and due to the difference in thermal properties, it was apparent that on a gram to gram comparison of these two samples, Nomex is the much preferred material for use onboard aircraft from a toxicological standpoint.

TABLE 2. THERMAL DEGRADATION PRODUCTS OF WOOL EVOLVING AT 330 C (mg/g/min).

CO_2	310	Toluene	8
H_2O	260	Pyridine	5
H_2S	22	Acetaldehyde	11
COS	14	Acetone	10
CS_2	8		
Ethane	34	Methylcyanide	40
Propene	16	Ethylcyanide	15
Isobutane	22	Isobutylcyanide	11
Butene	6	Butylcyanide	17

TABLE 3. THERMAL DEGRADATION RPODUCTS OF NOMEX EVOLVING AT 575 C (mg/g/min).

CO_2	458
H_2O	420
Acetaldehyde	27
HCN	47
Methylcyanide	38

For the next example I present the results of a similar investigation where two materials of the same chemical nature were compared. One sample was the untreated material and the other sample contained a finish.

Figure 7 represents the TGA trace of an Aramid batting which is a polyamide. The thermal properties of this material are very similar to Nomex which is also a polyamide polymer.

The first weight loss step observed at around 50 C is exclusively due to the evaporation of adsorbed water. The material remains stable up to about 400 C. Between 400 C and 475 C an appreciable breakdown step occurs with a weight loss of several percent. This is followed by a catastrophic decomposition with its maximum rate occurring at 540 C. At 600 C the entire material is consumed, leaving only a very small amount of residue.

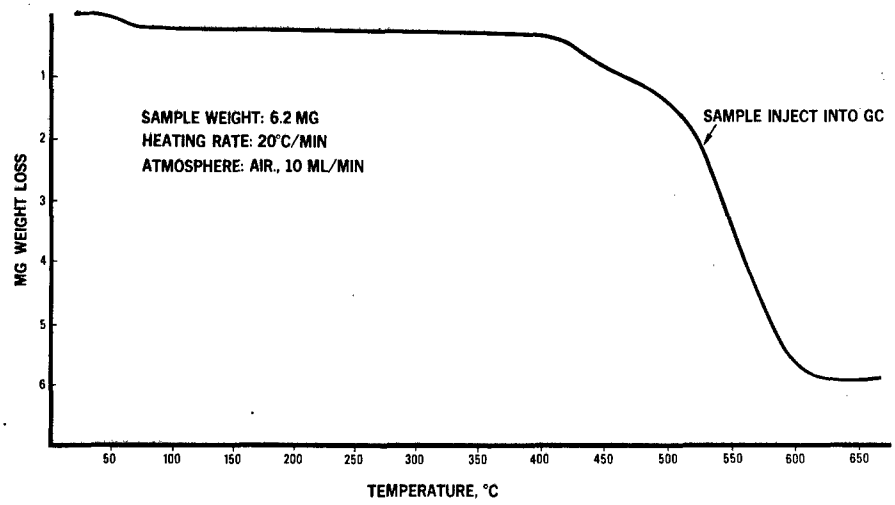


Figure 7. TGA trace of Aramid batting.

At the point where the sample temperature had reached 540 C, 2.0 ml of the gas surrounding the sample containing mostly air plus the evolving volatile oxidation/decomposition products was injected through the sampling loop into the gas chromatograph for analysis applying the same techniques that I described earlier.

Figure 8 represents the total ion plot from this GC sample of the Aramid batting material's decomposition products. The major components were identified as the oxidation products carbon dioxide and water. Acetaldehyde, methylcyanide, and a trace amount of an unidentified third material were the only decomposition products present in this sample.

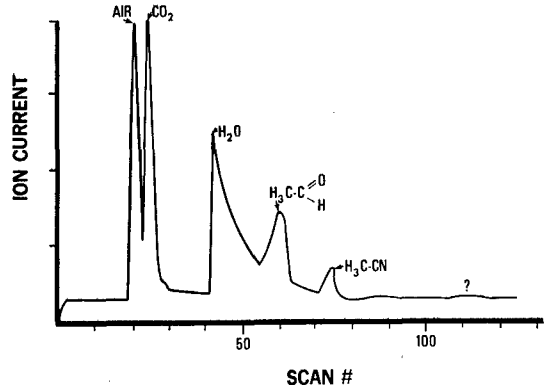


Figure 8. Total ion plot from GC sample of Aramid batting at 540 C.

The TGA trace for the Aramid fabric with finish is shown in Figure 9. This thermogram differs from that of the untreated Aramid sample. Following the initial water desorption, we observe a continuous, creeping, weight loss between 75 C and 420 C before a more pronounced decomposition sets in similar to that in the untreated sample. At 420 C a sample of the evolving decomposition products was injected into the gas chromatograph and analyzed in the usual manner.

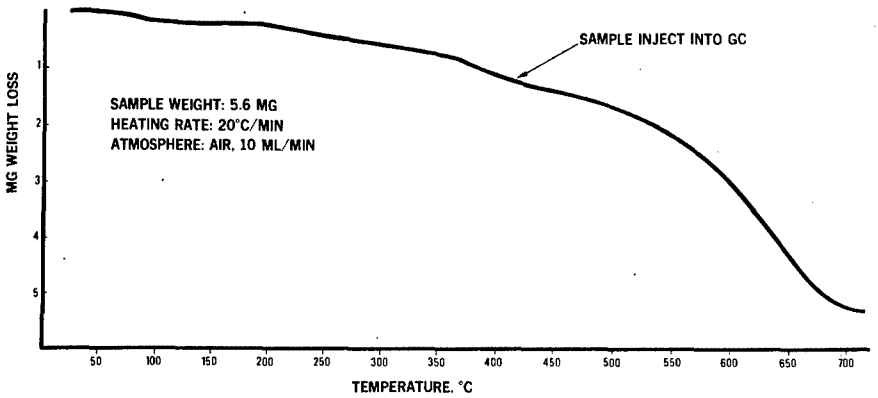


Figure 9. TGA trace of Aramid fabric with finish.

Figure 10 represents the obtained total ion plot with a surprising total of at least 17 peaks of various sizes. The chemical identity of the decomposition products represented by the peaks numbered 2 through 17 is listed in Table 4.

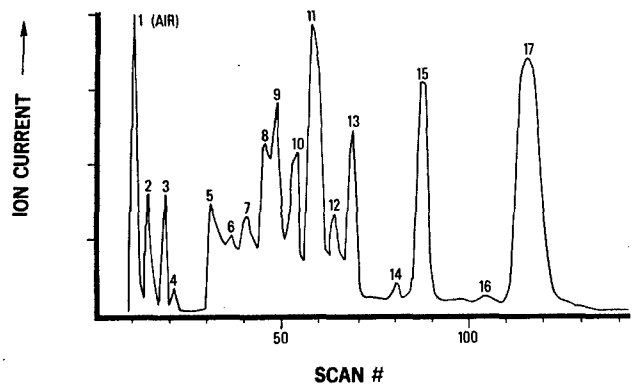


Figure 10. Total ion plot from GC sample of Aramid fabric with finish at 420 C.

TABLE 4. THERMAL DEGRADATION PRODUCTS OF ARAMID FABRIC WITH FINISH AT 420 C.

Peak #		Peak #	
2	Carbon Dioxide	10	Bromomethane
3	(Ethene)?	11	Ethanol
4	Ethane	12	Cyclobutane
5	Water	13	Bromoethane
6	Propylene	14	Vinyl Acetate
7	Chloromethane	15	Methyl Acrylate
8	Methanol	16	Benzene
9	Acetaldehyde	17	Ethyl Acrylate

Methylcyanide which was formed from the untreated sample is missing on this formidable listing. This is due to the difference in temperature at which these two GC samples were obtained for analysis, which was 540 C in one case and 420 C in the other. Further samples from the finished Aramid taken at the same temperature of 540 C also contained methylcyanide, but less of the other components. In addition to carbon dioxide, water, and acetaldehyde, which were also present in the oxidation/decomposition products formed by the untreated Aramid, the mixture of decomposition products from the finished Aramid fabric consists of aliphatic unsaturated, saturated, cyclic, and halogenated hydrocarbons and benzene, as well as alcohols and several esters in large quantities. All these compounds are formed from the applied finish material.

Both materials that were compared here are candidates for the same application. The finished material certainly has a more appealing aesthetic appearance and may have improved mechanical properties; however, based on toxicological evaluation of the analytical data, it is obvious that the untreated material offers the advantage of increased safety.

As a last example I would like to discuss the results of mass spectrometric thermal analysis performed on fluomine.

Figure 11 gives the chelate structure of this compound. Fluomine, or more accurately cobalt - bis(3-fluoro-salicylaldehyde)-ethylenediamine is able to reversibly adsorb about 4.4% oxygen. It is for this property that it is being considered for an oxygen delivery system onboard aircraft. Figure 12 shows the TGA trace obtained for this compound.

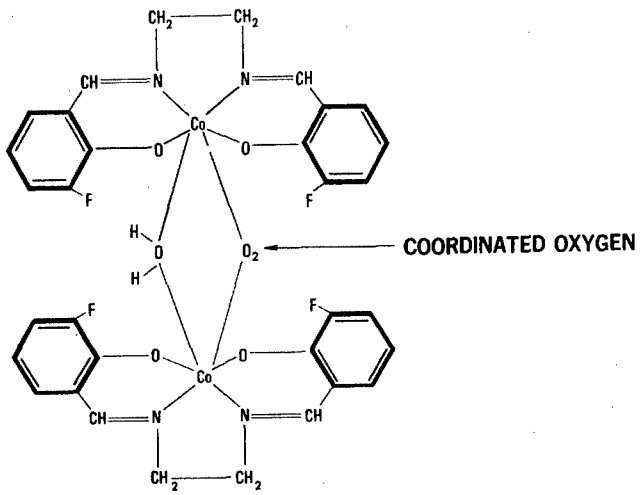


Figure 11. Chelate structure of fluomine.

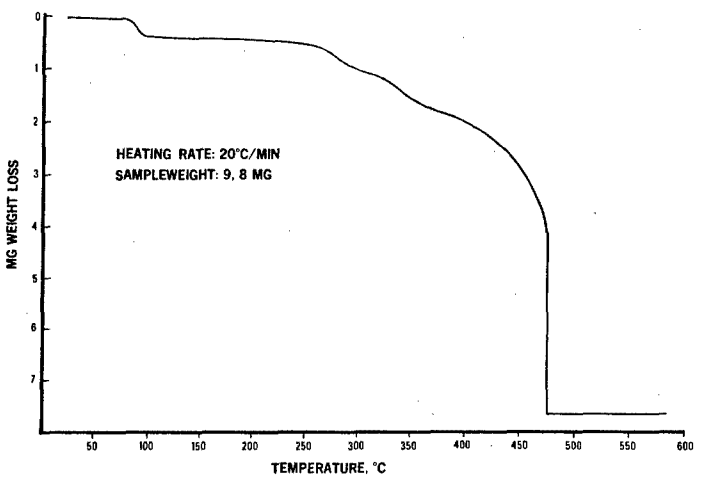


Figure 12. TGA trace for fluomine in air.

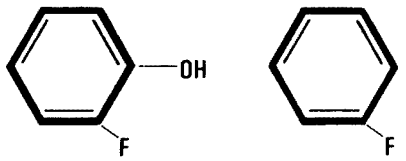
When fluomine is heated, a weight loss of from 4.0% to 4.33%, exclusively due to oxygen desorption, is observed at about 75 C to 90 C. Immediately following desorption, the same amount of oxygen can be readsorbed by the fluomine when air is blown over the cooling sample. However, when the cobalt complex is heated to only 175 C it loses its ability for oxygen absorption completely. It is interesting to note that all thermograms obtained showed no further weight loss at this temperature beyond that accounted for by oxygen desorption. From this it can be concluded that a shift in the physical matrix of the crystalline structure rather than thermally induced chemical decomposition of the compound is responsible for this drastic property change.

The proposed use of the fluomine involved cycles of heating and cooling. In order to investigate what would happen to this compound in case it gets exposed to even higher temperature due to a possible heater malfunction, the chemical was heated at a rate of 20 C per minute up to 580 C. Between 250 C and 425 C, 17.3% of the sample weight is lost. At about 425 C a very rapid exothermic degradation/oxidation began. This final decomposition step was complete at 475 C. An inorganic residue of 20.6% is left.

For the identification of the decomposition products that are formed at those temperature ranges, the gaseous breakdown products were introduced directly into the mass spectrometer and also sampled for prior separation by the gas chromatograph.

Table 5 is a listing of the decomposition products identified using these techniques. Ortho-fluorophenol and fluorobenzene are the predominant compounds found during the first breakdown step. The larger and more vigorously occurring final breakdown step almost exclusively yields the oxidation products carbon dioxide and water. The concentrations of hydrogen fluoride and of the nitrogen oxides vary somewhat for the two distinct decomposition steps but they are always present as are traces of hydrogen cyanide and carbon monoxide. The amount of 20.6% for the residue is in good agreement with the theoretical amount of 20.8% that one would expect for Co_3O_4, the mixed cobalt oxide most likely to be formed under the experimental conditions employed.

TABLE 5. THERMAL DEGRADATION PRODUCTS OF FLUOMINE.



HF CO_2 H_2O NO_X

HCN (TRACE) CO (TRACE)

Co_3O_4(RESIDUE)

Figure 13 shows the results obtained when a sample of fluomine was pyrolyzed in a pyrolyzer at a ramp speed of 1 C per millisecond to a final temperature of 325 C for 2 sec. The pyrolyzer was coupled directly to the GC/MS-System, which identified the four compounds listed in the bottom line of Figure 13 as pyrolysis products that were formed under these conditions.

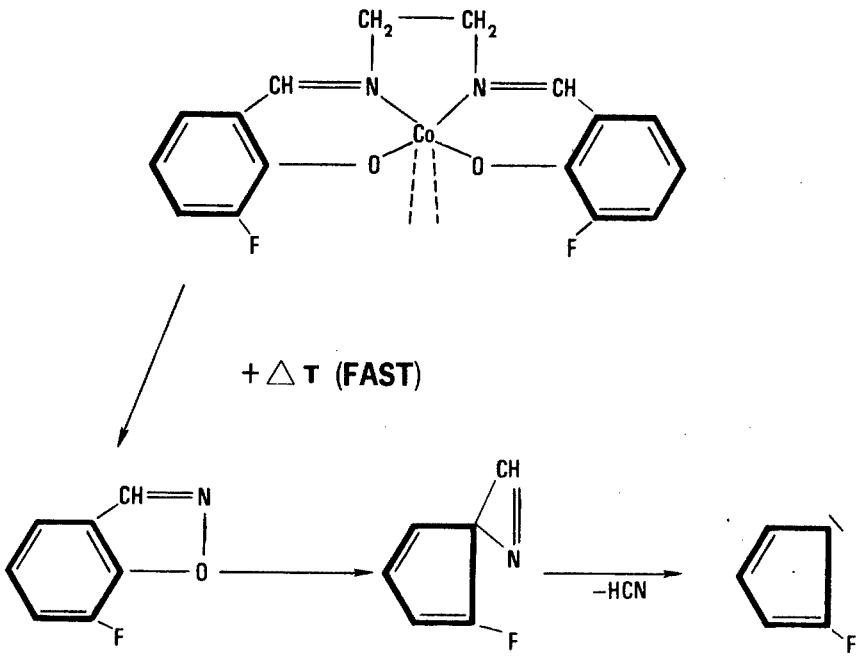


Figure 13. Pyrolysis products of fluomine.

In the upper portion of Figure 13 we see once more the structural arrangement of the fluomine molecule. The formation of 7-fluoro-benzo-isoxazole is not very surprising. The basic configuration is already present in the original fluomine molecule. After cleavage of the covalent and coordination bonds of the cobalt chelate structure, a stabilization by ring closure resulting in the formation of an isoxazole seems to be a likely reaction because of the aromaticity of the end product.

The formation of the spiro derivative can be explained by carbon monoxide (CO) elimination from the isoxazole.

This reaction is followed by expulsion of a hydrogen cyanide molecule leading to the ionic species that is seen in the lower right hand corner. The HCN molecule is already preformed in the spiro compound and this makes its elimination a very likely reaction to occur.

The isoxazole, the spiro compound, and also the ionic species could not be detected in the mixture of decomposition products that we analyzed from the TGA, possibly because they are too short-lived to be isolated under the conditions employed there. However, traces of CO and HCN were always present, which could mean that we identified their otherwise unknown precursors by using the supplementary technique of pyrolysis.

Taking all the results into account that we obtained employing the various analytical approaches that I discussed, our recommendation was that the fluomine could be used safely as an oxygen delivery system onboard aircraft only if a means of preventing accidental overheating beyond 140 C is provided.

These were but a few examples of our work, but they should suffice to demonstrate what we as chemists can do in the performance of mass spectrometric thermal analysis to aid the toxicologist in his job of hazard evaluation.

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Kleineberg, G. A., D. L. Geiger, and W. T. Gormley, (1974), "Rapid Determination of Kinetic Parameters for the Thermal Degradation of High Polymers Utilizing A Computerized Thermogravimetric Analyzer-Mass Spectrometer System," Die Makromolekulare Chemie, 175:483-495.

OPEN FORUM

DR. TERRILL (E. I. duPont de Nemours & Co., Inc.): I'd like to ask Dr. Kleineberg if animals are used in the evaluation of these materials?

DR. KLEINEBERG (6570 Aerospace Medical Research Laboratory): No, we have not used animals in these in-house studies yet but there are plans in the offing that we might go to that step.

DR. TERRILL: Well, I would not only add "might." I think you should. As a matter of fact, there are quite a few people working in this field of toxic off-gases or combustion products who have found over the past couple of years that this is a must, and that the mass spectral work, the analytical work, is a backup to the animal exposures.

DR. KLEINEBERG: It is not a matter that we aren't aware of. We are not doing animal exposures up to this point because it's a matter of logistics, of personnel, and of money. But I'm glad you brought this point up.

MR. BELL (University of Cincinnati): Dr. Kleineberg, would you please elaborate on the case history described at the beginning of your talk. You described an exposure to an abundance of alkyl nitriles as well as a small amount of hydrogen cyanide. In light of your analysis, what did the medical treatment include? Was it specifically for the cyanide?

DR. KLEINEBERG: I am unable to tell you details about the treatment given because I wasn't at the hospital at the time. We only know that the patient survived, and he later came to our laboratory and was very grateful for the information we were able to provide. I think that the information given to the hospital really aided in the proper treatment of the case. What the treatment was and what the symptoms were, I am not at liberty to tell you.

DR. A.THOMAS (6570 Aerospace Medical Research Laboratory): If we have a little time during the open forum, I think that Dr. Vera Thomas who gave the modeling paper kind of lost her punch line because of the shortness of the time for presentation. I would at least have her state what was the application of those findings.

DR. V. THOMAS (University of Miami): What we tried to do was correlate the effects of interrupted industrial exposure to continuous exposure. It is assumed that the effect would be proportional to the concentration. Now, if you would like to go one step further, if you determine that the metabolism is a first order reaction at low concentrations and you determine your rate of metabolism, then gradually increase your concentration of contaminant by a multiple of 5 or 10, you will probably find that metabolism will start to follow some sort of Michaelis-Menten kinetics. At some concentration, I assume, the metabolic rate will no longer be a first order reaction. The next question we will ask, is it related to some toxic effect? Is it possible that the rate of metabolism will change when you have continuous exposure? So it can signalize some changes in metabolism and probably its toxicity because the toxicity of most organic compounds that produce necrosis or organ toxicity or carcinogenicity is related to the metabolism. One thing I think is very important to point out is that we have here a noninvasive method to measure overall metabolism, not only metabolism of those metabolites which are excretable and found in the urine, but also the metabolites that stay in the body in the form of carbon binding. The other thing I didn't have time to draw to your attention is that you can more rapidly equilibrate partial pressure of the compound in the body by increasing the concentration in the inhaled air. The first thing with which we have to be concerned is the ratio of concentration in alveolar air to the concentration of the inhaled air. At the beginning of exposure taking the first breath, what goes into the lung and what is exhaled? It is the inhaled concentration multiplied by ventilation rate, and let's call that the alveolar ventilation. This is what is available for inflow into the lung. Now, what goes out from the lung? This is the concentration that we have in our lungs multiplied by ventilation rate plus what the blood has taken up, which is expressed as cardiac output multiplied by arterial concentration. The arterial concentration is immediately equilibrated with alveolar concentration, so it equals alveolar concentration multiplied by the partition coefficient for blood and air. As a rule, the inflow must be equal to the outflow because it has to reach an equilibrium, and so I will finish the equation with something that equals cardiac output, divided by alveolar ventilation times the partition coefficient for blood and air. This is what determines the ratio at the beginning of exposure. For example, if you expose a man to methylene chloride, the ratio usually is 1.95 for the cardiac output/alveolar ratio. The blood partition coefficient is around 7.

Now if you do all this mathematics, you find out that in the first breath of methylene chloride, you have actually been inhaling 11% of the exposure concentration. The lung has received only 11% of the concentration you inhale. Now, to compensate for that, you can increase the concentration in the inhaled air to speed up the equilibrium. This means the venous concentration will be equal to arterial concentration for nonmetabolized blood which is equal to 1. For metabolized blood, the concentration will depend on metabolic coefficients and alveolar ventilation. I think the resultant equation is something like I have shown on the blackboard. So, what do you do to speed up and reach the desired blood concentration of methylene chloride instead of waiting all day to equilibrate with partial pressure in the inhaled air? You increase the concentration in the beginning. Now, based on this premise, you can program the concentration in air to increase 5 fold if that is not too irritating and does not exceed some ceiling level. For a short time period, you can increase the concentration up to five fold. By increasing the inhaled concentration of methylene chloride, you unbalance the equation to the point where the expired air reaches the desired level to speed up equilibration of its partial pressure. The other thing I wanted to point out is that metabolism depresses the concentration in the tissues.

MR. VERNOT (University of California, Irvine): This question is for Dr. Thomas, also. There's some strong evidence that one of the major metabolic products of methylene chloride is carbon monoxide. I wonder whether you've considered this in your experimental design and whether this might have some implications about what you might do with this sort of work in the future.

DR. V. THOMAS: Yes, I would like very much in my next experiment with methylene chloride to arrange so that I will test this concentration dependence. I believe that I will encounter enzyme inhibition because carbon monoxide probably in situ in the liver inhibits cytochrome P-450, which we know are bound together. It will cause a problem and probably will cause a problem sooner if we used animals pretreated with phenobarbital. I would like very much to do these experiments.

DR. GAUME (Douglas Aircraft Company): Question for Dr. Lewis. In the TLV booklet, why is the skin TLV listed rather than the respiratory? Why don't they list a respiratory TLV also?

DR. LEWIS (National Institute for Occupational Safety and Health): For what particular compound are you talking about?

DR. GAUME: HCN. There is an air concentration.

DR. LEWIS: Are you correctly interpreting the skin notation? There is an air TLV concentration for every compound that has a skin notation. The word "skin" with a TLV value just denotes the fact that skin adsorption can be a significant contributory factor to the total body exposure. In other words, if it's practical, you should eliminate dermal contact totally.

DR. PETERING (University of Cincinnati): I'd like to ask a question of Dr. Lewis also. I'm interested in how successful the TLV committee has been in carrying out the philosophy which you expressed so elegantly in your slides of attaining a TLV which had a two to five fold safety limit. I'm specifically interested in the problems associated with cadmium. As I recall, a cadmium study was made with the criteria documents two or three years ago, and I don't believe the TLV limit for the workplace has yet been changed. Has it or not?

DR. LEWIS: Which study are you referring to? Do you mean the preparation of the documentation?

DR. PETERING: The documentation set up the probability of the necessity for reducing the TLV and yet there had been no action on that matter. I'm wondering how successful you are in carrying through the philosophy which you have expressed in other cases. That was one case in which, I think, there has been considerable delay. I don't know whether the TLV still has been changed.

DR. LEWIS: I want to make it perfectly clear that the ACGIH committee on Threshold Limit Values does not prepare criteria documents nor do I in my capacity as a NIOSH employee. There are several of the people here from NIOSH that are involved in criteria document preparation. The TLV committee, as you know, is a totally independent organization. Our TLV's have no direct relevance to the standards that are set by ORSD. Sometimes the committee uses the same numbers, and sometimes we have different numbers. It's a value judgment, so to speak. We solicit all the

material that we have. Once again, in my tenure on the TLV committee, cadmium has come up from time to time, particularly due to the impetus of Hector Blair, but we have not seen fit to change the TLV recommendation as such. I really can't speak for the NIOSH figure. I don't know whether Dr. Jacobson would care to speak about it or someone else from ORSD.

DR. MAC EWEN (University of California, Irvine): My question is to Dr. Lewis. I'd like to ask you something about the methodology or the approach to establishing the threshold limit values. For example, I recognize that many of them are based on experimental animal evidence of morphologic change either in one or more organs but many on the lung alone. By morphologic change, I mean irreversible anatomic injury. In view of our discussion earlier this afternoon about the more sensitive techniques of pulmonary change or pulmonary effects, would you say that a measurement of quantitative morphometric changes or electron microscopic changes such as the rounding up of mitochondria at the threshold limit would be suggestive of a need for downward revision for some of the TLV values?

DR. LEWIS: I'm glad you used the word suggest rather than mandatory. I would certainly concur that it would be suggestive. It would not necessarily be mandatory. These are the types of things that wave a red flag. Every time we have a TLV committee meeting, the members prepare an agenda of those action items that should be dealt with. The agenda items are promoted by recent findings whether it be in the pesticide field or whatever it is. As I said, the inhalation route is the primary route of occupational exposure. I always work on the premise that the lung is a target organ because it's the first organ that comes in contact with a chemical. And just relating to some of the discussion that took place in the previous forum session, I'd like to see animals looked at physiologically. You can look at rats and guinea pigs but there are limitations imposed so I prefer to use some dogs and monkeys in my experiments. I'd like to have preexposure values. I'd like to have serial physiological measurements throughout the duration of the study and at the termination of the study. And in other cases, we work with effects such as the peripheral neuropathy induced by MBK. I think it's very important in toxicological experiments to carry extra groups of animals to see whether these toxic properties are reversible. This is something that is seldom done.

MR. VERNOT: This is for Dr. Lewis and is prompted by your last statement. What's the significance of reversibility of toxic signs?

DR. LEWIS: Well, it's a prognosis as to whether or not man is going to have a continuous health problem and all of the other legal, social, economic factors that interact with that. It is very encouraging to me that in our animal experimentation regardless of whether we used 100 ppm or 1000 ppm of MBK, we had a reversion of the neuropathy and finally obtained normal nerve conduction velocities. It would also be important to know if some of those terminal lung lesions that Mr. Moorman referred to from dust exposures were reversible with time. Remember in the TLV booklet, we have a number of substances which we call nuisance dusts. The Federal Air Standard now is 15 mg/m^3 but the TLV is 10 mg/m^3. The TLV committee has lowered its value but it hasn't been changed by legislation. Mr. Moorman has looked at coal dust, both the dust that comes from the mine and that which has been sterilized by heat showing that you still get the peripheral airway abnormalities in sterilized dust, meaning it's not the infectious agents that are carried on the dust that are causing this peripheral disease. He's also looked at polyurethane foam dust and found the same thing. So far, all the dusts that have been looked at in this manner show the same response. Now, this opens up another Pandora's box. I made the plea for more sensitive tests in toxicologic assessment. That is a two edged sword. We are seeing very, very sensitive tests being employed in behavioral toxicology such as alterations in the electroencephalogram. We're getting very sophisticated and sensitive in pulmonary function testing. We're reaching the point, like we did for the sensitivity of analysis of contaminants in the atmosphere, where somebody's going to have to make judicious judgment as to whether or not these things are just adaptive responses to a chemical stimulant that have no toxicological consequences or whether they truly reflect an adverse effect.

COL. STEINBERG (HQDA, Washington): Speaking as a member of the TLV committee, I think there is one thing that hasn't been brought out that I think is significant. In the establishment of a TLV by ACGIH in contrast to the preparation of a NIOSH criteria document is the ability or permissiveness for using unpublished data. So when one speaks of animal effects, quite often in addition to the published literature, the experience

of industry in manufacturing the chemical and the controls that they've applied are available to us which oftentimes is a very significant source of information in the establishment of a TLV. In addition, I'd like to mention the use of analogies which is a practice that draws much criticism. In the past there were often no inhalation data available, particularly in pesticides, so one would have to use feeding data and draw analogies as to what might have happened. This isn't as far-fetched as it may seem because the effects are usually the same; there is very little change in most of the pesticides in the gut. So, again in developing these TLV's, a great deal of experience is drawn from the members of the scientific community who are in fact, often in industry. Oftentimes you see that notation "private communication" for what is called a reference which unfortunately is exactly that, a private communication.

DR. LEWIS: I concur. I didn't bring some of that out due to the time factor and also because some people might question data that is derived from industry. I don't want to open up that box. The point is we do get burned occasionally when we set TLV's by analogy and methylbutylketone is an example of that.

DR. STERNER (University of California, Irvine): Last week I attended a meeting of the American Academy of Occupational Medicine, the American Academy of Industrial Hygiene in Houston. There was a symposium on the "socially acceptable risk" and there were representatives from labor, representatives from industry, and representatives from the government. The elephant looked just like an elephant when we were finished, but obviously everyone had found a different portion of the elephant. It's going to be a problem that's with us from now on. The data that we put in on the biological side is only going to be one element in the equation but a very important element and we'd better have it as reliable as we can get it. But the question then becomes, are we going to permit an individual to have a 1% defect in his liver function, for example, if we could measure it that way? We can't. But it is going to be just this kind of decision that society has to make, and in which we are going to have an extremely important input. Hopefully, as reliable as that can be.

SESSION III

GENERAL TOXICOLOGY

Chairman

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AMRL-TR-75-125

PAPER NO. 11

CURRENT TECHNIQUES OF EVALUATING CARDIODYNAMIC FUNCTION

Alan M. Harris, Captain, USAF, VC
Michael L. Horton, Captain, USAF, VC
Ralph N. Terpolilli, 2nd Lt, USAF
Roger C. Inman, Major, USAF, VC
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Toxic effects of inhaling the low molecular weight halogenated alkanes chlorobromomethane (CH_2BrCl), difluorochlorobromomethane ($CBrClF_2$), and bromotrifluoromethane ($CBrF_3$) have been documented by this laboratory (Van Stee and Back, 1969, 1971, 1972; Van Stee et al., 1973, 1974, 1975). The toxic effects as well as recommendations for use of the halocarbons and tentative exposure criteria have been compiled in a comprehensive review for the Air Force (Van Stee, 1974).

The majority of exposures to CH_2BrCl, $CBrClF_2$, and $CBrF_3$ were conducted using anesthetized, surgically instrumented dogs. Under these conditions relatively high concentrations of the compounds could be used without encountering resistance by the animal. The concentrations were 27-75% $CBrF_3$, 4-12% $CBrClF_2$, and 0.3-1% CH_2BrCl. Although the experiments were controlled as carefully as possible, the influence of anesthesia, surgical stress and trauma, and time on the open-chest preparations could not be fully evaluated in terms of an actual exposure situation. A new procedure was, therefore, designed to make the exposure environment more

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realistic, to eliminate concurrent surgical factors from the experiments, to confirm or deny previous results and to evaluate the tentative exposure criteria cited in the review.

This procedure was designed to evaluate any compound of interest with possible cardiodynamic effects. Interest expressed by the armed services made the aforementioned halocarbons the first compounds to be studied. The Army currently employs a $CBrF_3$ extinguisher in helicopters and tanks and is interested in the practicality and safety of switching to a $CBrClF_2$ hand-held model. The Air Force uses $CBrF_3$ as a total flooding system in the C-5A Galaxy and is interested in finding a replacement for the hand-held CH_2BrCl extinguisher used throughout the service. CH_2BrCl has inherent problems of vapor toxicity, inadequacy in extinguishing Class A fires (solid combustibles), corrosivity to aircraft (it forms HCl and HBr when combined with water - even air moisture) and adverse effects on many materials because of its effectiveness as a solvent. Current attention is focused on the combination of $CBrF_3$ and $CBrClF_2$ as a safer, more effective extinguisher. The extinguisher contains $CBrClF_2$ at 61.47%, $CBrF_3$ at 30.25% (weight basis) and a surfactant foaming agent.

To study these compounds, mongrel dogs weighing at least 25 kg are chosen on the basis of docile behavior and maintained in the colony vivarium where diet and physical conditions are stabilized. Since cooperation of the animals during the experiment is of paramount importance, they are repeatedly brought to the exposure room for acclimation to the surroundings and to the personnel. Over a period of weeks, this has proven to be so successful an endeavor that even dogs which were intractable initially became friendly and cooperative.

During the period of acclimation, three operations are scheduled for each dog. The first is the preparation of a carotid loop. Two parallel skin incisions are made in the neck 3-5 cm apart (distance depends on elasticity of the skin and surgical judgement), lateral to the trachea. The skin flap is kept moistened with a saline soaked sponge while the carotid artery is dissected free from the sheath. The skin flap is then closed around the artery. After placing penrose drains subcutaneously, the neck incision is closed. The drains are removed after the swelling subsides. Since this procedure requires much post-surgical attention and a long healing period,

an alternative was sought. Currently, a polyvinyl catheter is being inserted into the internal thoracic artery and threaded to the base of the heart at the time of thoracic surgery. This is to be done in addition to the loop until it can be proven useful over long periods of time. In this way the number of procedures, hours of aftercare and length of recovery time can be reduced. During the experiment the carotid loop (or internal thoracic catheter) provides access to arterial blood for blood gas and chemical analysis and, in conjunction with a jugular catheter, provides the capability of performing Stewart-Hamilton indicator-dilution studies with indocyanine green.

The second operation, creation of a chronic tracheostomy, facilitates the exposure of conscious animals to gaseous compounds. An elliptical skin flap is removed from the neck over the trachea. A rectangular incision is then made in the tracheal rings and 4-5 of them are removed depending on width. The skin is sutured edge to edge to the incised cartilage and allowed to heal. The removal of the segments of skin and cartilage together with daily insertion of an endotracheal tube prevent closure of the tracheostomy.

The final operation entails a thoracotomy at the left fifth intercostal space and the implantation of the remaining cardiac monitoring devices. An electromagnetic flow transducer is placed around the left coronary circumflex artery for the determination of blood flow. A polyvinyl or silastic catheter is placed in the great coronary vein at the ostium of the coronary sinus, providing access to blood that has passed through myocardium exclusively, for blood gas and chemical analysis. An implantable pressure transducer is then placed through the left ventricular free wall into the left ventricle in order to measure left ventricular pressure and contractility; heart rate is also determined with this instrument. Prior to closure, a catheter is inserted into the internal thoracic artery as previously described. The leads from the instruments and the catheters are brought out the dorsum of the dog between the scapulas. Following 7-10 days recovery period the animals are ready for the experiment. A jacket has been specially designed to protect the instruments between experiments (Horton et al., 1975). The jacket is made of sturdy material with pores for air circulation. The pouch on the back is constructed of bullet-proof nylon to prevent chewing of the leads within it.

The entire experimental procedure per se requires no more than a few hours time with the exception of blood chemical analysis. Before the experiments start, standards are made for blood halocarbon determination by head space analysis with a gas chromatograph. At the same time the exposure gas concentrations are prepared in a mylar bag and the indocyanine green dye for the Stewart-Hamilton curves, syringes and other equipment are readied for the experiment. All instruments that are to contact dog blood are sterilized in advance. Upon completion of preexposure preparation, the dog is placed on the table and the back pouch on the jacket is opened to unfasten the leads. Once the jacket is removed, catheters are inserted in the carotid loop (when used) and jugular vein and in turn connected to the dye syringe (venous) and to the densitometer (arterial). An endotracheal tube, moistened with xylocaine, is then inserted into the trachea through the tracheostomy, the cuff is inflated and a one-way system is attached. The inspiration port is connected to the gas bag and the expiration port is attached to a vacuum system to remove expired gases from the environment. Finally, the flow and pressure transducers are connected (Figure 1).

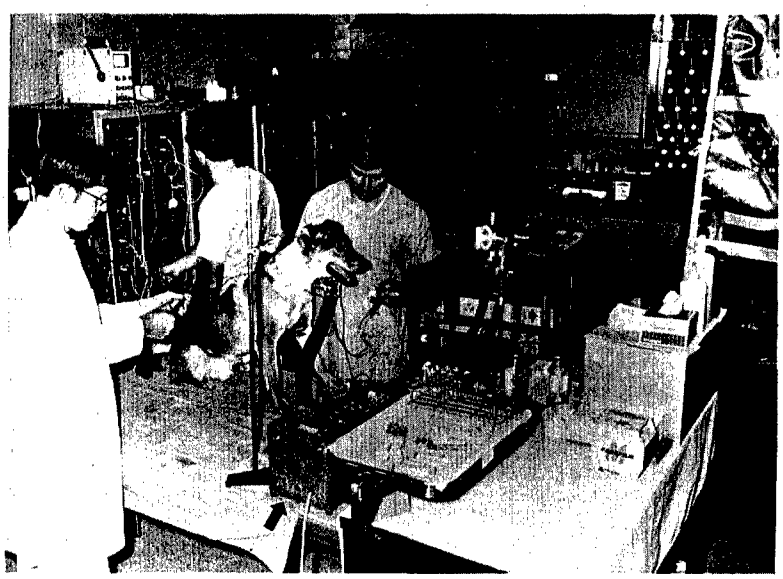

Figure 1. Chronically-instrumented dog experiment. Mylar exposure bag (extreme right) is connected to the endotracheal tube in the dog. Blood is withdrawn from the coronary sinus (left), while data are recorded on the Electronics for Medicine physiograph (center). Indocyanine green dye injection is made via the jugular catheter (right) while arterial blood from the carotid loop passes through a densitometer to a syringe on a Harvard Infusion/Withdrawal Pump (arrow).

All data (Table 1) are collected as preexposure controls, after a 30-minute exposure and following a 10-minute recovery period. Non-esterified fatty acid determinations are not made because they did not change significantly with any compound during the anesthetized dog experiments (Van Stee et al., 1975). Similarly, since glucose varied significantly only with exposures to $CBrF_3$ during those experiments, it is now measured only during experiments involving this compound. After the experiment is completed the dog is returned to the vivarium until needed again. When the animal is no longer useful, it is euthanized, the heart is removed and India ink is injected into the left circumflex coronary artery at the site of the flow probe. The stained tissue is dissected away and weighed allowing determinations to be stated in units/gm perfused myocardium. Data are calculated in the same fashion as in previously reported experiments (Van Stee et al., 1975).

TABLE 1. DETERMINATIONS.

Blood Halocarbon Concentration	Arterial Lactate/Pyruvate
Arterial PO_2	Cor Sinus Lactate/Pyruvate
Cor Sinus PO_2	Arterial Base Excess
Arterial O_2 Content	Cor Sinus Base Excess
Cor Sinus O_2 Content	Arterial Glucose
Myocardial O_2 Extraction	Cor Sinus Glucose
Arterial PCO_2	Myocardial Glucose Extraction
Cor Sinus PCO_2	L Cor Circumflex Art Blood Flow
Arterial Bicarbonate	Mean Arterial Blood Pressure
Cor Sinus Bicarbonate	Cardiac Output
Arterial pH	Mean Transit Time
Cor Sinus pH	Central Venous Vol (Pulm Blood Vol)
Arterial Lactate	Total Peripheral Resistance
Cor Sinus Lactate	Heart Rate
Myocardial Lactate Extraction	Stroke Volume
Arterial Pyruvate	Stroke Work
Cor Sinus Pyruvate	Peak dP/dt
Myocardial Pyruvate Extraction	$\frac{\text{Peak dP/dt}}{P}$

This method is being employed to study $CBrF_3$ and $CBrClF_2$ toxicologically as a mixture at two different concentrations. The high exposure is 8-12% $CBrF_3$ with 1.5-2% $CBrClF_2$; the low concentrations are 5-8% $CBrF_3$ with 0.8-1.2% $CBrClF_2$. CH_2BrCl is being investigated at 0.8-1.0%. These values, taken from the tentative exposure criteria are considered practical and probably high for an actual human exposure.

Significant results of the studies with anesthetized dogs are shown in Table 2. It is noteworthy that these exposures occurred at relatively high gas concentrations and were performed with anesthetized, open-chested animals as opposed to the chronically-instrumented dogs.

TABLE 2. SIGNIFICANT EFFECTS FROM EXPERIMENTS WITH ANESTHETIZED DOGS

	$CBrClF_2$	$CBrF_3$	CH_2BrCl
Elevation coronary sinus PO_2 and O_2 content	X		X
Elevation arterial and coronary venous glucose concentration		X	
Decreased myocardial lactate extraction	X		
Decreased coronary venous pyruvate			X
Decreased arterial pyruvate	X		
Prolongation mean transit time	X		
Decreased dp/dt max	X		
Decreased dp/dt/P			X
Decreased coronary circumflex arterial blood flow	X		

Initial experiments with the chronically-instrumented dogs dealt with combinations of $CBrF_3$ and $CBrClF_2$. Analyses of variance have been performed on the data currently available from these exposures. To date, in contrast to the experiments using anesthetized animals, no significant changes have been detected between the control and exposure periods in any of the variables studies (Tables 3 and 4). This must be considered only as preliminary information until several more trials are completed. A

continued lack of statistically significant changes would indicate that the effects noted with the anesthetized dogs probably resulted from the higher gas concentrations used in the exposures. This would not be surprising since a dose-response relationship is basic to any administered compound. Furthermore, these results lend credence to the tentative exposure criteria referred to previously with a satisfactory margin of safety; these combinations would be relatively safe during an exposure of limited duration. It is interesting to note that by the end of the recovery period, halocarbon blood level concentration had decreased to only a trace, or about 1/8-1/9 of the exposure levels according to the limits of the gas chromatograph.

TABLE 3. MEAN VALUES OF DETERMINATIONS FROM CHRONIC DOG EXPOSURES TO 5-8% $CBrF_3$ AND 0.8-1.2% $CBrClF_2$*

Measurement	Pre-exposure	During exposure	Post-exposure
Blood Halocarbon µl/ml			
$CBrF_3$	0	5.23 (±2.22)**	trace
CH_2BrCl	0	5.18 (±5.33)	trace
Arterial PO_2:torr	84 (±10)	77 (±10)	90 (±16)
Cor Sinus PO_2:torr	25 (±4)	26 (±3)	28 (±6)
Arterial PCO_2:torr	30 (±4)	30 (±7)	31 (±5)
Cor Sinus PCO_2:torr	36 (±3)	32 (±12)	39 (±6)
Arterial O_2 Content: ml/l	162 (±14)	173 (±25)	179 (±29)

* Pre- and post-exposures were to ambient air only.
** ( ) One SD

TABLE 3 (continued)

Measurement	Pre-exposure	During exposure	Post-exposure
Cor Sinus O_2 Content:ml/l	53 (±21)	68 (±22)	76 (±35)
L Cor Circ Art Flow:ml/min/g	0.53 (±0.22)	0.61 (±0.50)	0.36 (±0.18)
Art pH	7.42 (±0.03)	7.42 (±0.05)	7.41 (±0.04)
Cor Sinus pH	7.38 (±0.03)	7.38 (±0.05)	7.37 (±0.04)
Art Base Excess:mEq/l	-3.19 (±2.34)	-3.87 (±1.83)	-3.84 (±1.98)
Cor Sin Base Excess: mEq/l	-2.94 (±0.76)	-5.55 (±3.04)	-1.64 (±2.38)
Myocardial O_2 Extraction:μl/min/g	55 (±26)	64 (±52)	24 (±0)
Art Lactate:mM/l	0.63 (±0.13)	0.70 (±0.26)	0.62 (±0.25)
Cor Sin Lactate:mM/l	0.57 (±0.40)	0.57 (±0.30)	0.62 (±0.45)
Myocardial Lact Extract:μM/min/g	0.05 (±0.06)	0.11 (±0.09)	0.04 (±0.03)
Art Pyruvate:μM/l	42 (±10)	57 (±11)	46 (±14)
Cor Sinus Pyruvate:μM/l	38 (±8)	38 (±23)	44 (±18)

TABLE 3 (continued)

Measurement	Pre-exposure	During exposure	Post-expousre
Myo Pyruvate Extract:nM/min/g	5 (±1)	16 (±17)	1 (±4)
Art Lact/Pyruvate	15.6 (±3.2)	12.5 (±3.9)	13.1 (±3.0)
Cor Sin Lact/Pyruvate	15.8 (±6.2)	14.1 (±3.2)	15.8 (±4.9)
Art Glucose:mM/l	5.92 (±0.16)	5.80 (±0.21)	5.95 (±0.45)
Cor Sin Glucose:mM/l	6.07 (±0.75)	6.81 (±2.43)	7.10 (±3.51)
Myo Gluc Extraction:μM/min/g	-0.13 (±0.31)	-0.38 (±0.58)	-0.53 (±1.27)
Max dP/dt:torr/sec	2259 (±421)	2363 (±836)	2375 (±593)
Max dP/dt/P:sec^{-1}	41 (±12)	39 (±9)	40 (±9)
Art HCO_3^-:mEq/l	26.7 (±11.9)	18.6 (±2.6)	19.0 (±2.0)
Cor Sin HCO_3^-:mEq/l	20.2 (±1.1)	17.8 (±4.9)	22.8 (±2.4)
Mean Art Bld Press:torr	120 (±41)	124 (±47)	125 (±44)
Cardiac Output:ml/min/g	60.1 (±30.2)	41.7 (±15.3)	47.7 (±18.5)

TABLE 3 (continued)

Measurement	Pre-exposure	During exposure	Post-exposure
Mean Transit Time:sec	6 (±1)	7 (±1)	6 (±3)
Central Venous Vol:ml	308 (±89)	268 (±65)	237 (±107)
Total Periph Resist:pr units	44 (±32)	56 (±25)	48 (±15)
Heart Rate:beats/min	100 (±15)	100 (±18)	106 (±20)
Stroke Vol:ml	32.35 (±10.16)	24.02 (±7.09)	24.81 (±5.64)
Stroke Work:g-m/g	0.901 (±0.401)	0.668 (±0.270)	0.734 (±0.268)

TABLE 4. MEAN VALUES OF DETERMINATIONS FROM CHRONIC DOG EXPOSURES TO 8-12% $CBrF_3$ AND 1.5-2% $CBrClF_2$*

Measurement	Pre-exposure	During exposure	Post-exposure
Blood Halocarbon µl/ml			
$CBrF_3$	0	4.98 (±3.51)	trace
CH_2BrCl	0	3.92 (±3.36)	trace
Arterial PO_2:torr	82 (±12)	70 (±18)	88 (±12)

TABLE 4 (continued)

Measurement	Pre-exposure	During exposure	Post-exposure
Cor Sinus PO_2:torr	24 (±3)	28 (±7)	30 (±9)
Arterial PCO_2:torr	30 (±6)	29 (±5)	30 (±3)
Cor Sinus PCO_2:torr	37 (±4)	34 (± 8)	38 (±5)
Arterial O_2 Content:ml/l	167 (±30)	167 (±43)	167 (± 18)
Cor Sinus O_2 Content:ml/l	56 (±15)	78 (±37)	75 (±34)
L Cor Circ Art Flow:ml/min/g	0.79 (±0.51)	0.45 (±0.15)	0.46 (±0.28)
Art pH	7.42 (±0.04)	7.40 (±0.02)	7.40 (±0.01)
Cor Sinus pH	7.36 (±0.02)	7.36 (±0.02)	7.36 (±0.01)
Art Base Excess:mEq/l	-4.32 (±0.37)	-4.93 (±1.28)	-4.46 (±0.92)
Cor Sin Base Excess:mEq/l	-3.93 (±0.89)	-4.62 (±3.93)	-3.41 (±2.05)
Myocardial O_2 Extraction: µl/min/g	92 (±61)	41 (± 16)	45 (± 34)

* Pre- and post-exposures were to ambient air only.
** ( ) One SD

TABLE 4. (continued)

Measurement	Pre-exposure	During exposure	Post-exposure
Art Lactate:mM/l	0.55 (±0.25)	0.49 (±0.14)	0.50 (±0.10)
Cor Sin Lactate:mM/l	0.57 (±0.17)	0.46 (±0.22)	0.47 (±0.15)
Myocardial Lact Extract:μM/min/g	0.06 (±0.04)	0.09 (±0.05)	0.06 (±0.05)
Art Pyruvate:μM/l	39 (±19)	36 (± 10)	32 (±22)
Cor Sinus Pyruvate:μM/l	42 (±10)	34 (±10)	35 (±17)
Myo Pyruvate Extract:nM/min/g	1 (±19)	1 (±3)	-2 (±11)
Art Lact/Pyruvate	18.0 (±9.4)	18.3 (±8.4)	24.4 (±19.4)
Cor Sin Lact/Pyruvate	12.9 (±5.6)	14.7 (±8.2)	17.3 (±11.2)
Art Glucose:mM/l	5.60 (±0.52)	5.73 (±0.50)	5.91 (±0.43)
Cor Sin Glucose:mM/l	5.68 (±0.48)	5.63 (±0.41)	5.82 (±0.37)
Myo Gluc Extraction:μM/min/g	-0.18 (±0.26)	-0.05 (±0.22)	-0.10 (±0.12)
Max dP/dt:torr/sec	2938 (±734)	2456 (±1127)	2696 (±755)

TABLE 4. (continued)

Measurement	Pre-exposure	During exposure	Post-exposure
Max dP/dt/P:sec^{-1}	41 (±6)	40 (±8)	35 (±10)
Art HCO_3^-:mEq/l	18.5 (±1.6)	17.9 (±2.2)	18.7 (±1.4)
Cor Sin HCO_3^-:mEq/l	20.6 (±1.3)	19.4 (±4.6)	21.1 (±2.6)
Mean Art Bld Press:torr	135 (±33)	135 (±55)	124 (±31)
Cardiac Output:ml/min/g	84.2 (±58.1)	68.7 (±23.2)	56.2 (±26.7)
Mean Transit Time:sec	4 (±2)	4 (±2)	5 (±2)
Central Venous Vol:ml	281 (±117)	307 (±172)	277 (±135)
Total Periph Resist: pr units	31 (±10)	29 (±15)	34 (±17)
Heart Rate:beats/min	118 (±27)	119 (±29)	131 (±74)
Stroke Vol:ml	38.50 (±9.25)	42.41 (±6.77)	34.83 (±8.06)
Stroke Work:g-m/g	1.288 (±0.854)	0.989 (±0.194)	0.729 (±0.095)

One dog did manifest severe convulsions during exposure to the high combination of $CBrF_3$ and $CBrClF_2$; these values were not considered in the analysis, except for the preexposure controls. The blood halocarbon levels were no higher than those of other dogs at this same concentration leaving the etiology of the convulsions unexplained, except for the possibility of individual increased susceptibility. It is because of this, however, that studies will be made using a slightly higher concentration of gases providing that the dogs will tolerate the exposure. At no time during this or any other exposure did the animals exhibit cardiac arrhythmias. The occurrence of convulsions or excitement would limit the usefulness of the preparation as any other effects would be masked.

There have not been sufficient exposures to CH_2BrCl alone to draw any conclusions at this time.

CONCLUSION

The chronically-instrumented dog preparation has been created to approximate human exposure to halocarbons more realistically than has been accomplished previously. At this time there is no indication of the effects noted in prior experiments with higher gas concentrations using anesthetized animals.

Canine models for cardiodynamic toxicological effects have been useful as a basis for extrapolation to the human situation. One must, however, remain cognizant of species differences in interpreting such information. One method that would facilitate the understanding of human reaction based on animal data would be to use a primate model. Plans are being formulated for the preparation of chronically-instrumented baboons for just this reason. Coupled with the results from the canine and other species experiments, the primate tests will lend further support to the application of this information to the human situation.

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AMRL-TR-75-125

PAPER NO. 12

MEASUREMENT OF THE INTERACTION OF OZONE AND NITROGEN DIOXIDE

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INTRODUCTION

The University of California, Irvine (UCI) has instituted a program for investigation of the interaction of air pollutant gases and aerosols. This program was drawn up because of the considerable evidence (McJilton et al., 1973; Amdur and Underhill, 1968; Frank et al., 1964) that aerosols can potentiate the toxic action of gases or vapors. Before tests could begin on the effects of combining pollutant gases and aerosols, it was necessary to determine if there are interactions among the gaseous pollutants themselves. The simplest initial approach to investigation of interaction of toxic gases appeared to be the observation of effects of combining two common air pollutants, ozone (O_3) and nitrogen dioxide (NO_2). The toxic characteristics of the individual gases have been investigated quite extensively in recent years. A review of some of the O_3 work done in the last 3 or 4 years will illustrate this and also serve to catalog many of the techniques used in studies on lung irritant gases.

Bruch and Schlipkoetas (1973) exposed mice to 0.86 ppm O_3, 8 hours/-day, 5 days/week for 10 months. They noted marked hyperplasia of the Clara cells and conspicuous thickening of the basement membrane between epithelial and endothelial lining of the alveoli. The Type 1 pneumocytes seemed to be most sensitive to O_3.

Stephens et al. (1973) exposed dogs to 3 ppm O_3, 8 hours/day for 18 months. Using transmission electron microscopy (TEM) they found that the endoplasmic reticulum of Type 2 cells was frequently dilated and contained an electron-dense substance. There was a reduction in the lamellar membranes of the Type 2 cells. Stephens et al. (1974) used rats and concentrations of 0.5 and 0.9 ppm O_3. After 2 hours exposure at 0.5 ppm, Type 1 cells were damaged. After 6-10 hours, a loss of ciliated cells from the terminal bronchioles was seen. Type 2 cells were very resistant and proliferated to repair the loss of Type 1 cells after 48 hours of exposure. Mitochondria in the Type 1 cells swelled to a great degree before the cells sloughed off.

Schwartz et al. (1974) exposed rats to 0.2, 0.5 and 0.8 ppm O_3, 8 hours/day for 7 days. By light microscopy they saw accumulation of alveolar macrophages along with thickening and cellular infiltration of the interalveolar septa. Examination by scanning electron microscopy (SEM) revealed macrophage infiltration of the alveoli and damage to the epithelial cells of the terminal bronchiole. TEM demonstrated that damage to the Type 1 cells was confined to the proximal alveoli.

Bartlett's group (1974) studied pulmonary growth and elasticity in young rats continuously exposed to 0.2 ppm O_3 for 30 days. Lung weight was not affected by this exposure but lung volume, measured after fixation at 20 cm H_2O transpulmonary pressure was increased by 16%, probably due to a decrease in lung elasticity. Chow and Tappel (1972) exposed rats to 0.7 ppm O_3 continuously for 5 days and measured increases in lipid peroxidation products, primarily malonaldehyde as well as activities of glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase (G-6-PD). They proposed an enzymatic protective mechanism with G-6-PD providing the NADPH necessary for the hydrolytic enzymes.

DeLucia's group (1972) performed acute exposures of rats to 2 ppm O_3 for 4 to 8 hours and chronic exposures to 0.8 ppm continuously for 10 days. Following acute exposure, they found that both protein and nonprotein lung sulfhydryl levels fell as did levels of sulfhydryl containing enzymes such as G-6-PD, glutathione reductase, and cytochrome-C reductases related to succinate and NADH. Lung sulfhydryl was maintained at normal levels under chronic exposure while G-6-PD and cytochrome-C reductases increased.

After 5 days continuous exposure of rats to 0.7 ppm O_3, Dillard et al. (1972) found increased activities of lysosomal hydrolases. They attributed the increases to infiltration of lung by macrophages during the inflammatory response induced by O_3. Macrophages have a greater number of lysosomes than other pulmonary cells. Mustafa and coworkers (1973) noted a decrease in lung mitochondrial oxygen consumption on acute (2 ppm, 8 hours) exposure and an increase on chronic (0.8 ppm, 10-20 days) exposure. They theorized that the increase on chronic exposure might be related to proliferation of the Type 2 alveolar epithelial cells which have a large population of mitochondria.

Castleman, Dungworth and Tyler (1973) studied lung acid phosphatase localizations histochemically and cytochemically after rats were exposed to 0.7-0.8 ppm O_3 continuously for 7 days. The enzyme was found distributed in the cytoplasm of terminal airway epithelium and interalveolar septa in exposed animals, whereas it was limited to lysosomes and other vacuolar elements in control animals. This supports the hypothesis that the lysosomal membrane is damaged by O_3 exposure. Goldstein's group (1974) investigated the bactericidal and clearance properties of the rat lung after 4-hour exposure to 2.5 ppm O_3. They found that phagocytic ingestion of staphylococcus aureus was only slightly impaired while bacterial clearance was completely suppressed.

At the time of planning this research, the Toxic Hazards Research Unit of the University of California, Irvine did not yet have available scanning or transmission electron microscopes or scientists trained in the sophisticated methods of lung preparation for proper light microscopy. We also lacked personnel competent to perform histochemical or cytochemical tests on lung tissue or to handle the radioactive areosols necessary in lung bacterial killing and clearance experiments. Therefore, we decided to proceed with the study using rat wet lung weight increases after 4-hour exposure to the toxic gases as measures of effect. This technique was introduced to our laboratory by Dr. E. J. Fairchild in 1967. We proposed to measure the threshold concentrations of the individual gases, and to determine whether the thresholds were significantly changed when rats were exposed to mixtures of the gases. After measurement of the coefficient of variation of rat lungs in a group, the smallest difference in group means of 20 animals having statistical significance was calculated as 5%.

MATERIALS AND METHODS

The first problem which required solution in the investigation of the effects of low levels of ozone and nitrogen dioxide was analytical. The requirements of a chemical analytical procedure for chamber atmospheres were:

1. Continuous analysis so that the chamber pollutant concentration would be known at all times during the exposure.

2. Rapid response so that any change in chamber concentration would be quickly detected and remedied.

The THRU chemistry department, therefore, adapted standard colorimetric procedures for O_3 and NO_2 to the Technicon AutoAnalyzer continuous analyzer. The Lyshkow (1965) modification of the Saltzman method was found satisfactory for the analysis of NO_2 alone and when O_3 was present. The standard EPA iodometric method for O_3 (Federal Register, 1971) was successfully automated and used in the early studies with O_3 as the sole contaminant. However, it could not be employed in the presence of NO_2 which interfered. A Dasibi Ozone Monitor was borrowed from UCI and used in exposures to mixtures. Since this instrument measures UV light absorbed by O_3, no interference is given by NO_2. Experiments performed on pure O_3 mixtures demonstrated that identical results were given by iodometric and Dasibi procedures.

NO_2 was delivered from calibrated mixtures of 1% NO_2 in N_2 pressurized to about 1000 psi. Ozone was generated using Sander Model III ozonizers. Pure oxygen had to be used as a source rather than air to avoid formation of some NO_2.

The following procedure was used to obtain wet lung weights from rats. A critical requirement is that one individual performs a given function throughout the procedure, thus minimizing variability between animals.

1. Bend S-shaped hooks from straight pins (1 needed per animal).
2. Weigh several S-hooks (10) on an analytical balance and determine average weight.

3. Slightly anesthetize animal with 0.1 cc Uthol (sodium pentobarbital) and exsanguinate via femoral arteries.
4. Open thoracic cavity to expose lungs, heart, etc.
5. Tie off trachea about 1 mm below level of thyroid.
6. Cut trachea at the level of thyroid.
7. Cut dorsal aorta and posterior vena cava.
8. Remove trachea, lungs and heart, stripping away esophagus.
9. Tie off pulmonary artery and vein, including heart and remnants of dorsal aorta and posterior vena cava as close to lungs as possible.
10. Excise heart and attached blood vessels directly above ligature.
11. Cut away excess connective tissue and ends of suture thread.
12. Wash lungs by dipping several times in normal saline and blot dry with gauze pads.
13. Insert S-hook through trachea and obtain weight on analytical balance.

Since the edematous effects of acute exposure to deep lung irritants are sometimes delayed, the experiments were designed to take this into consideration with measurements of lung weight made at varying times after exposure until a maximum was reached. We planned to use this sacrifice time routinely in all subsequent exposures. As it turned out, the time required to produce maximum increase in lung weight was different for the mixtures than for the pure compounds and may have varied slightly with concentration of the pure materials. Although rat lungs were weighed immediately after sacrifice and after drying at 100 C for 24 hours to yield measurements of wet and dry lung weights and lung water, the wet lung weights were more precise indicators of edema than lung water. This might have been expected since wet lung is measured with one weighing while lung water requires two. Also, lung water and lung solids increased in approximately the same percentage in exposed animals over controls. This indicates that lung edema fluid produced by exposure to irritating gases contains approximately the same ratio of water to solids as normal rat lungs.

One of the assumptions upon which this study was based was that rats of equal body weights would have equal lung weights, and that the lungs would increase equally in weight after exposure to identical concentrations of irritant gases. However, when 2 groups of rats from different shipments were given 4-hour exposures to mixtures of 2.5 ppm O_3 and 15 ppm NO_2, these assumptions did not hold. The data are shown in Table 1.

TABLE 1. EFFECT OF EXPOSURE TO A MIXTURE OF O_3 AND NO_2 ON RAT LUNG PARAMETERS

Group A	Wet Wt. Gms.	Dry Wt. Gms.	Lung H_2O Gms.	Body Wt. Gms.	N
Controls	1.4890	0.3462	1.1428	253.1	10
Sac. Immed.	1.5762	0.3632	1.2120	253.8	10
Sac. 24 Hrs.	1.6179	0.3969	1.2210	251.5	10
Group B					
Controls	1.0979	0.2649	0.8330	252.3	10
Sac. Immed.	1.3778	0.2977	1.0801	256.4	10
Sac. 24 Hrs.	1.3569	0.3083	1.0486	245.8	10

Comparison of the two control groups clearly shows that Group B had significantly lighter lungs than Group A even though they were the same strain and age and their body weights were almost identical. The increase in lung weight after exposure was greater in the animals with light lungs in both a relative and absolute sense. Although this was a rare occurrence, it illustrates that the variation among groups is probably larger than anticipated. With few exceptions, the mean body weights of the rats used in this study ranged from 240 to 300 grams and the control lung weights from 1.3 to 1.7 grams.

RESULTS AND DISCUSSION

The differences between lung weights of control animals and those exposed for 4 hours to various concentrations of O_3 or NO_2 are listed in Table 2. Table 3 contains the data for mixtures of O_3 and NO_2.

TABLE 2. EFFECT OF A SINGLE 4-HOUR EXPOSURE TO O_3 OR NO_2 ON RAT LUNG WEIGHTS

Gas Conc. ppm	Postexposure Time to Sacrifice, Hrs.	N	Lung Weight Difference from Controls	
			Grams	Percent of Control Lung Wt.
O_3				
2.5	0	18	0.0317	2.43
2.5	24	20	0.1784	13.16
1.25	0	5	0	0
1.25	48	5	0	0
1.25	96	5	0	0
1.25	168	5	0.0667	3.66
1.9	0	10	0.0290	2.04
1.9	0	10	0.0068	0.44
1.9	24	10	0.1566	10.35
4.6	18	5	0.6184	35.67
4.6	24	10	0.6118	43.95
NO_2				
10.6	0	5	0	0
16.2	0	5	0.0482	3.14
15.4	0	10	0	0
15.4	24	10	0.0809	6.14
25.7	0	10	0.0262	1.75
25.7	24	10	0.0209	1.39
26.9	0	10	0	0
26.9	24	10	0.1047	6.93
30.1	0	10	0.2333	15.28
30.1	24	10	0.3106	20.35
15.7	0	10	0.07882	4.85
15.7	24	10	0	0
46.1	0	10	0.3645	22.87
46.1	24	10	0.2760	17.32
30.0	0	10	0.1659	10.45
30.0	24	10	0.0898	5.67

TABLE 3. EFFECT OF A SINGLE 4-HOUR EXPOSURE TO MIXTURES OF O_3 AND NO_2 ON RAT LUNG WEIGHTS

Gas Conc. ppm		Postexposure Time to Sacrifice, Hrs.	N	Lung Weight Difference From Controls	
				Grams	Percent of Control Lung Wt.
Mixture					
NO_2	O_3				
15.3	2.5	0	10	0.1878	12.55
15.3	2.5	24	10	0.1289	8.65
15.3	2.5	0	10	0.2789	25.49
15.3	2.5	24	10	0.2590	23.59
7.5	1.25	0	10	0	0
7.5	1.25	24	10	0.0626	4.34
15.5	1.25	0	10	0	0
15.5	1.25	24	10	0	0
7.4	2.5	0	10	0.1070	6.98
7.4	2.5	24	10	0.2053	13.40
27.3	1.7	0	10	0.2380	15.05
27.3	1.7	24	10	0.0888	5.61

Examination of Table 2 reveals that there is a fairly well defined effect threshold for O_3 somewhere between 1.25 and 1.9 ppm. However, this does not appear to be the case for NO_2 since the reproducibility of lung weight increase due to exposure is not as good as for O_3. This makes it difficult to analyze the mixture data in Table 3 on the basis of threshold effect, except for the conclusion that there is no obvious synergism. Apparently, exposure to O_3 alone caused an edematous response which maximized 24 hours after exposure, while NO_2 lung weight values 24 hours after exposure differed little from those obtained immediately postexposure. Most of the time, exposure to the mixture led to maximum lung weights immediately after exposure. Although the experiment was designed as an examination of threshold effects, the data in the tables indicate that there is a relationship between concentration and lung weight increase. When the maximum percent lung weight increase obtained after any O_3 exposure is

plotted versus concentration, as in Figure 1A, a very good linear relationship is noted. The data are more scattered in the case of NO_2 (Figure 1B), but here too concentration and lung weight increases appear to be linearly related. The plots are least square lines with a correlation coefficient of 0.98 for the O_3 line and 0.84 for NO_2. The NO_2 line can be transformed to the O_3 line under the relationship of equivalent O_3 concentration, ppm = 0.66 + 0.053 NO_2 concentration, ppm. Points from pure O_3 exposures and from pure NO_2 exposures plotted as equivalent O_3 concentrations will lie about this line. If O_3 and NO_2 are additive with respect to edema formation, mixture points plotted as equivalent O_3 concentrations should also lie about this line. In Figure 2, the equivalent O_3 concentrations calculated from the mixture values in Table 3 are plotted against the maximum lung weight increases obtained after exposure. All these points lie below the line, implying that there is no synergism between O_3 and NO_2 with respect to edema production and that there may be some antagonism. When only the O_3 concentrations are plotted against percent lung weight increase, the points do lie about the line, yielding an alternative explanation that only the O_3 is edemagenically active in the mixture. However, this appears less likely than the possibility of antagonism since NO_2 alone is edemagenic.

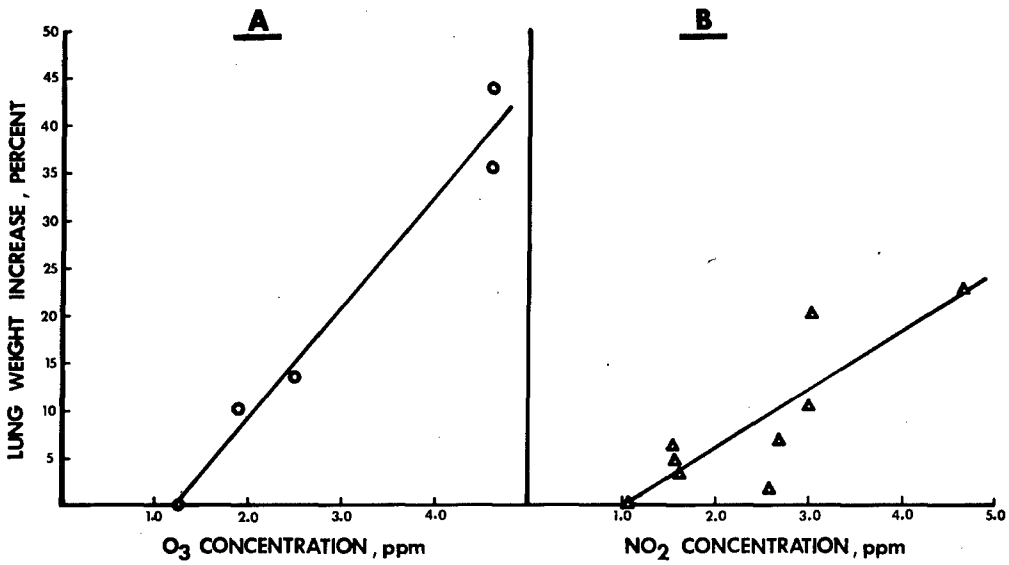

Figure 1. Effect of O_3 or NO_2 concentrations on rat lung weight.

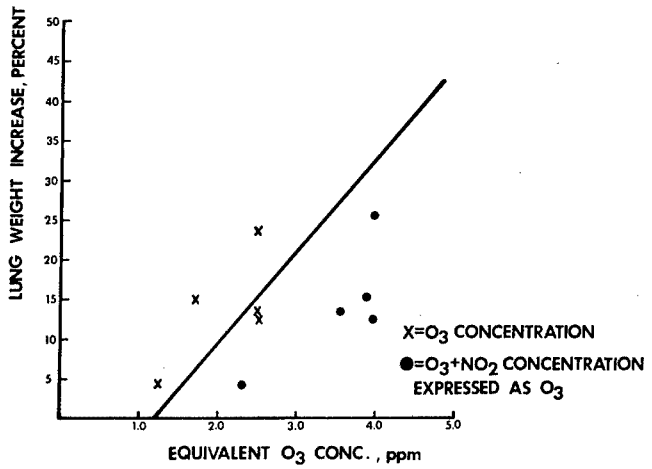

Figure 2. Relationship between effective mixture concentration and lung weight, assuming additivity of O_3 and NO_2.

It is possible that any antagonism between the 2 toxic gases may result from their reaction:

$$2NO_2 + O_3 \longrightarrow N_2O_5 + O_2$$

Obviously, the contaminants were reacting in the chambers during exposures since it was necessary to introduce amounts of O_3 and NO_2 which would have given concentrations of 10 and 45 ppm individually in order to obtain 1.7 and 27 ppm in the mixture. Since N_2O_5 does not appear to have significant edemagenic effects at the concentrations generated, the effect is one of removal of the toxic components. Possibly the reaction between O_3 and NO_2 continues in the airways leading to the alveoli and decreases their deep lung concentrations below those measured in the chamber. However, as the concentrations of the reactant gases O_3 and NO_2 are decreased to ambient pollution levels (0.3 ppm and below), the rate of the reaction will decrease and antagonism due to disappearance of the toxic gases will lessen; therefore, one might expect the interaction of O_3 and NO_2 to approach additivity at ambient levels.

Early reported work on the toxicology of mixtures of NO_2 and O_3 was done by Stokinger (1957) who noted that the lethalities of some mixtures were less than that of O_3 alone. He ascribed this result to the

removal of the highly toxic O_3 by NO_2 to form N_2O_5. Goldstein et al. (1974) exposed mice to mixtures of the gases at low concentrations and found no synergism or antagonism in depressing bactericidal activity in mice. His concentrations, 7 ppm and lower for NO_2 and 0.4 ppm and lower for O_3, were sufficiently low that any slight antagonism because of reaction to form N_2O_5 was not detectable by his technique.

Freeman's group (1974) performed exposures of rats to a mixture containing approximately 1 ppm of each gas. Over a period of 60 days, they found that the histopathological changes which occurred were those to be expected from O_3 exposure at this level. This is not surprising since 0.8 ppm of NO_2 alone caused only marginal effects. In the same investigation Freeman also exposed animals to 2.5 ppm NO_2 and 0.25 ppm O_3 and found early (24-48 hours) evidence of peripheral epithelial hypertrophy and desquamation of Type 1 cells. This was characteristic of O_3 exposure as contrasted to the more proximal bronchiolar effects seen on exposure to NO_2. In both these exposures the concentrations of the gases were probably too low for significant reaction of O_3 and NO_2 within the chamber or respiratory tract of the animals.

It is possible to compare results obtained by different investigators using the relative potencies of O_3 and NO_2 found by them. These relative potencies are remarkably similar over a wide range of concentrations and with widely different techniques. Goldstein et al. (1974) found the effect thresholds of O_3 and NO_2 to be 0.4 and 7 ppm, respectively, for depression of bactericidal activity to give a relative potency ratio of O_3 to NO_2 of about 18. Based on survival time during exposure to relatively high, subacute concentrations and on cellular responses at lower levels, Freeman et al. (1974) noted that similar effects were elicited by approximately a 20-fold difference in concentration between O_3 and NO_2. Alarie (1973) observed that O_3 was about 10 times more effective than NO_2 in causing a given increase in respiratory rate of guinea pigs. Finally, our own data (Figure 1) indicate that 14 times as much NO_2 as O_3 is required to give a 15% increase in rat lung weight after a 4-hour exposure.

We believe that our results, obtained using a relatively simple technique, are in agreement with those obtained in investigations utilizing more sophisticated techniques both in the areas of relative potencies of O_3 and NO_2 and their toxicological interaction.

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AMRL-TR-75-125

PAPER NO. 13

METABOLIC EFFECTS OF SIMPLE HYDRAZINE COMPOUNDS

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INTRODUCTION

The hydrazines received their greatest attention as spacecraft and missile propellants, but a multitude of industrial applications require the bulk of the more than 20 million pound worldwide hydrazine production. Such extensive use carries with it an environmental and industrial hazard potential that continues to demand investigation. We have been concerned with the impact of the hydrazines in four general areas of metabolic study: glucose homeostasis, amine metabolism, the sequence leading to synthesis of spermidine and spermine, and intermediary energy metabolism, which is the area I propose to emphasize here.

We try to deal with effects in these categories from three viewpoints. We want to know what the hydrazines are capable of doing and what can be expected of them as hazards in the real world. Somewhat more academically we are also interested in ways these compounds can help us learn about the biology of the organism.

The principal findings reported here show that subacute treatment with monomethylhydrazine (MMH) can cause a profound interference with glycolysis in intact animals, and that hydrazine apparently interferes with the flux of acetate carbon into fatty acid synthesis and subsequent emergence for oxidation. Hydrazine decreases tolerance to a single administration of glucose, but MMH does not. On the other hand, if MMH is administered slowly over several hours, it causes hyperglycemia at moderate continuous glucose loading. Chronic exposure to MMH at doses as low as

0.6 μmoles/kg/hr obstructed methylamine metabolism. Hydrazine was generally less effective, and oxidation of putrescine was less sensitive. Zero effect doses for the observed parameters of carbohydrate metabolism are on the order of 0.1 LD_{50}.

METHODS

All studies reported here utilized male Sprague-Dawley rats at weights of 310-330 grams. Substrate was infused to each rat through an indwelling intestinal cannula inserted about 5 mm from the pylorus. Glucose samples were drawn through an indwelling cannula in the posterior vena cava. Toxicants were infused subcutaneously, except single acute doses given intraperitoneally.

Basic radiorespirometric procedures were described in a preceding conference (Wang and Dost, 1969). Raw data recorded on punched tape is now processed by a Hewlett-Packard 9821 programmable calculator with punched tape reader, teletype interface, and plotter. In studies with labeled glucose and intermediates, intoxicant was infused continuously, subcutaneously, for 14 hours; the substrate was infused from hour 4 to hour 25. Glucose was infused at a rate of 150 mg/hr/rat; other substrates were accompanied by sufficient glucose to provide total carbon input equivalent to 150 mg glucose. The rate of $^{14}CO_2$ output was automatically measured every three minutes and these values averaged every 15 minutes. Figure 1 is a 15 minute plot of three control and three treated animals, to show the quality of the data. Figures 2 and 3 are plotted through averages of 2-4 animals at the hourly points.

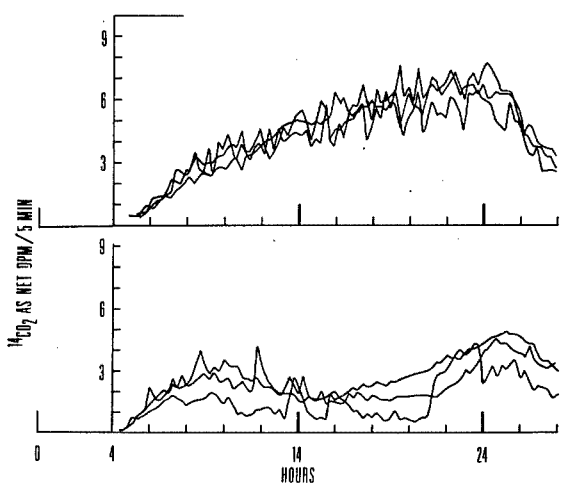

Figure 1. Nature of raw radiorespirometric data. Effect of MMH on oxidation of glucose-6-^{14}C. Top, control data; bottom, MMH, 0.036 μmole/kg/hr. Each trace represents rate of $^{14}CO_2$ output of a single animal at 15 minute intervals, with points connected without curve fitting. All data normalized to convention of relative input rate equal to 10^4 DPM/5 min.

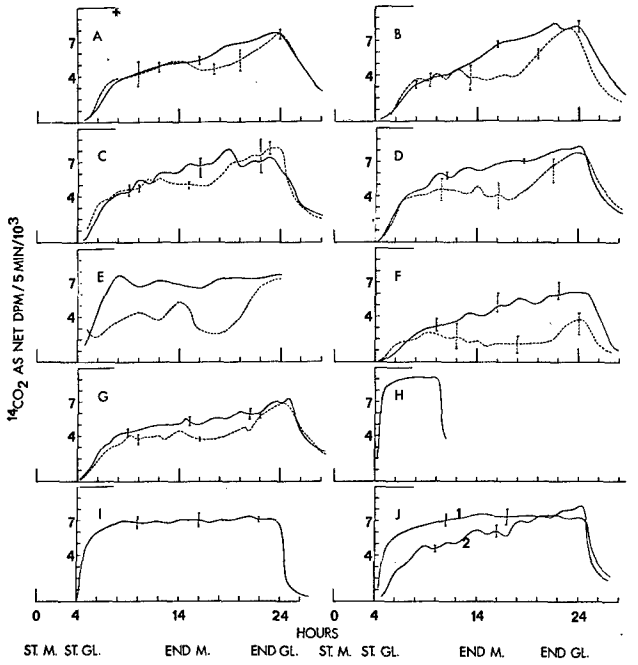

Figure 2. Effect of MMH on oxidation of variously labeled ^{14}C-glucose. MMH administered from time zero at 0.036 mmole/kg/hr to hour 14, total dose 0.5 mmole/kg. Glucose infused 150 mg/hr, hours 4 to 24. A. $^{14}CO_2$ output from rats catabolizing glucose-1-^{14}C; B. -2-^{14}C; C. -3-^{14}C; D. 3,4-^{14}C; E. Carbon 4 calculated by difference from data on glucose-3-^{14}C and -3,4-^{14}C; F. glucose-6-^{14}C; G. Fructose-1-^{14}C; H. Na bicarbonate-^{14}C; I. Na glutamate-1-^{14}C; J. Na pyruvate-1- and -2-^{14}C. Each curve is average of 2-4 animals. Bars indicate ranges of values. Curves have been smoothed by using rate measurements at hourly intervals only. All data normalized to convention of relative input rate equal to 10^4 DPM/5 min.

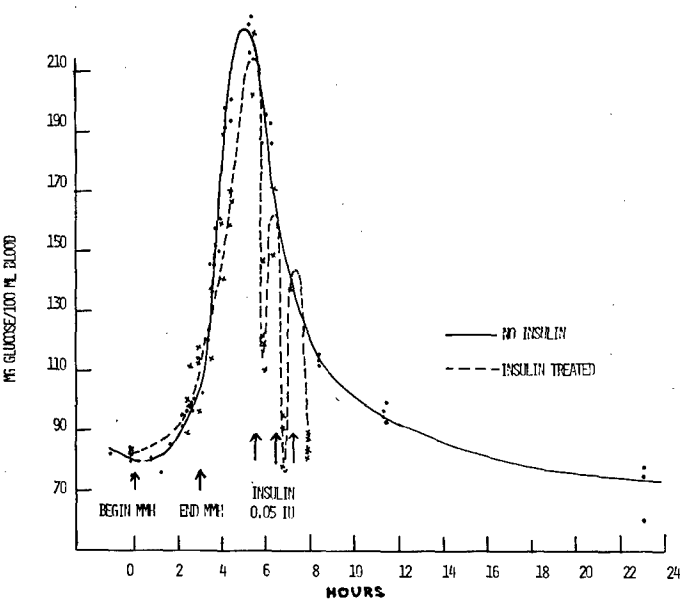

Figure 3. Effect of single injections of 0.05 I.V. crystalline insulin on MMH induced hyperglycemia. Glucose infused intraintestinally 150 mg/hr from 6 hours prior to time zero through hour 22. MMH infused subcutaneously 0.1 mmole/kg/hr. Each point represents a single blood sample.

Chronic toxicants were administered subcutaneously while animals were confined 8 hours daily in restraint devices made from acrylic tubing. Animals were returned to cages for free access to food and water. Rats are unusual in that they appear to accept such restraint without stress; they consistently enter the chambers without hesitation after previous experience, and frequently reenter if released immediately after removal. Capability for oxidation of methylamine-^{14}C and putrescine-^{14}C was tested periodically during the intoxication schedule by injecting a single 10 mg intraperitoneal dose of labeled substrate and measuring the total percentage recovery of $^{14}CO_2$ over a six-hour period during the daily infusion of intoxicant.

Blood glucose was measured with a Beckman Glucose Analyzer. Glucose tolerance was tested by administering 0.5 LD_{50} or less of toxicant over a one-hour period, followed immediately by 500 mg of glucose infused into the intestine over a 14-minute period to simulate gastric mixing and discharge.

Glucokinase was measured by the method of DiPietro and Weinhouse (1960). The glucokinase and/or hexokinase phosphorylation of glucose is coupled in this assay to the oxidation of glucose-6-phosphate formed in the reaction and simultaneous reduction of $NADP^+$ to NADPH by glucose-6-phosphate dehydrogenase. Because the K_m for glucokinase is about $10^{-2}M$ glucose and about $10^{-4}M$ glucose for hexokinase, determination in the presence of high glucose concentration will provide the total activity of both enzymes. At low glucose concentration only hexokinase is appreciably active, and glucokinase activity is obtained by difference. Activity is proportional to NADPH formation and is expressed as change in OD_{340}/mg protein/minute, during the initial stages of the reaction.

Pyruvate kinase activity was measured by the method of Weber et al. (1965). The reaction forms pyruvate from phosphoenol-pyruvate, and the subsequent lactic dehydrogenase reaction with accompanying oxidation of $NADH_2$ provides the measurement index. In this case disappearance of $NADH_2$ is expressed as decrease in OD_{340}/gm protein/minute.

Results of each series of assays were analyzed by application of a t-test to ascertain differences between data from treated and untreated animals.

RESULTS AND DISCUSSION

Glucose Catabolism

The intact animal system we study has the disadvantage of reflecting the sum of similar processes throughout the organism. If there is a singular inhibition in a given tissue or organ, it may not be visible when measured simultaneously with normal function in the remainder of the animal, and in this way we potentially lose both sensitivity and selectivity. It is not unreasonable to assume within limits, however, that a given process will be as vulnerable in one tissue as another, given uniform disposition of the toxic agent. Intact animal preparations do also have the advantage of intact control systems, and they can be observed over extended time courses.

Furthermore, the information derived can be applied on the basis of existing knowledge to special subsystems. The erythrocyte, with its unusual dependence on glycolysis for derivation of energy, or the brain, which is capable of oxidizing only glucose, are examples. Since the hydrazines are known for their apparent central nervous effects, effects upon brain metabolism may be of crucial importance.

In studying the labeled CO_2 pattern of untreated animals (Figure 2), note that in spite of the long period of continuous infusion, the system never quite equilibrates nor does $^{14}CO_2$ production reach the rate of label infusion during the period of observation. It must be recognized that while the glucose input of 150 mg/hr is approximately equal to the carbon turnover rate for the animals, only a portion of the CO_2 emerging from any animal arises directly from glucose at any given time, and that while the total ^{14}C input (corrected to 10^4 DPM/5 min; Figure 2) is used as a reference in quantitating $^{14}CO_2$ output, the output will rarely equal input.

There are two stages of activity. The system first comes to an initial metabolic equilibrium over a period of about six hours, after which a phase of slower increase in $^{14}CO_2$ becomes visible. During the latter period carbon moves into other metabolic pools, eventually re-emerging as $^{14}CO_2$ more slowly than carbon which stays in the "mainstream." Most of that effect is probably movement through acetyl CoA, in and out of fatty

acid synthesis and oxidation, although at this time we cannot rationalize the existence of the same behavior of carbons 3 and 4, which should not become components of acetate except through extensive recycling.

The character and dynamics of the oxidation of glucose and other substrates by intact animals can be in part illuminated by noting the way in which infused Na bicarbonate-^{14}C (Figure 2H), Na-glutamate-1-^{14}C (Figure 2I), and NA pyruvate-1- and -2-^{14}C (Figure 2J) are converted to $^{14}CO_2$. Bicarbonate-^{14}C labels the extracellular bicarbonate and CO_2 pool, and is quickly equilibrated with respiratory CO_2 through primarily physical-chemical processes. Consequently it reaches equilibrium rapidly and output is more or less constant at about 90% of input, very gradually approaching 100% over many hours, not shown in this short-term example (Figure 2H). Glutamic acid enters the tricarboxylic acid cycle readily through transamination to α-keto-glutarate and is not subject to diversion into a secondary pool; accordingly, the rate of $^{14}CO_2$ formation from glutamate (Figure 2I) constitutes a steady state within the time frame of these experiments. The first carbon of pyruvate emerges in much the same pattern (Figure 2J-1), since it is lost in decarboxylation. The second carbon (Figure 2J-2) labels acetyl-CoA and follows the biphasic pattern characteristic of glucose.

These experiments are intended to provide qualitative information of major deficit rather than highly quantitative data which can be analyzed to detect small differences among groups of animals. Small differences are not considered biologically significant in these circumstances, however refined the statistical treatment may be.

A dose rate of 36 μmoles MMH/kg/hr for 14 hours (approximately acute LD_{50}) caused marked changes in catabolism of glucose. Glucose-1-^{14}C conversion was decreased slightly (Figure 2A) and C-2 (Figure 2B) and C-3 (Figure 2C) were somewhat more depressed. The conversion of the 3, 4-^{14}C label hindered substantially (Figure 2D) and that of C-6 (Figure 2F) was severely slowed. Data for carbon-4 (Figure 2E) was calculated by difference between the data for conversion of glucose-3-^{14}C and glucose-3, 4^{14}C to $^{14}CO_2$.

In considering these data, it must be remembered that the C-4, C-5, C-6 fragment of glucose must remain intact through glycolysis and the pentose cycle. Even in all of the shuffling in the pentose cycle, with formation of 5 carbon products and 4, 5, and 7 carbon intermediates, the "bottom" half of the molecule stays together, and only in decarboxylation of pyruvate does it come apart. Of every three moles of glucose to enter the pentose cycle, one C-4, 5, 6 fragment will enter triose metabolism as glyceraldehyde 3-phosphate. If glycolysis is impeded, the only oxidation of C-6 will be the limited amount allowed through the obstruction and that 1/3 which follows the pentose cycle. Carbon 1 of glucose can be quickly oxidized in the pentose cycle, on the other hand, and C-2 and C-3 can be cycled to successively emerge as carbon 1 on fructose-6-phosphate, which isomerizes to glucose-6-phosphate and passes in turn through the pentose cycle to also be oxidized.

These data from labeled glucose alone suggest severe glycolytic interference but do not rule out a simultaneous slowdown in the citric acid cycle. That possibility was highly doubtful, however, because glutamic acid, aspartate, and pyruvate all were metabolized without interference by MMH treated rats.

With the lesion localized within the broad scheme of glycolysis there was then need for a label which would at least localize the problem within either the metabolism of 6 carbon intermediates or in the triose sequence, from glyceraldehyde phosphate and dihydroxyacetone phosphate (DHAP) "downward."

Fructose-1-^{14}C is an appropriate label at this point because it enters glycolysis as DHAP and does not enter the hexose pool. Fructose metabolism was almost unaffected by MMH (Figure 2G), indicating that triose metabolism was not affected and that the blockade rested in the relatively short segment of reaction of 6 carbon intermediates. [MMH treated animals became peculiarly intolerant of fructose, however. Several animals which were given 50 mg fructose and 100 mg glucose/hr died after 4-8 hours of infusion, with no evident impairment of fructose metabolism until death. Published accounts indicate a rather high capacity for fructose assimilation in several species (DiPietro, 1964; Rauschenback and Lamprecht, 1964; Ashby, 1975) and no other MMH treated animals have shown such effects.]

It was clear from the relatively limited interference with oxidation of C-2 and C-3 of glucose under intoxication that the recycling of those carbons from fructose-6-P to glucose-6-P was reasonably functional. That left as the presumed site of inhibition the primary glycolytic regulatory enzyme, phosphofructokinase (PFK), and that is where we are convinced the damage lies.

In vitro assays of PFK activity have been equivocal but suggest a substantial inhibition. The enzyme is present in small amounts and it has some notoriety as a difficult system to measure; we are not yet prepared to make firm statements about it. However, glucokinase, pyruvate kinase (PK), and hexokinase are all clearly inhibited by MMH treatment, but the latter two have such a great capacity that no functional change should be seen even at 50% inhibition (Table 1). PK, for example, is present in about 30 times greater activity than PFK (Weber et al., 1967).

TABLE 1. EFFECT OF SUBACUTE MMH UPON GLYCOLYTIC ENZYME ACTIVITIES. MMH INFUSED SUBCUTANEOUSLY FOR 13 HOURS PRIOR TO SACRIFICE. GLUCOSE (0.036 mMole/kg/hr) INFUSED INTRAINTESTINALLY FOR 9 HOURS PRIOR TO SACRIFICE.

Expressed as Δ OD_{340}/mg protein/min

	Control	MMH treated	Difference	%Δ	P
Pyruvate kinase	2.84±0.35 (N = 7)	1.65±0.58 (N = 6)	1.19	-34%	>0.99
Glucokinase	0.146±0.054 (N = 6)	0.099±0.036 (N = 6)	0.047	-32%	>0.90
Hexokinase	0.134±0.034 (N = 7)	0.069±0.035 (N = 6)	0.065	-49%	>0.975

The respective enzyme activities were also evaluated in liver homogenates after MMH was added but no inhibition was evident. Such a difference suggests that regulatory changes remote from the tissue may be responsible for changes under intoxication. The nature of the changes suggests that the possibility of impaired insulin release ought to be explored. Weber et al. (1966), in reviewing the role of insulin in glycolysis, quote numerous studies showing that glycolytic enzymes decrease in insulin deficiency and normalize with added insulin. We have made a preliminary evaluation of MMH influence on insulin levels and found lowered insulin in many treated animals but in no controls. This finding is not entirely surprising, since hydrazine blocks insulin release from pancreas (Aleyassine and Lee, 1971). In addition, MMH induced hyperglycemia responds to physiological amounts of endogenous crystalline insulin (Figure 3).

Hydrazine intoxication leads to a different story. The effects of hydrazine are similar on all the carbons of the molecule (Figure 4). There was a general leveling of the second phase of metabolism discussed earlier, in which carbon presumably enters a slower metabolic pool. An apparent true steady state was established over the entire infusion period which persisted for at least the ten hours of glucose administration after hydrazine was stopped. The implication is that the effect occurs at some point following triose formation, since the 3 and 4 carbons of glucose emerge in about the same pattern in treated and untreated animals, indicating that there is no defect prior to pyruvate decarboxylation. Metabolic behavior of pyruvate-2-^{14}C and of fructose-1-^{14}C, both of which label the acetate pool, and both of which are metabolized in the characteristic two-phase pattern, are changed by hydrazine in the same manner that it changes glucose oxidation. Consequently, we believe that the presumed usual flux of acetate carbon into fatty acid synthesis, with subsequent oxidation, was interrupted. The nature of the effect on acetate metabolism is not yet clarified, and we have in vitro labeling experiments in progress which should clarify the problem.

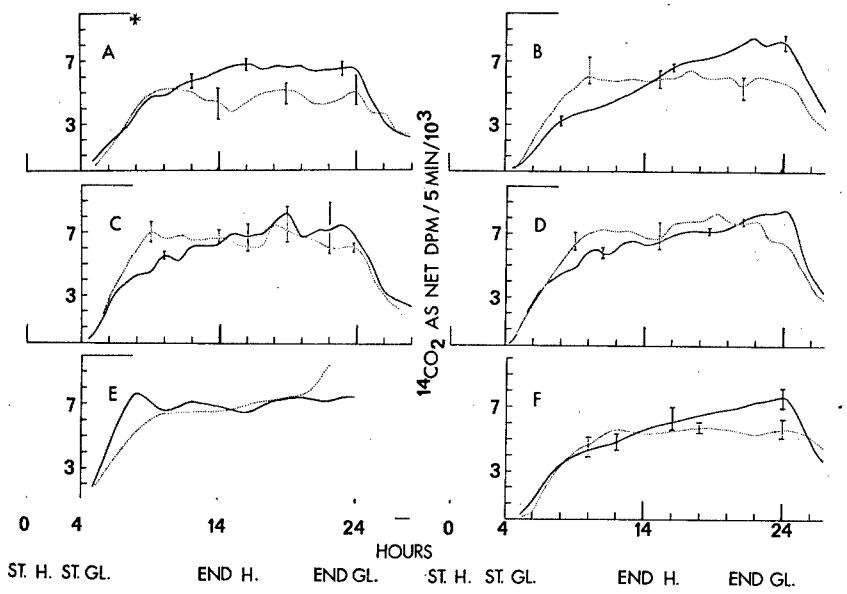

Figure 4. Effect of hydrazine on oxidation of variously labeled ^{14}C-glucose. Hydrazine administered from time zero at 0.143 mmole/kg/hr to hour 14; total dose 2.0 mmole/kg. Glucose infused 150 mg/hr, hours 4 to 24. A. $^{14}CO_2$ output from rats catabolizing glucose-1-^{14}C; B. -2-^{14}C; C. -3-^{14}C; D. -3,4-^{14}C; F. glucose-6-^{14}C. Each curve is average of 2-4 animals. Bars indicate ranges of values. Curves have been smoothed by using rate measurements at hourly intervals only. All data normalized to convention of relative input rate equal to 10^4 DPM/5 min.

Glucose Homeostasis

Availability of exogenous glucose influences the blood glucose response to hydrazine and MMH, but there appears to be a dependence upon the rate of administration of both glucose and the intoxicant. In 36-hour fasted animals, 1 mmole hydrazine/kg/3 hours (0.5 LD_{50}) caused a modest depression of blood glucose. A similar response appeared in fasted animals receiving up to about 150 mg glucose/kg/hr (Figures 5 and 6). MMH caused a slight and erratic hyperglycemia during fasting, but when glucose was infused at a 150 mg/hour the blood glucose concentration rose sharply for several hours in most animals (Figures 5 and 6). The effect of MMH, when extended over a period of 6-7 hours, was similar. In some cases

there appears to be a relationship between blood glucose changes and observed convulsive activity. In the example shown in Figure 7, a log plot of the data shows a loss of regulation at the time convulsions began. Glucose tolerance tests, on the other hand, using a single 500 mg glucose load injected directly into the small intestine, show little response after MMH, but hydrazine treated animals clearly lose regulatory capability (Figure 8).

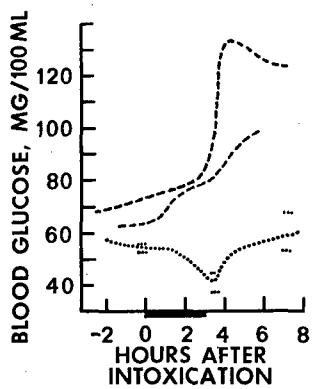

Figure 5. Effects of hydrazine (····) (1.0 mmole/-kg/3 hr) and MMH (----) (0.3 mmole/kg/3 hr) on blood glucose during 36 hour fast. MMH effect highly variable; two examples shown. Hydrazine effect is average of four experiments.

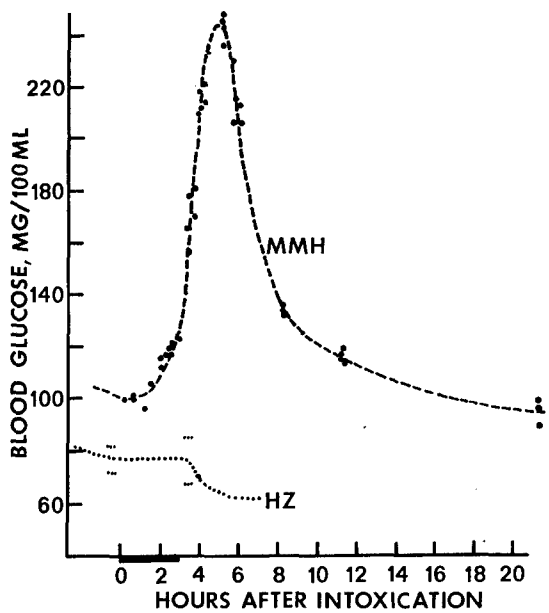

Figure 6. Effects of hydrazine (····) (1.0 mmole/kg/3 hr) and MMH (----) (0.3 mmole/kg/3 hr) on blood glucose during continuous infusion of 150 mg glucose /hr, intraintestinally. Glucose infusion started 6 hours prior to time zero, continued through hour 22. A single typical animal is used to illustrate the effect of MMH. Hydrazine effect is average of four experiments.

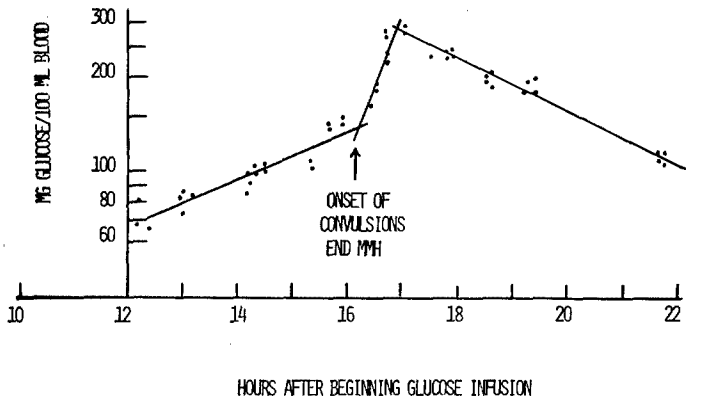

Figure 7. Log plot of blood glucose during MMH intoxication. Glucose infused 150 mg/hr from time zero. MMH infused 0.05 mmole/kg/hr, hour 9-16. Each point represents a single blood sample.

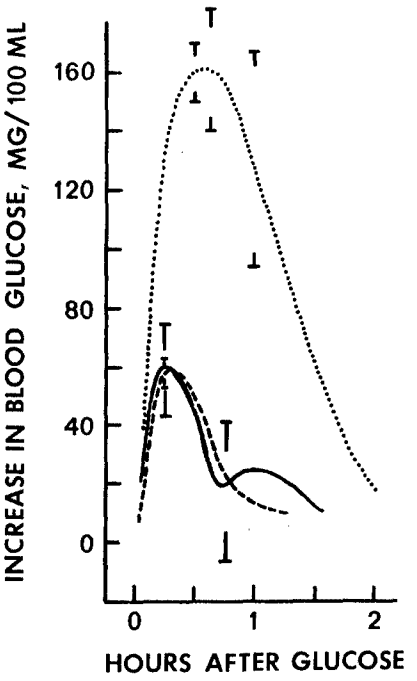

Figure 8. Glucose tolerance following hydrazine (····) (1.0 mmole/kg) and MMH (----) (0.25 mmole/kg), both infused subcutaneously over one hour prior to challenge with 500 mg glucose infused intraintetinally over 8-10 minutes. Untreated (——). Each curve represents average of five experiments. Bars indicate range of values.

It has been the traditional view that hydrazine accelerates glycogen mobilization. In a full liver with 6-10% glycogen, depletion proceeds at a normal rate until the glycogen content is below 1%, but instead of leveling off as normal animals do, depletion continues (Figure 9). The reason is that hydrazine totally blocks glycogen deposition at the dose used (Table 2) and outflow is not replaced. This effect is almost certainly in the pathway of glycogen synthesis from glucose-6-phosphate, since radiorespirometric data show there is no impairment of glucose phosphorylation of entry to cells. The well established failure of gluconeogenesis implied or identified by Izume and Lewis (1926), Fortney et al. (1967), and Ray et al. 1970) and blockage of phosphoenolpyruvate carboxykinase (Ray et al., 1970) in hydrazinized animals are probably not responsible for failure of glycogen synthesis from exogenous glucose and may not be the entire cause of blocked glycogen synthesis from amino acids. Izume and Lewis (1926) showed that the hyperglycemia resulting from large doses of lactate was not blocked by hydrazine, but that glycogen formation from lactate was stopped, indicating that the entire scheme between glucose as such and the mitochondrial barrier was not obstructed.

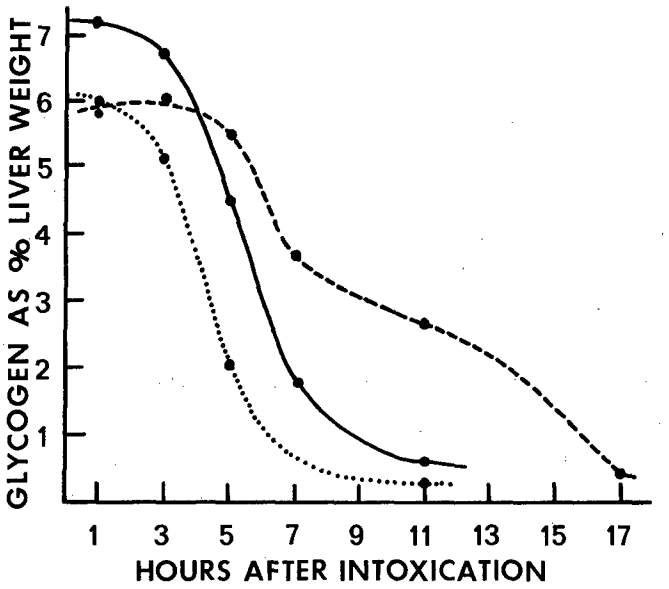

Figure 9. Glycogen depletion following acute hydrazine (····) (1.0 mmole/kg) and MMH (----) (0.25 mmole/kg). Each group was administered 500 mg glucose/hr for 14 hours. Intoxicants administered at hour 14. Untreated (——).

TABLE 2. INHIBITION OF LIVER GLYCOGEN DEPOSITION FOLLOWING VARYING ACUTE DOSES OF HYDRAZINE

Hydrazine dose	1.0	0.66	0.44	0.30	0.20	Control
Glycogen* deposition, % liver wt	0.9±.41 (5)	2.4±.08 (3)	4.1±.74 (5)	5.2±.9 (5)	6.1±.3 (3)	5.9±.69 (5)

Hydrazine, 1.0 mmole/kg, I.P. followed by glucose, 500 mg/hr for 14 hours.
*Mean ± standard deviation.
( ) = Number.

At 0.2 mmole/kg, which is about 0.1 LD_{50}, the interference with glycogen synthesis in our experiments is no longer perceptible, and the dose response is more or less linear between the two extremes (Table 2). Acute treatment with MMH has no effect on glycogen synthesis although it seems to slow mobilization by a few hours.

Amine Metabolism

In the intact animal we know of no system as sensitive to the hydrazines as the oxidation of methylamine and putrescine. The toxicologic significance of such systems as these which can be totally blocked without apparent hardship to the animal is a subject for debate, but there appears to be a considerable potential for development of these parameters as means for detecting intoxication by hydrazines. Exposure to 0.0006 mmole MMH/kg/hr, subcutaneously 8 hours daily, 5 days a week, for three weeks, causes a sharp decrease in methylamine oxidation (Figure 10). Hydrazine is somewhat less effective. Putrescine oxidation is less sensitive than that of methylamine, and at the lowest dose hydrazine actually caused a considerable increase in turnover; 1,1-dimethylhydrazine seems still less effective than hydrazine.

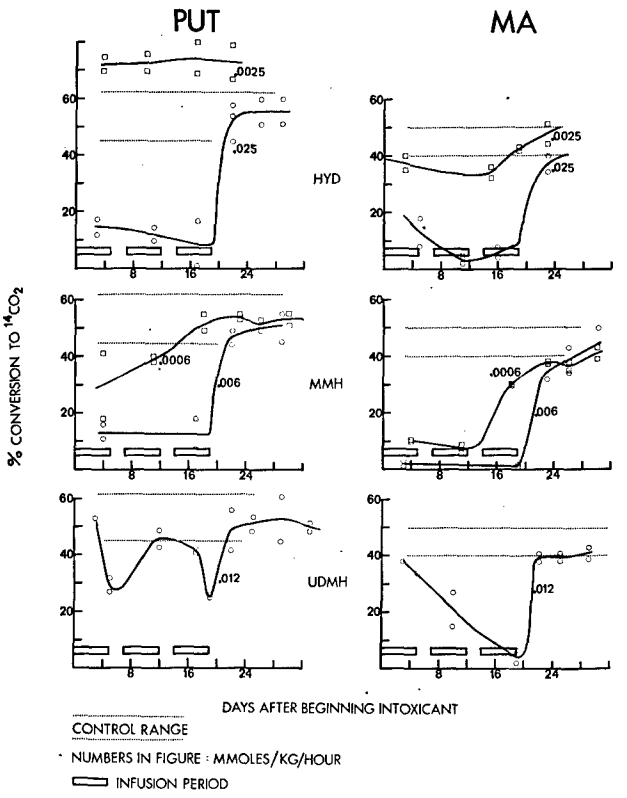

Figure 10. Effect of hydrazine, MMH and UDMH at low dose rates on oxidation of putrescine and methylamine. Each agent infused subcutaneously 8 hours daily, 5 days weekly for 3 weeks. Each point represents % recovery of $^{14}CO_2$ over six hours after a single intraperitoneal dose of ^{14}C-labeled amine. Dotted lines show limits of eight control values.

The lowest dose rate for MMH that was used can be translated into vapor phase exposure (if we can be permitted to consider MMH an ideal gas and to ignore MMH degradation on surfaces). If we assume a 70 gm man with 6000 ml ventilation/minute and a parallel dose response between rat and man, at 0.6 μmole/kg/hr the exposure will be about 15.5 mg/work day, which amounts to a little less than 5.5 mg/M^3. Surface reaction will raise the effective concentration, and activity with faster respiration will lower it.

CONCLUSIONS

A concern was expressed earlier that we must estimate the performance of these agents in the real world. It is entirely proper to question the significance of these findings, since the experiments described represent high doses of 0.5-1.0 acute LD_{50}, usually given over several hours. All of the effects I mentioned disappeared with reduction of the

dose to 0.1 to 0.25 LD_{50}. From that it is easy to suggest that these agents are no major concern. Keep in mind, however, that many of these effects were in a highly defended system, and were evident after only a modest fraction of the total treatment was received. The animals were well, and rested, without diabetes, without thyroid or adrenal dysfunction. Beyond that, we have no information about that very special case, the brain, which burns only glucose except under deep starvation. Other tissues catabolize fatty acid and ketones; glucose is often probably a second choice.

The brain is highly dependent on glucose and may be highly vulnerable, but we still know little about its functional metabolic impairment by the hydrazines, or about what residual effects which may follow.

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AMRL-TR-75-125

PAPER NO. 14

TOXICITY OF SOLID ROCKET MOTOR EXHAUST - EFFECTS OF HCl, HF AND ALUMINA ON RODENTS

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INTRODUCTION

The firing of certain solid fuel rocket engines has been shown to result in the emission of large quantities of hydrogen chloride (HCl), hydrogen fluoride (HF) and submicron sized particles of aluminum oxide (alumina). The possible exposure of Air Force personnel and local residents to these combustion products prompted a rodent lethality study to determine the degree of hazard posed by such exposures. Although much acute toxicity data on HCl and HF has been generated in this laboratory, no information existed on effects resulting from simultaneous exposure to these two compounds plus alumina particles.

Three series of 60-minute inhalation exposures were performed in an attempt to classify the toxicity to rats and mice of combinations of the three materials. First, single contaminant exposures were undertaken to determine the dose-response relationship of HCl and that of HF. Second, concurrent exposures to both HCl and HF were performed to discover if they act in an additive, less than additive, or more than additive manner as indicated by rodent mortality. The third series of experiments comprised the duplication of exposure concentrations and conditions of selected concurrent HF and HCl exposures with the addition of high concentrations of alumina dust. Any interaction of the alumina to cause an alteration in toxicity would be indicated by greater or lesser animal mortality.

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MATERIALS AND METHODS

Test animals consisted of groups of ten male CFE (Sprague-Dawley derived) rats weighing between 250 and 325 grams and groups of 10 CF-1 (ICR derived) mice with weights ranging from 25 to 32 grams.

Animals were observed for toxic signs and mortality during the exposure and for a period of 14 days postexposure. A representative number of test animals that died following treatment or were sacrificed after the 14-day observation period were submitted for gross and histopathological examination.

The exposure chamber was a modified Longley type utilizing a sliding cage drawer to permit rapid insertion and withdrawal of test animals from the contaminant equilibrated chamber. Partially dried air with a relative humidity of approximately 23% was metered at a rate of 11 cfm to one quadrant of the Longley chamber having a volume of 22.1 cubic feet.

Hydrogen fluoride vapor was supplied to the exposure chamber from steel tanks of the pure liquid material (Matheson Gas Products) or from gas cylinders of a 1% concentration HF in dried nitrogen. Metering of the contaminant was accomplished with a Teflon® flowmeter (Mace 16032) or a Hastings Raydist Mass Flowmeter (Model LF-20K).

A glass flowmeter was used to meter HCl vapors from a steel cylinder of the pure liquid material (Matheson Gas Products).

Anhydrous aluminum oxide powder of the gamma form with an upper particle size limit of 1.4 micron was acquired from a commercial source (Research Organic/Inorganic Chemical Corp.). Delivery of the alumina particles was accomplished with a modified fluidizing dust generator (Figure 1) of the type described by Drew and Laskin (1971). Modifications consisted of a more powerful fan motor and the pressurization of the fluidizing chamber. This enabled the generation of much higher dust concentrations for longer periods than possible with the unmodified device.

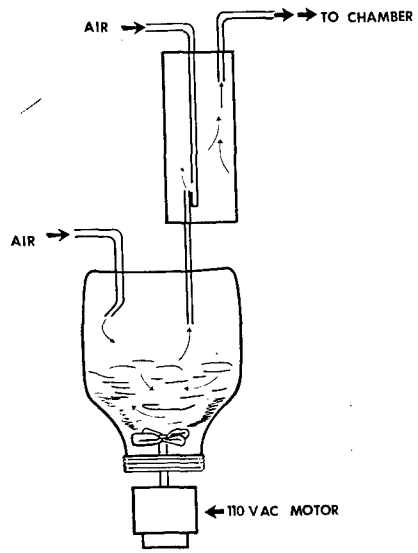

Figure 1. Alumina dust generator.

Continuous analysis of HF and HCl concentrations in the exposure chamber were provided by utilizing specific ion electrodes. Known volumes of chamber atmosphere were mixed in a gas scrubber column with known amounts of an aqueous reagent absorber. The solution was then passed through a flow cell containing the ion and reference electrodes. Calibration of the electrodes was done prior to every exposure.

The analysis of alumina exposure concentrations was performed by a gravimetric method. Samples of the chamber atmosphere were drawn at a rate of 10 liters per minute through a sample head (Gelman Model 1220) containing two membrane filters having a pore size of 0.45 and 0.2 micron (Gelman, 47 mm, type GA-6 and type GA-8). Alumina concentrations, expressed in milligrams per cubic meter, were determined 12 times for each one hour exposure.

Statistical analysis of mortality data was accomplished using the BMD03S Biomedical Computer Program, Biological Assay - Probit Analysis Method.

RESULTS AND DISCUSSION

Single Contaminant Exposures

The 60-minute LC_{50} values for rats and mice exposed to HCl vapors alone are 3124 ppm and 1108 ppm, respectively. Mortality response data for individual exposures are shown in Table 1.

TABLE 1. MORTALITY RESPONSE OF RATS AND MICE EXPOSED TO HCl VAPORS FOR 60 MINUTES

RATS		MICE	
Concentration (ppm)	Mortality Ratio	Concentration (ppm)	Mortality Ratio
1813	0/10	557	2/10
2585	2/10	985	3/10
3274	6/10	1387	6/10
3941	8/10	1902	8/10
4455	10/10	2476	10/10
LC_{50} & 95% C.L.= 3124 ppm (2829-3450)		LC_{50} & 95% C. L. = 1108 ppm (874-1404)	

Toxic signs noted during exposure to HCl included increased grooming, irritation of eyes, mucous membranes and exposed skin. A rapid, shallow breathing pattern and fur discoloration to a yellow-green were noted by the end of 60 minutes.

Necropsy of animals dying during or after exposure revealed pulmonary congestion and intestinal hemorrhages in both rats and mice, with rats also exhibiting thymic hemorrhages.

Mortality data for rats and mice exposed to various concentrations of HF vapors alone are shown in Table 2. LC_{50} concentrations were 1395 ppm for rats and 342 ppm for mice.

TABLE 2. MORTALITY RESPONSE OF RATS AND MICE EXPOSED TO HF VAPORS FOR 60 MINUTES

RATS		MICE	
Concentration (ppm)	Mortality Ratio	Concentration (ppm)	Mortality Ratio
1087	0/10	263	0/10
1108	2/10	278	1/10
1405	3/10	324	7/10
1565	8/10	381	6/10
1765	10/10	458	9/10
LC_{50} & 95% C. L. = 1395 ppm (1302-1495)		LC_{50} & 95% C. L. = 342 ppm (315-372)	

Symptoms of rats and mice during exposure included eye and mucous membrane irritation, respiratory distress, corneal opacity and erythema of exposed skin.

Pathological examination of rats dying during or after exposure showed pulmonary congestion, intraalveolar edema and some cases of thymic hemorrhage. Mice exhibited pulmonary congestion and hemorrhage.

HCl and HF Simultaneous Exposures

The purpose of the next series of experiments was to determine if any toxic interactions exist from simultaneous exposures to HCl and HF. By defining a dose response relationship, it can be shown whether the combination results in additive, less than additive, or more than additive effects. The mortality response of rats obtained from simultaneous exposure to various concentrations of HCl and HF is shown in Table 3.

TABLE 3. MORTALITY RESPONSE OF RATS EXPOSED FOR 60 MINUTES TO COMBINATIONS OF HCl AND HF VAPORS

HCl, ppm	HF, ppm	Mortality
1292	493	80%
1366	580	50%
1421	610	30%
1564	570	40%
1505	640	10%
1685	786	60%
1845	823	80%

Figure 2 is a graphic representation of the mortality response of rats obtained from exposure to HCl and HF singly and in combination. The line marked HCl is the probit regression line obtained from plotting percent mortality (converted to probits) versus the log of the average concentration during the exposures to HCl alone. The line marked HF is the probit regression line for the HF only exposures. The equations of the probit regression lines were obtained from the computer printouts during statistical analysis and are as follows for rats:

$$\text{Probit response} = 4.9\ (\ln \text{ of HCl conc.}) - 34.426 \quad (1)$$

$$\text{Probit response} = 6.481\ (\ln \text{ of HF conc.}) - 41.928 \quad (2)$$

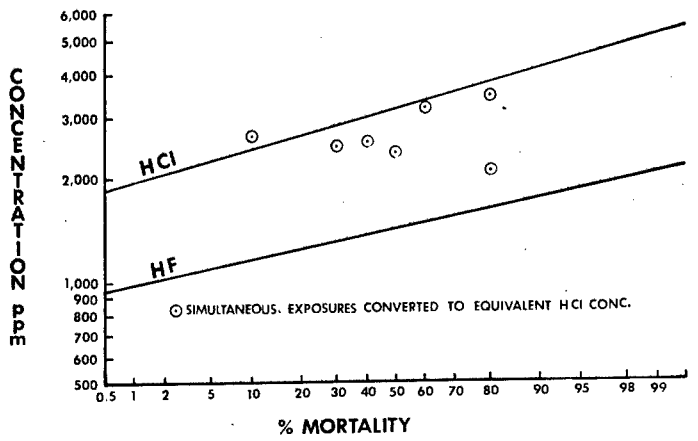

Figure 2. HCl and HF probit regression lines for rats.

Analysis of the simultaneous exposure data was performed by the use of these equations to convert HF concentrations to the equivalent HCl concentration necessary to produce the same mortality response. The addition of the actual HCl concentration plus the concentration of HF expressed as its equivalent HCl concentration equals a total effective HCl concentration. For example, a concentration of 786 ppm HF and 1685 ppm HCl was achieved during a simultaneous exposure and produced 60% mortality in rats. The probit response for an exposure of rats to HF alone was calculated by inserting the log of 786 into equation 2. The obtained probit response (1.2803) was then substituted into the HCl equation and the equivalent concentration of HCl was calculated to be 1460 ppm.

The point plotted from the total 3145 ppm (1685 ppm + 1460 ppm) giving a 60% mortality plus the other points derived from the above method are represented by circles in Figure 2.

Note that the points are distributed along and slightly below the probit regression line for HCl. This indicates that the effects of HCl and HF are almost entirely additive. A distribution of combination exposure points that was markedly above or below the HCl probit regression line would indicate less than additive or more than additive effects respectively. Additivity is not surprising considering the similar chemical nature and target organs of the two compounds.

Table 4 shows the mortality response of mice exposed to HCl and HF concurrently.

TABLE 4. MORTALITY RESPONSE OF MICE EXPOSED FOR 60 MINUTES TO COMBINATIONS OF HCl AND HF VAPORS

HCl, ppm	HF, ppm	Mortality
555	169	20%
816	197	20%
816	197	20%
547	230	60%
808	267	40%
875	284	20%
911	290	70%
1090	332	80%

Figure 3 depicts graphically the dose response data for mice exposed to HCl and HF singly and in combination.

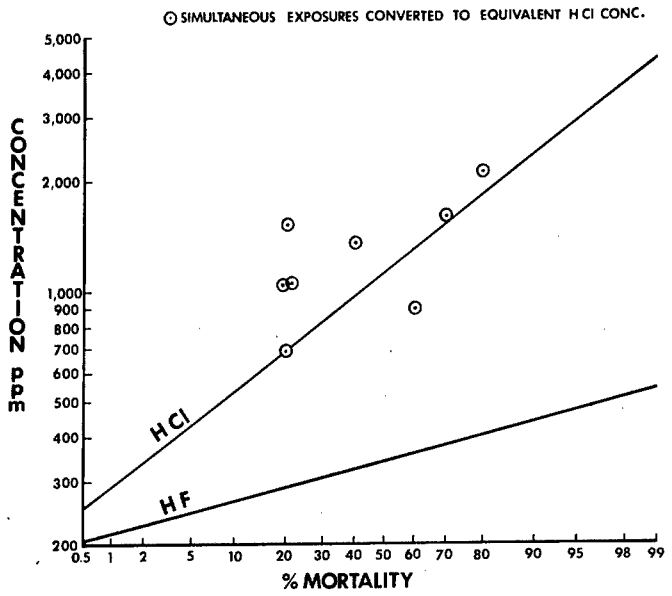

Figure 3. HCl and HF probit regression lines for mice.

The equations of the probit regression lines for mice are as follows:

$$\text{Probit response} = 1.737\ (\ln \text{ of HCl conc.}) - 7.177 \quad (3)$$

$$\text{Probit response} = 5.075\ (\ln \text{ of HC conc.}) - 24.618 \quad (4)$$

As in the rat data, the circles in Figure 3 are plots of the total equivalent HCl concentration versus mortality during simultaneous exposures. Again, the points are distributed along the HCl probit regression line. Therefore, additivity of effect of HCl and HF for mice is also indicated.

Gross and histopathological examination of rats and mice exposed to combinations of HCl and HF revealed no additional sites of damage from those seen of single exposures.

Alumina Exposures

At this stage of investigation, an exposure of 10 rats and 10 mice to an average 478 mg/m^3 of alumina dust for 60 minutes was performed. Symptomatology included vigorous grooming during the first quarter of the exposure, followed by signs of mechanical irritation to the eyes and nasal passages as indicated by half closed eyes and sneezing-like activity. No toxic effects were apparent immediately postexposure, nor in the 14 days following. All animals survived and had normal weight gains. Gross and histopathological examination at the end of the postexposure observation period showed normal tissue with no changes attributable to the exposure.

Spectrographic analysis of lung tissue from control animals and from rats and mice sacrificed immediately after the exposure to alumina dust revealed that a significant amount of alumina had been deposited in the lungs of exposed animals.

The last series of exposures investigated the change in toxicity, if any, produced by the addition of high concentrations of alumina dust to an atmosphere containing HCl and HF gases. Although HCl and HF act primarily as pulmonary irritants and mainly affect upper respiratory tissue, it is possible that the gases could be adsorbed onto the alumina particles and carried into the more inaccessible parts of the lung.

An apparent positive or negative effect of alumina to produce rat mortality greater or lesser than seen in the duplicate exposure without alumina is represented by a plus or minus sign in Table 5. The same information for mice is shown in Table 6.

Each exposure pair was conducted on the same day using animals from the same lot, with contaminant and analysis chemicals from the same batch.

Gross and histopathological examination of animals exposed to all three materials showed the same type of damage as exposure to HCl and HF combinations.

Our results indicate that the addition of alumina dust to HCl and HF combination exposures had no net effect on rodent mortality.

TABLE 5. MORTALITY RESPONSE OF RATS EXPOSED TO HF, HCl AND ALUMINA FOR 60 MINUTES

HCl Conc. ppm	HF Conc. ppm	Al_2O_3 Conc. mg/m^3	Mortality	Apparent Alumina Effect
875	284	-	0	
911	290	232	0	
1421	610	-	30%	
1366	580	505	50%	+
1564	570	-	40%	
1505	640	538	10%	-
1845	823	-	80%	
1685	786	610	60%	-

TABLE 6. MORTALITY RESPONSE OF MICE EXPOSED TO HF, HCl, AND ALUMINA FOR 60 MINUTES

HCl Conc. ppm	HF Conc. ppm	Al_2O_3 Conc. mg/m^3	Mortality	Apparent Alumina Effect
375	151	-	0	
428	147	121	0	
555	169	-	20%	
541	175	128	0	-
816	197	-	20%	
816	197	156	20%	0
875	284	-	20%	
911	290	232	70%	+
1564	570	-	100%	
1505	640	538	100%	

SUMMARY

In summary, the purpose of this investigation was to determine the degree of hazard posed by simultaneous acute inhalation of HCl, HF and alumina dust. LC_{50} concentrations for rats and mice were determined for 60-minute exposures to HCl alone and to HF alone. Combination exposures to various concentrations of HCl and HF vapors provided information that could be analyzed by probit analysis techniques. It was shown in exposures of rodents to both corrosive gases simultaneously that the mortalities produced were due to a physiologically additive effect. That is, one compound did not potentiate nor antagonize the effects of the other. The addition of alumina dust to atmospheres containing HCl and HF vapors did not increase nor decrease rodent mortality.

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AMRL-TR-75-125

PAPER NO. 15

TOXICITY OF HIGH DENSITY JET FUEL COMPONENTS

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INTRODUCTION

A new aircraft fuel has been developed for extending the flight range before refueling. The fuel designated JP-9 is a mixture of three primary ingredients, namely, RJ-4, RJ-5, and methylcyclohexane. RJ-4 and RJ-5 are high density hydrocarbons yielding a greater BTU output per unit volume than conventional jet aircraft fuels. They also have a higher viscosity which causes pumping or flow problems at low temperatures which is the reason for the addition of methylcyclohexane to the mixture. The precise composition of the JP-9 fuel is not fixed but will be tailored for use in specific aircraft systems. Although no toxicity data are available for JP-9 fuel, it is not meaningful to evaluate the entire mixture for two reasons: first, the actual mixture has not been set, and second, methylcyclohexane is extremely volatile in comparison with the other constituents and would dominate the vapor exposure mixture, thus masking the effects of RJ-4 and RJ-5.

The acute and chronic toxicity studies on methylcyclohexane have been reported by Treon et al. (1943). Acute exposures of rabbits to inhaled concentrations of methylcyclohexane above 10,000ppm ($\approx$ 40 mg/liter) caused significant weight loss, narcosis and convulsions while a concentration of 15,227 ppm was fatal in slightly over one hour. Repeated, daily 5-hour exposures to rabbits to concentrations of 1162 ppm or lower for periods up to 10 weeks produced no measureable or observable signs of toxicity.

Some of the physical chemical properties of RJ-4, RJ-5, and methylcyclohexane are shown in Table 1. RJ-4 is a mixture of isomers of perhydro di (methylcyclopentadiene). RJ-5, also known as "Shelldyne H", is a mixture of reduced dimers of bicycloheptadiene. Noticeable in Table 1 are the very low vapor pressures of RJ-4 and RJ-5 relative to that for methylcyclohexane. The vapor pressures of RJ-4 and RJ-5 are approximately 100 and 1500 times less, respectively, than methylcyclohexane. This information is basic to the consideration of the hazard (the probability of injury in use) of these fuels.

TABLE 1. PHYSICAL CHEMICAL PROPERTIES OF RJ-4, RJ-5, AND METHYLCYCLOHEXANE

	RJ-4	RJ-5	MCH
Empirical Formula	$C_{12}H_{20}$	$C_{14}H_{20}$	C_7H_{14}
Molecular Weight	164	188	98
Boiling Point (F)	431	522	213
Vapor Pressure (70 F)	0.354 mm Hg	0.025 mm Hg	42 mm Hg
Density (70 F)	0.925 g/ml	1.0813 g/ml	0.7660 g/ml

To examine the acute inhalation hazard and to obtain experimentally determined saturation concentrations as an aid in the selection of vapor levels for the chronic study, groups of six rats each were exposed for 6 hours to essentially saturated vapors (MacEwen and Vernot, 1974) of each compound. No adverse effects were seen during exposure. Pathologic examination after 14-day postexposure observation showed no abnormalities. Peroral doses of 4 g/kg RJ-5 in corn oil were not lethal to a group of 3 rats; however, 2 of 3 mice succumbed to a 250 mg/kg dose (MacEwen and Vernot, 1974).

The toxicity of RJ-4 and RJ-5 has not been reported previously and it was, therefore, necessary to conduct chronic inhalation studies with these materials to evaluate their potential health hazard.

Accordingly, concentrations of 0.15 mg/l RJ-5 and 2 mg/l RJ-4 were selected for the 6-month chronic exposure of 4 animal species. The levels chosen are slightly below saturation vapor pressures so that condensation on chamber surfaces would not occur.

METHODS

Each experimental group and the unexposed chamber controls consisted initially of 4 female and 4 male beagle dogs, 50 male CFE rats, 40 female CF-1 mice, and an uneven mixture of male and female Macaca mulatta monkeys, 4 per chamber.

Each group of animals was housed in separate Thomas Domes operated with nominal airflows of 40 CFM at a slightly reduced pressure, 725 mm Hg, to avoid leakage of the hydrocarbons. Temperatures were controlled at 72 + 2 F and relative humidity at 50 + 10%. Exposures were conducted on a 6 hour/day, 5 day/week schedule. No exposures were made on weekends and holidays. Upon completion of the daily exposures, the chambers containing RJ-4 and RJ-5 were purged with air for 30 minutes before lifting the dome tops. Cleaning of the chambers was done and residual food replaced with fresh supplies at this time.

Although expected to be low, the toxicities of the chemicals under study are unknown except for the minimal acute animal information mentioned earlier. Personnel working with these materials avoided skin contact and inhalation. The vapor generation apparatus and chemical supplies were in ventilated hoods and the areas were no smoking zones.

The chamber concentrations of RJ-4 and RJ-5 were continuously monitored using flame ionization hydrocarbon analyzers. The generation and monitoring techniques were identical to those used during the JP-4 toxicity study (MacEwen and Vernot, 1974).

To measure the chronic toxicity of RJ-4 and RJ-5, a limited number of parameters were selected, with the view toward increasing the variety of tests should the basic battery reveal trends or deleterious effects during the course of the study.

All exposed animals were observed for signs of toxic stress as well as mortality. Gross and histopathologic examinations were made on all dead animals. Body weights of dogs, monkeys and rats were measured on a biweekly schedule. Table 2 shows the reduced battery of clinical hematology and chemistry tests performed on blood samples taken from dogs and monkeys on a biweekly basis. A complete battery of clinical laboratory tests

was performed at the start and at the completion of the exposures. These tests included, in addition to those shown in Table 2, creatinine, chlorides, cholesterol, BUN, total inorganic phosphorus, bilirubin and serum triglycerides. At final blood sampling or sacrifice of the large animals, additional blood samples were drawn for identification and refrigerated storage of serum. These "banked" serum samples were stored until histopathology reports were received and reviewed. Twenty rats and mice from each of the study groups were retained for one-year observation following exposure termination in the event of any postexposure effects from RJ-4 and/or RJ-5 inhalation. All remaining animals were sacrificed at exposure termination and submitted for gross and histopathologic examination. Major organs were taken from 20 rats from each group and weighed to allow for comparison of mean organ weights and organ to body weight ratios.

TABLE 2. CLINICAL BLOOD TESTS PERFORMED ON RJ-4, RJ-5 EXPOSED AND CONTROL DOGS AND MONKEYS

HCT	Sodium	Calcium
HGB	Potassium	Glucose
RBC	Albumin/Globulin	Alkaline Phosphatase
WBC	Total Protein	SGPT
		Differential Cell Count

RESULTS

A curious effect occurred in the rats exposed to RJ-4. Diarrhea was evident in the majority of the rats at 10 weeks of exposure and continued throughout the duration of the exposure portion of the study. Frequent postexposure observation of surviving rats revealed gradual alleviation of this condition. At 14 weeks postexposure, there were no signs of diarrhea.

There were six deaths during the 6 months of exposure. One male monkey died during the seventh week of exposure to RJ-5. Pathology revealed death was due to gastric dilatation of unknown etiology, but believed to be unrelated to exposure. One mouse in each of the exposure groups was sacrificed at 4 and 16 weeks of exposure due to accidental injuries. Remaining mortality was limited to control rodents. One mouse and one rat died of pneumonia at 1 and 24 weeks respectively. One rat was sacrificed at 10 weeks because of abnormal behavior indicative of middle ear infection.

Mean body weights of exposed monkeys obtained on a biweekly schedule were normal when compared with control weights taken on the same time schedule. However, weight depressions were noted for rats and dogs exposed to RJ-4 and RJ-5.

The growth rate of rats is shown in Figure 1. Noticeable is the apparently subnormal gain from 2 weeks forward for both exposed groups. The mean weights of theRJ-4 exposed animals were statistically different from control values at all time periods. However, the weights were only about 5% less than that of the controls. This is certainly an indication of stress, but of no great importance biologically. Although at several time periods the mean weights of the RJ-5 exposed rats were 10-12 grams less than control, statistical calculations revealed no significant differences from control weights. The odors of RJ-4 and RJ-5 were very noticeable and objectionable even after purging of the chambers after each exposure period. It was theorized that the chemical odors were causing appetite suppression in rats resulting in weight gain suppression. To test this theory, food consumption measurements were made over a 3-day period during the 10th week of exposure. The daily results were variable, but overall information suggested there was no real difference in food consumption between control and exposed rats.

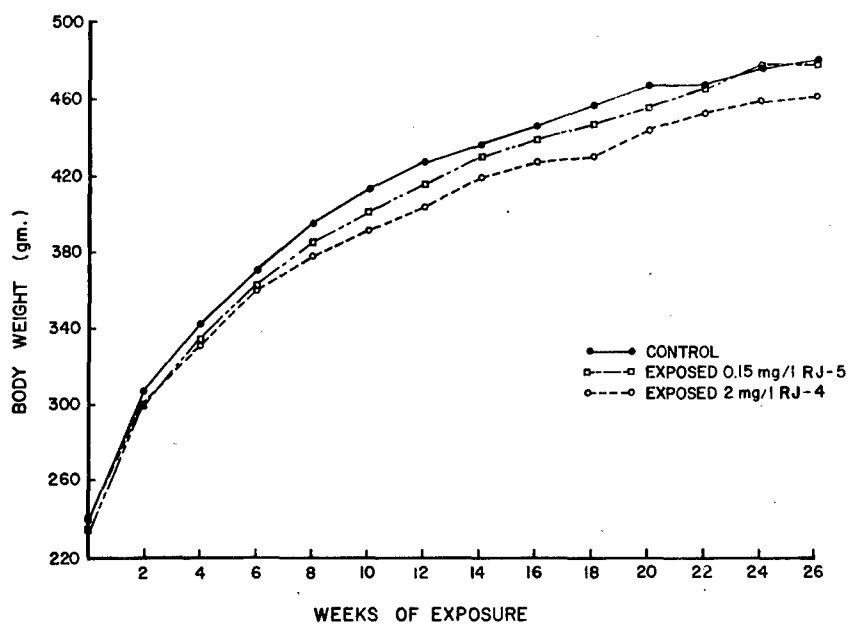

Figure 1. Mean body weight change in rats exposed to JP-9 Constituents (RJ-4 and RJ-5).

Dog mean body weights are shown in Figure 2. Both exposed groups of dogs gained less weight than controls throughout the course of the study. At first glance, it would appear that weight depression was greater for the RJ-5 exposed dogs. This is not the case in that the RJ-5 group weighed 0.5 kilograms less than the RJ-4 group at the beginning of the study. An examination of initial and final group mean body weights revealed that the controls, the RJ-5 and the RJ-4 groups gained 2.10, 1.22, and 0.98 kilograms, respectively. Therefore, comparable subnormal weight gains occurred for dogs exposed to RJ-4 and RJ-5.

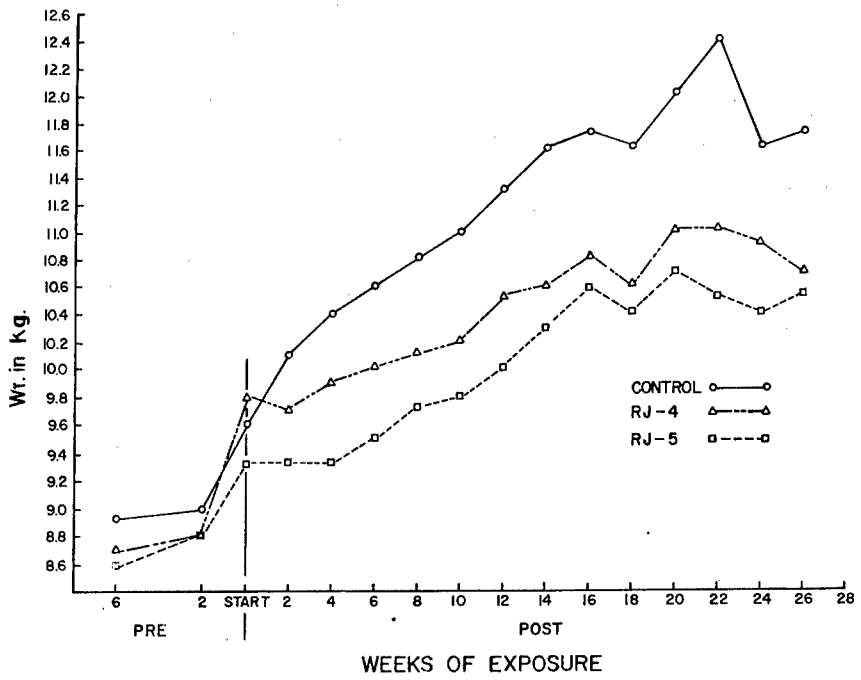

Figure 2. Mean body weight change in dogs exposed to JP-9 Constituents (RJ-4 and RJ-5).

Biweekly clinical blood test results collected on dogs and monkeys showed no abnormalities or trends to adverse hematological effect.

There was no abnormal change in organ weights in rats exposed to RJ-5. Mean organ weights and organ to body weight ratios are shown in Table 3 for rats exposed to RJ-4 and controls. No toxicological significance is attached to the lung weight difference between RJ-4 exposed and control rodents in that body weights of RJ-4 rodents were also significantly lower than controls. However, mean liver and kidney weights as well as the ratios for the RJ-4 exposed rats are statistically higher than control values.

TABLE 3. THE EFFECT OF 6-MONTH CHRONIC INHALATION EXPOSURE TO RJ-4 ON RAT ORGANS[1]

	Mean Organ Weight, Grams		Organ/Body Weight Ratio*	
Organ	RJ-4	Control	RJ-4	Control
Liver	15.61**[2]	12.96	3.536**[2]	2.820
Kidney	3.50**[2]	3.20	0.792**[2]	0.674
Lung	1.92**[3]	2.16	0.434**[3]	0.471
Spleen	0.84	0.91	0.188	0.198
Heart	1.47	1.55	0.333	0.339

* Grams/100 grams body weight x 10^{-2}.
** Statistically different from control at 0.01 level.
[1] N = 20.
[2] Significantly higher than control.
[3] Significantly lower than control.

Gross pathology results for animals sacrificed at exposure conclusion revealed no changes attributable to RJ-4 or RJ-5 exposure. There were no significant histopathology findings in monkeys and mice. However, acute inflammation was noted in the lungs of dogs and rats exposed to RJ-4 and RJ-5. This information is shown in Table 4. It can be seen that lung lesions were restricted to male RJ-4 and female RJ-5 exposed dogs while 8 of 20 RJ-4 exposed rats and 6 of 20 RJ-5 exposed rats showed bronchopneumonia only.

TABLE 4. LUNG HISTOPATHOLOGY IN DOGS AND RATS EXPOSED TO JP-9 CONSTITUENTS (RJ-4 AND RJ-5)

	RJ-4 Exposed			RJ-5 Exposed			Controls		
	Dogs		Rats	Dogs		Rats	Dogs		Rats
Condition	♂	♀	♂	♂	♀	♂	♂	♀	♂
Bronchopneumonia	3/4	0/4	8/20	0/4	2/4	6/20	0/4	0/4	2/20
Bronchitis	1/4	0/4	1/20	0/4	3/4	0/20	0/4	0/4	1/20
Abscess	0/4	0/4	0/20	1/4	0/4	0/20	0/4	0/4	0/20

Cause of death in groups of 20 rats and mice during the 12-month postexposure observation period, almost without exception, was due to pneumonia. Table 5 shows the number of rodent survivors at sacrifice, one-year postexposure. Gross pathology results for the few remaining rats, 2 RJ-5 exposed and 3 controls, revealed chronic respiratory infection in all cases. Nodules, tumor like lesions, were seen on the lungs of exposed and control mice. One control mouse, 3 RJ-4, and 5 RJ-5 exposed mice showed these lesions. Histopathology results are not complete and, therefore, cannot be reported here.

TABLE 5. JP-9 STUDY SURVIVORS AT ONE-YEAR POSTEXPOSURE

Group	Rats	Mice
RJ-4 Exposed	0/20	12/20
RJ-5 Exposed	2/20	14/20
Control	3/20	12/20

SUMMARY AND CONCLUSIONS

No lethality occurred in four animal species during 6-month inhalation exposures to near saturation concentrations of RJ-4 and RJ-5. Dogs and rats in both exposure groups experienced body weight depressions relative to their controls. Weight depressions proved to be statistically lower than controls except for rats exposed to RJ-5 vapor. Mean liver and kidney weights

as well as their ratios were significantly elevated in RJ-4 exposed rats when compared with control data. Histopathology which included oil-red-o staining failed to reveal any fat deposition or abnormal alterations in liver and kidney tissue which could account for increased organ weights in RJ-4 rats. Histopathologic findings in exposed monkeys and mice showed no abnormalities that were treatment related. However, for dogs and rats, considering acute pulmonary inflammation as a group, i.e., abscess, bronchopneumonia, and bronchitis, the frequencies suggest respiratory irritation with the probability of secondary bacterial inflammation. The results of clinical hematology and chemistry tests performed on dogs and monkeys provide evidence that no kidney, liver or hematological toxicity occurred from chronic exposure to RJ-4 or RJ-5 vapors.

The results of this study demonstrate the low order of toxicity of JP-9 constituents exhibited in experimental animals. Kidney and liver hyperplasia in RJ-4 exposed rats and pulmonary irritation in dogs and monkeys exposed to RJ-4 and RJ-5 emerge as the salient results of this study. Although the reasons for organ hyperplasia in rats is not clear, it is of little toxicological significance in that there was no tissue destruction or alteration. The latter finding of respiratory irritation should be considered relative to possible human experience of chronic exposure to RJ-4 or RJ-5. In this regard, certain factors must be considered. Due to their low vapor pressures, the inhalation hazard (the probability of injury in use) is extremely low. The odors of RJ-4 and RJ-5 are extremely objectionable and it is, therefore, doubtful that workers would tolerate concentrations far less than those used in this study for any substantial time period. Furthermore, as constituents of JP-9 fuel, the toxicity of the mixture would be largely that of methylcyclohexane. However, both RJ-4 and RJ-5 individually show a low order of toxicity in experimental animals and are judged to be of a low inhalation hazard to man.

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AMRL-TR-75-125

PAPER NO. 16

SUBACUTE STUDIES OF OZONE AND SULFURIC ACID MIST AND THEIR MIXTURES

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The biological effects of sulfuric acid and ozone have been investigated in experimental animals by several investigators. Recent synergism studies involving acid mist, sulfur dioxide and fly ash have been reported by Alarie et al. (1973, 1975). These studies evaluated long term effects in both cynomolgus monkeys and guinea pigs. Pulmonary function and lung morphology changes were seen in monkeys exposed to 2.43 and 4.79 mg/m^3, whereas no abnormalities were noted in guinea pigs exposed to 0.10 or 0.08 mg/m^3 of sulfuric acid mist. Several synergism studies involving ozone and other edemagenic gases have been reported (Goldstein et al., 1974; Alpert and Lewis, 1971). No cross-tolerance was reported in either mice or rabbits for ozone and nitrogen dioxide and no synergism was reported in mice for infection with aerosols of Staphylococcus aureus. A recent report of exposures to ambient levels of ozone (Dungworth et al.,

1975) emphasizes that one 8 hour exposure evaluated 40 hours later exhibits identical pulmonary damage as a continuous 48 hour exposure. The threshold level for the reported damage to the respiratory bronchioles was between 0.1 and 0.2 ppm ozone in both monkeys and rats. The purpose of this study was to evaluate the acute effects of sulfuric acid mist, ozone and their combination in rats and guinea pigs so that proper levels of acid mist and ozone could be used in a long term study to determine possible synergistic effects between acid mist and ozone.

MATERIALS AND METHODS

Exposure Chambers

The exposure chambers are 1.3 cubic meter stainless steel and glass (Young and Bertke, Cincinnati, Ohio) chambers described by Hinners et al. (1968). The chambers operate at a slight negative pressure (0.5 inches of water) with an air flow of 200-300 liters per minute. Air entering the chambers was filtered and heated to 70 F and maintained at approximately 55 percent relative humidity. A schematic diagram of one chamber is shown in Figure 1.

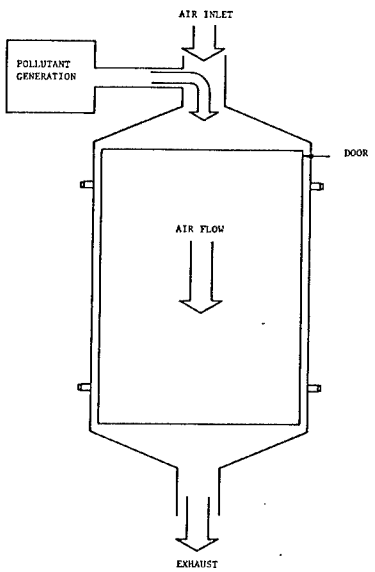

Figure 1. Exposure chamber.

Experimental Animals

Male Charles River (Sprague-Dawley)or Fisher rats weighing 150 to 200 grams and Charles River (Hartley strain) female guinea pigs weighing 250 to 300 grams were used for this series of short term exposures. The animals were group housed in stainless steel cages and were fed appropriate commercially prepared dry food and water ad libitum. For exposures, the animals were transferred to exposure cages and did not receive food or water during the exposures. The animals were observed daily for abnormal clinical signs and mortality. Body weights were recorded initially and periodically throughout the exposures.

Pollutant Generation

Ozone was generated from 100% oxygen in a Model C2P-3 ozone generator (PCI Corporation, Stamford, Conn.). Sulfuric acid mist was generated from 2M sulfuric acid in an all glass nebulizer (Alarie et al., 1973). A particle size of 1 micron was maintained in the acid mist chambers as determined by a Model DCI-6 stainless steel cascade impactor (Delron Research Products, Powell, Ohio).

Analytical Procedures

Ozone concentration was monitored using a Model 8002 ozone analyzer (Bendix Corporation, Baltimore, Maryland) connected to a Model 680 strip chart recorder (Hewlett-Packard, High Point, North Carolina). Ozone concentrations were periodically checked by standard wet chemical methods and the ozone analyzer was calibrated using the procedure described in the Federal Register, 36(4), 8196(1971). Sulfuric acid mist samples were collectedon glass fiber filters hourly from each chamber and were analyzed by titration using Thorin as the indicator (NIOSH). The details of these procedures have been published (Cavender, 1975).

Terminal Procedures

Peripheral blood samples are collected from each animal for hematological evaluation. Animals from each experimental group were sacrificed after two or seven exposure days. Each animal was sacrificed using barbiturate anesthesia followed by exsanguination. Five animals from each exposure group underwent a complete necropsy on each sacrifice day. Lung and brain weights were recorded and the following tissues were processed for histopathological examination: brain, nasal cavity, trachea, lung, peribronchial lymph node, heart, liver, kidney, and spleen. At least some animals of each group were used for lung biochemistry determinations and macrophage counts. The details of these procedures have been reported (Cavender, 1975).

RESULTS

There were neither gross lesions nor significant differences in macrophage counts, hematology values, blood chemistry data, lung biochemical parameters or lung to body weight ratios from exposed animals. There were no effects seen in rats exposed to sulfuric acid mist at any of the concentrations used in this series of studies. No effects were seen in guinea pigs exposed to sulfuric acid mist at concentrations ranging from 5 to 9 mg/m^3. No death occurred in the guinea pigs exposed to 10 and 20 mg/m^3; however, definite exposure-related microscopic alterations were seen in the lungs from the guinea pigs exposed to these two levels. Exposure-related microscopic alterations were seen in the lungs from both rats and guinea pigs in all ozone exposures.

Histopathology

The sulfuric acid mist exposure-related microscopic alterations in the lung were characterized by a diffuse regional, nonsuppurative alveolitis. The alveolitis was not necessarily associated with terminal bronchioles and consisted of an infiltration of mononuclear macrophages into the alveolar septa and an accumulation of alveolar macrophages in the lumen of the alveoli. Varying degrees of septal edema were present at these exposure levels. Focal areas of hemorrhage were seen occasionally. A normal section of rat lungs is shown in Figure 2. In almost every exposed animal,

a definite line of demarcation (Figures 3 and 4) which did not necessarily coincide with any anatomical structure was present between normal and lung tissue affected by the inhalation of sulfuric acid mist. Few, if any, changes were seen in the small airways. Occasionally, a minimal infiltration of mononuclear macrophages could be seen in the walls of the terminal bronchioles.

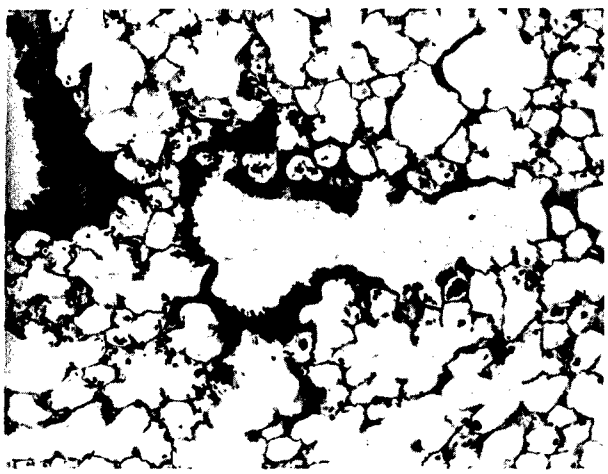

Figure 2. Respiratory bronchiole in an acid exposed guinea pig lung.

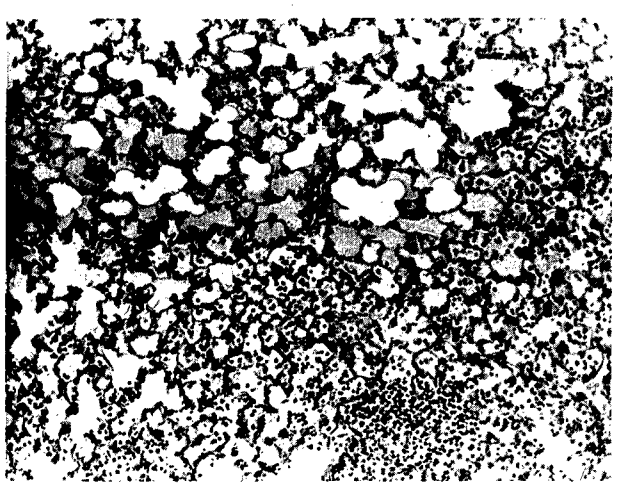

Figure 3. Alveolitis in an acid exposed guinea pig lung.

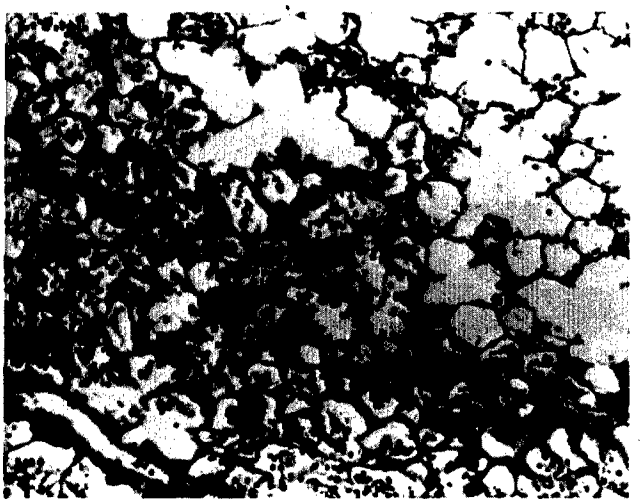

Figure 4. Line of demarcation between affected and normal tissue in an acid exposed guinea pig lung.

In both rats and guinea pigs, the ozone lesion was primarily confined to the terminal bronchioles and proximal alveoli. In the bronchioles, the microscopic alterations after two exposure days were characterized by bronchiolar epithelial hypertrophy and hyperplasia. Alveolitis characterized by pneumonocyte hyperplasia, alveolar wall thickening, and accumulation of alveolar macrophages was present in the alveoli adjacent to terminal bronchioles. Pulmonary edema was present to varying degrees in all of the animals. These lesions are shown in Figures 5 and 6. A moderate to marked loss of cilia in the bronchi of guinea pigs was also evident at 2 ppm ozone. A loss of cilia and a definite reduction in the number of goblet cells were exposure-related microscopic findings in the trachea of guinea pigs. At 2 ppm the exposure-related microscopic alterations were just as severe after seven exposure days, while there was a reduction in degree of severity of the lesion after seven days in animals exposed to 1 ppm.

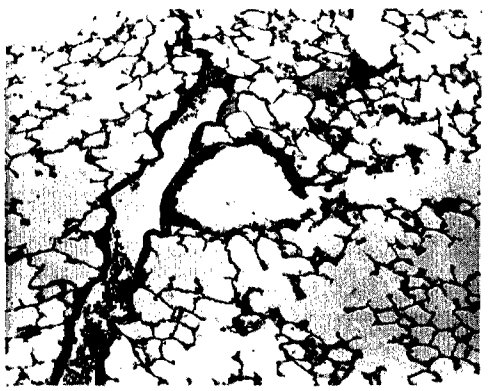

Figure 5. Alveolar duct in an ozone exposed guinea pig lung.

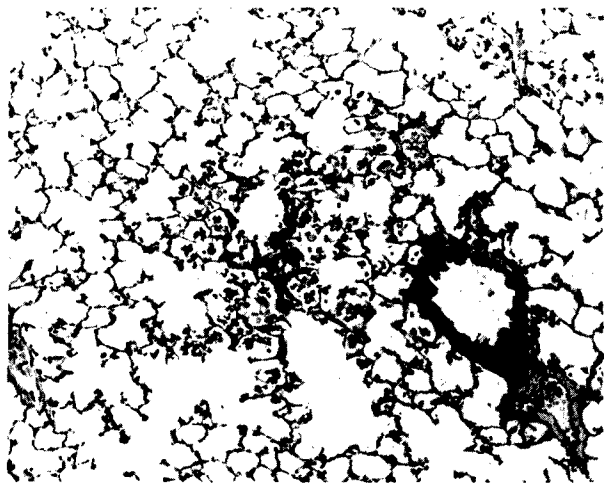

Figure 6. Cross section of a respiratory bronchiole in an ozone exposed guinea pig lung.

DISCUSSION

No synergistic effects between ozone and sulfuric acid mist were seen in either rats or guinea pigs in any of these subacute exposures. The lesions for each pollutant have been characterized. These lesions occur in separate and distinct regions of the deep lung. The sulfuric acid lesion in guinea pigs is extremely interesting because the lesion is a true alveolitis and because of regional location of the lesion within the lung. The significance of the effect and the line of demarcation between normal lung tissue and the affected area is not known. The irritant response in guinea pigs is well known. When bronchoconstriction occurs to such a degree that the guinea pigs show respiratory distress, perhaps the air flow patterns dictate the regional deposition pattern seen in these animals. Higher dose levels produce death before the microscopic lesion is manifested, while lower dose levels do not produce respiratory distress and no regional microscopic lesion is seen.

ACKNOWLEDGEMENTS

Thanks are kindly extended to Dr. R. Handy of the Research Triangle Institute for chemical analyses, Dr. J. L. Williams of Becton, Dickinson Research Center and Dr. D. Swift of Johns Hopkins University for their assistance in the aerosol physics involved in generating sulfuric acid mist,

Patricia Jeffreys of Becton, Dickinson Research Center for her assistance in the animal exposures, and Edna Beck of Becton, Dickinson Research Center for the preparation of this manuscript. These studies have been supported by Contract Number NIH-NIEHS-NOI-ES-4-2164 from the National Institute of Environmental Health Sciences.

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AMRL-TR-75-125

PAPER NO. 17

MAMMALIAN TOXICITY OF FLUOMINE DUST

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INTRODUCTION

Fluomine [cobalt-bis (3-fluorosalicylaldehyde)-ethylenediimine], when activated, is capable of selectively absorbing oxygen from air and upon heating will release pure molecular oxygen. There are several chelates which are capable of absorbing molecular oxygen including salcomine [bis(salicylaldehyde) ethylenediimine cobalt (II)], the parent compound of fluomine. However, fluomine far exceeds the capabilities of the others to absorb oxygen while in a solid form. This oxygen-scavenging property renders it useful as a possible component in life support systems for high altitude flights. The structure of fluomine is shown in Figure 1.

A search of the current literature failed to reveal any information concerning the mammalian toxicity of this compound. However, Coon et al. (1942) reported on the toxicity of salcomine powder inhalation on mice. They reported 1 of 6 mice dying following inhalation of 390 mg/m^3 dust for 5.5 hours and 4 of 6 mice dying after 6 hours at 1000 mg/m^3. Exposure to salcomine dust resulted in severe irritation to the tracheo-bronchial system and the lungs. The lungs were hyperemic and contained edema fluid in the peripheral portions of the lobules.

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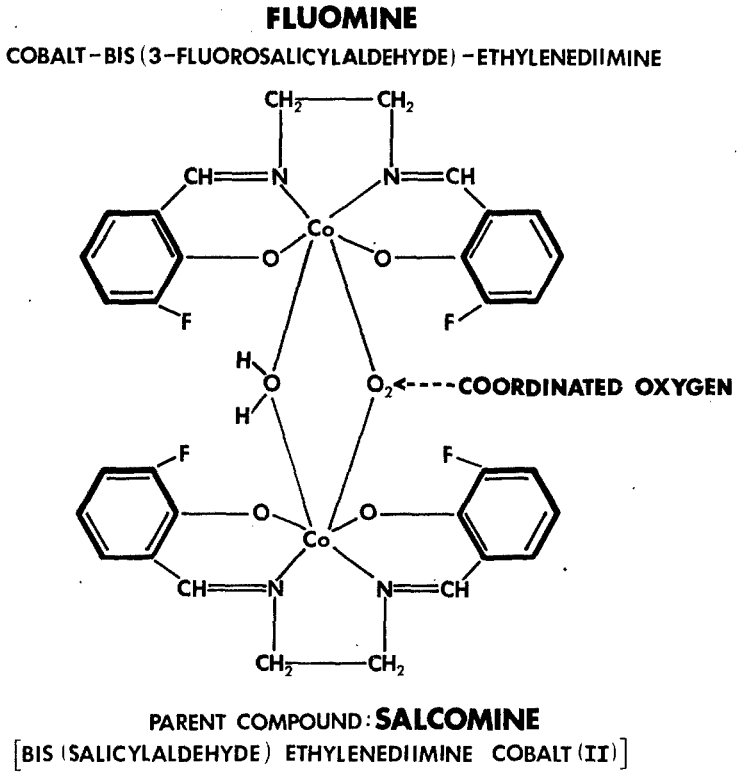

Figure 1.

METHODS AND MATERIALS

Eye Irritation

The compound was tested for eye irritation using New Zealand Albino rabbits and varying doses of fluomine suspended in normal physiological saline. Twenty-four hours prior to use, the rabbit eyes were examined for corneal lesions using a 1% solution of fluorescein dye.

Equal numbers of left and right eyes were tested using the following method. The lower lid was pulled away from the eye and 0.1 ml of the fluomine suspension was instilled into the lower conjunctival sac. The lid was held open for a few seconds then raised to close with the upper lid. The eyes were not washed following dosing.

The eyes were examined at 1, 24 and 72 hours and again at 7 days after application of the fluomine. Staining with fluorescein was done on any of the eyes that showed irritation after 24 and 72 hours and 7 days. Grading of eye irritation was done following the standard method of Draize (1944) which provides a maximum total numerical score derived from the sum of corneal, conjunctival and iris irritation.

Skin Irritation

A patch-test method was done to measure the degree of primary skin irritation of fluomine on the intact and abraded skin of New Zealand albino rabbits. All rabbits were clipped of hair on the backs and flanks 24 hours prior to exposure. The abrasions were minor incisions through the stratum corneum, not sufficiently deep to disturb the derma or to produce bleeding. These were made in a "tic, tac, toe" pattern with a syringe needle used to make the incisions.

One-half gram of fluomine was applied to each area, the intact skin and the abraded skin, and covered by a one-inch square of surgical gauze, two layers thick. The gauze patches were held in place with strips of elastoplast tape. The entire area was then covered with a rubber dental dam strip, secured with more elastoplast tape. The patches remained in place on the rabbits for 24 hours. During that time, the rabbits wore leather restraining collars to prevent disturbance of the patch area, while allowing the rabbits freedom of movement and access to food and water during the test period.

After 24 hours, the wrap and patches were removed and the test areas evaluated for irritation using the Draize table (Table 1) as a reference standard. Readings were also made at 72 hours (48 hours after the first reading). The values for erythema and eschar formation were added to the combined values for edema formation. The mean of these values is the primary irritation score.

TABLE 1. EVALUATION OF SKIN REACTIONS*

	Value[1]
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely preceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely preceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extended beyond exposed area)	4

[1]The "value" recorded for each reading is the average value of the six animals subject to the test.
*Draize et al. (1944).

Oral Toxicity

Fluomine was administered orally to rodents as a suspension in corn oil. The suspensions were kept in a turbulent state prior to dosing by stirring on a magnetic stirring platform. Glass syringes with 18 gauge intubation needles were used to administer the suspensions to the rodents. The animals were fasted for at least 16 hours prior to dosing to allow for more uniform absorption since the amount of food in the stomach varies greatly from animal to animal in the unfasted condition.

Five rats and five mice were dosed at each level and the LD_{50} with its 95% confidence limits was calculated using the moving average interpolation method of Weil (1952) and the method of Finney (1952) for the probit analysis. Deaths which occurred during the 14 days immediately following the administration of the single dose were included in the final mortality tally.

Inhalation

Groups of 10 rats and 10 mice were exposed to varying concentrations of airborne fluomine particles for one-hour and six-hour periods and observed for 14 days postexposure to determine mortality and/or signs of toxic stress. The concentrations were established using a Wright Dust Feeder® and adjusting gear ratios to vary the amount of suspended dust entering the 120 liter chamber. A constant flow of 20 liters of air per minute was maintained through the chamber. Concentrations were measured by gravimetric sampling using a 47 mm Metricel® filter (type GA-6) with a mean pore size of 0.45 μ.

A particle size analysis was done on the dust sampled from the 120 liter inhalation chamber using a plastic overlay on a microphotograph similar to the procedure of Vooren and Meyer (1971). The chamber air sample was drawn through a filter at 5 liters/minute (370 cm/minute), a rate calculated to be capable of collecting particles up to 35 microns in diameter.

After sampling, the filter was covered with emersion oil and placed under the microscope. Photographs taken of the filter and of a stage micrometer were enlarged to the same degree. The particles were then measured using a plastic overlay on which concentric circles had been drawn corresponding to particle diameters of 1, 2, 5, 10 and 20 microns. These measurements were calculated directly from the microphotograph of the stage micrometer. A pinhole was made in the center of the circles of the template, enabling the technician to pierce each particle image as counted.

A total of 455 particles were measured by the above method (Table 2). No attempt was made to classify the particles below one micron in diameter and they were included in the one micron group. Seventy-one percent of all particles were five microns or less in diameter and, therefore, of respirable size.

TABLE 2. PARTICLE SIZE DISTRIBUTION OF FLUOMINE DUST

Particle Size Range (Microns)	Number of Particles	Percentage of Particles	Cumulative Percentage of Particles
1	88	19.3	19.3
2	94	20.7	40.0
2-5	104	22.9	62.9
5	40	8.8	71.7
5-10	50	11.0	82.7
10	22	4.8	87.5
10-20	37	8.1	95.6
>20	20	4.4	100.0

A similar examination of particle size was done on fluomine which had been ground in a mortar and used for a high concentration level dust exposure and for the guinea pig inhalation-sensitization study. The results of this particle sizing are shown in Table 3. A total of 381 particles were measured with approximately 82% of these within the respirable range.

TABLE 3. PARTICLE SIZE DISTRIBUTION OF FLUOMINE DUST AFTER GRINDING IN A MORTAR

Particle Size Range (Microns)	Number of Particles	Percentage of Particles	Cumulative Percentage of Particles
1	132	34.6	34.6
2	93	24.4	59.0
2-5	71	18.6	77.6
5	16	4.2	81.8
5-10	37	9.7	91.5
10	13	3.4	94.9
10-20	15	3.9	98.8
>20	4	1.0	99.8

Sensitization (Intradermal)

A modified Landsteiner (1937) technique was used to determine whether fluomine would cause an antigen-antibody reaction in male albino guinea pigs. The experimental group consisted of 18 guinea pigs. Another group of three guinea pigs was used to determine the primary irritation properties of the compound.

The primary irritation test consisted of injecting 0.05 and 0.10 ml quantities of a 0.1% suspension of fluomine in peanut oil into the closely clipped scapular and sacral areas of the three guinea pigs. Similar injections of peanut oil alone were also made. The injection sites were examined at 24 and 48 hours. The I.D. injections of the 0.1% suspension caused inflammatory reactions resulting in this concentration being considered a primary irritant. A repeat of the process using a 0.01% suspension did not cause a response that could be considered as irritating, and thus this concentration was used for the intradermal sensitization regimen.

The sensitization test was started on a Monday with the guinea pigs being weighed and clipped on the scapular areas. 0.05 ml of the 0.01% suspension of fluomine was injected intradermally into the upper right scapular area of each animal. A similar injection of peanut oil was made concurrently into the upper left scapular area. The injections were examined at 24 and 48 hours and the numerical scores (determined as shown in Table 4) recorded.

TABLE 4. GRADING OF SKIN REACTIONS IN THE GUINEA PIG SENSITIZATION TEST

The product of the width and length of the wheal (in mm) is multiplied by the following reaction scores:

0 = needle puncture, no wheal
1 = very faint pink
2 = faint pink
3 = pink
4 = red
5 = bright red
6 = edema, <1 mm in height
7 = edema, >1 mm in height
*8 = necrosis, <1 square mm
*9 = necrosis, >1 square mm

*The product of width and length of the necrotic area multiplied by 8 or 9 is added to the numerical value of any of the foregoing reactions that are present.

Doses of 0.1 ml of the same dilutions (freshly prepared) were then injected into the clipped dorsal lumbo-sacral areas of guinea pigs the following Wednesday, Friday, Monday, etc., until a total of seven injections were administered. Care was taken to ensure that repeated doses were not injected into the same site.

The guinea pigs were rested for three weeks (incubation period), weighed, and given a challenge dose of 0.05 ml of the 0.01% suspension into the lower right scapular area. A control injection of peanut oil was made into the lower left scapular area. The reactions were again read after 24 and 48 hours.

The grading system is designed so that the intensity of the skin reaction is represented by a proportionate numerical value and also that any reaction elicited by the vehicle (peanut oil) is subtracted from the reaction elicited by the test substance and vehicle combined.

Animals

The animals used in these experiments were 25 to 30 gram CF-1 (ICR derived) mice, 250 to 400 gram CFE (Sprague-Dawley derived) rats, and 450-600 gram albino short-hair (Hartley strain) guinea pigs. All animals were obtained from Carworth Farms, Incorporated. Quality control studies on all species during the quarantine period showed the animals to be in good health.

EXPERIMENTAL RESULTS

To define the effects of fluomine dust, a range-finding toxicity study was performed on rodents. With the exception of one inhalation exposure and the inhalation sensitization study, the fluomine was used in the same form as received. The LD and LC 50% values were based on 14-day observation periods.

Oral Toxicity

To determine the oral LD_{50} in rats and mice, fluomine was administered as a suspension in corn oil. The oral LD_{50} of fluomine in the male rat is 187 mg/kg while in the male mouse it is 123 mg/kg as shown in Tables 5 and 6. Most deaths occurred during the first 24 hours postexposure with the latest deaths occurring at 7 days postexposure.

TABLE 5. ACUTE ORAL TOXICITY OF FLUOMINE IN MALE MICE

Dose, mg/kg[1]	Mortality Ratio[2]	Days to Death[3]
400	5/5	1, 1, 1, 1, 4
200	5/5	1, 1, 1, 4, 4
100	1/5	6

LD_{50} and Confidence Limits = 123(93-167) mg/kg

[1]Administered as a suspension in corn oil.
[2]Number died over number dosed.
[3]"1" indicates any death within 24 hours after dosing.

TABLE 6. ACUTE ORAL TOXICITY OF FLUOMINE IN MALE RATS

Dose, mg/kg[1]	Mortality Ratio[2]	Days to Death[3]
400	5/5	1, 1, 3, 5, 7
200	3/5	1, 5, 7
100	0/5	-
50	0/5	-

LD_{50} and Confidence Limits = 187(129-270) mg/kg

[1]Administered as a suspension in corn oil.
[2]Number died over number dosed.
[3]"1" indicates any death within 24 hours after dosing.

Gross examination of animals that died within 24 hours of dosing revealed distended blood filled stomachs in mice but not in rats. Microscopic examinations showed areas of necrosis of the lymphocytes within the germinal centers of the spleen. This was seen in both rats and mice examined. This lesion is not normally found in our rodents and is considered to be a result of the chemical insult.

Eye Irritation

Into one eye of each of six rabbits was instilled 0.1 ml of a 33% (w/w) suspension of ground fluomine in saline with the other eye serving as a control. Under these conditions, fluomine proved to be extremely irritating to the conjunctivae, causing marked chemosis and considerable discharge. Due to severe swelling of the conjunctivae and nictitating membranes, examination of corneal and iris tissue was impossible except in one case where definite corneal opacity was noted at 72 hours.

This was followed by the instillation of a 3% (w/w) suspension into the eyes of six rabbits. A comparison of the results of the two tests is shown in Table 7. As can be seen in the table, the 1-hour and 24-hour responses are comparable for the two mixtures tested. The values after 24 hours, however, indicate the higher concentration resulted in a degree of irritation of increased duration and severity. No residual fluomine particles were noted after 24 hours in those eyes treated with the 3% suspension. Undissolved fluomine was observable in eyes receiving the higher dose up to 7 days after treatment. The difference noted is probably due to the amount of retained particles capable of solubilizing in the ocular fluid and serving to provide a constant source of chemical irritation.

TABLE 7. IRRITATION FROM INSTILLATION OF FLUOMINE IN RABBIT EYES

Concentration of Suspension	Draize[1] Scores			
	1 Hour	24 Hours	72 Hours	7 Days
33%	4.3	12.7	23.3	52.5
3%	1.3	8.7	1.0	0

[1]Draize et al., 1944.

The characteristics of the irritation indicated that it was chemical in nature rather than mechanical abrasion, which implied that some solubilization of the fluomine was occurring to provide the irritant. In order to test this hypothesis, fluomine was milled with physiological saline for

1.5 hours and filtered to yield a 0.71 g/100 ml solution. This solution was tested for eye irritation potential by instillation of 0.1 ml into one eye of each of six rabbits. Examinations at 1 hour, 72 hours, and 7 days were negative, indicating that the solution, as tested, was nonirritating to the eyes.

The result cast some uncertainty on the original interpretation of fluomine eye irritancy residing in the soluble portion, but it is possible that the fluomine solution was quickly washed out of the eye while the solids were retained on the conjunctiva providing a continuous source of soluble material. However, mechanical abrasion cannot be ruled out as a contributing factor. Further testing would be necessary to define the relative contributions of both forms to eye irritation.

Skin Irritation

Rabbit skin irritation tests show fluomine to be a mild to moderate irritant, more potent in areas of abrasion. This is consistent with the findings of extreme irritation to the eye.

Inhalation

Six-hour and one-hour acute inhalation exposures were done on groups of 10 rats and 10 mice in a 120 liter plexiglas chamber. The measured exposure concentrations and the resulting mortality are shown in Table 8. The six-hour LC_{50} was 112 mg/m^3 for rats and 473 mg/m^3 for mice. The one-hour LC_{50} for rats was 712 mg/m^3 while it was not possible to generate high enough concentrations of fluomine to produce deaths in mice at this time period.

Gross examination of the rats that died following exposure revealed fluomine particles in the trachea and in the lungs. Microscopic examination of the animals revealed that the entire mucosa of the nasal cavity showed erosion and sloughing of the epithelium with a severe, purulent inflammatory response. The trachea also showed an intense inflammation with necrosis of the epithelium which extended downward into the subadjacent glandular tissue which was also necrotic.

TABLE 8. RESPONSE OF RATS AND MICE TO SIX AND ONE-HOUR INHALATION EXPOSURES TO FLUOMINE DUST

Rats		Mice	
Conc., mg/m^3[a]	Mortality Ratio[b]	Conc., mg/m^3[a]	Mortality Ratio[b]
	Six Hours		
407	10/10	464	6/10
185	10/10	195	1/10
104	3/10	94	1/10
49	0/10		
LC_{50} & 95% C.L. = 112(81-163)mg/m^3		LC_{50} & 95% C.L. = 416(222-780)mg/m^3	
	One Hour		
890[c]	9/10	890[c]	0/10
750	7/10	750	0/10
507	0/10	507	0/10
LC_{50} & 95% C.L. = 712(639-792)mg/m^3			

(a) Concentration measured gravimetrically.
(b) Number died over number dosed.
(c) This exposure was done with milled fluomine dust.

The lungs showed signs of perivascular edema, intraalveolar edema, and congestion with diffuse focal areas of lymphoid hyperplasia. Larger air spaces were often filled with a proteinaceous material. The liver showed periacinal congestion with dilation of the sinusoids.

Sensitization

The guinea pig intradermal sensitization study was done using a 0.01% suspension, one-tenth the standard concentration, of fluomine in peanut oil. Sensitization responses were obtained in 16 of 18 animals with a high mean reaction score indicating that fluomine is a potent sensitizer for guinea pigs by the intradermal route.

Since the normal route of exposure to this material is by inhalation, guinea pigs were exposed to a series of inhalation exposures to see if a sensitization response would be elicited. This was performed following the same regimen as the previous sensitization study, with inhalation exposures substituted for the intradermal injections. Milled fluomine powder was used in this series of exposures.

Table 9 lists the number of animals exposed and the concentrations generated during this study. The first 2-hour exposure of guinea pigs to 30 mg/m^3 fluomine dust resulted in the deaths of six animals. New animals replaced these prior to the next exposure of 12 mg/m^3 for two hours. Following this, eight more of the original group died. The exposure time was then reduced to one hour for the remainder of the exposures without further loss of animals. To determine whether sensitization response could be elicited after only a few exposures, an additional group of three guinea pigs was added for the last three inhalation exposures.

TABLE 9. INHALATION EXPOSURE REGIMEN FOR GUINEA PIG SENSITIZATION STUDY

Date	Exposure Time, Hrs.	Fluomine Conc., mg/m^3	No. of Guinea Pigs in Group 1	2	3
11-7	2	30	20		
11-9	2	12	14	6	
11-12	1	12	6	6	
11-14	1	12	6	6	
11-16	1	12	6	6	3
11-19	1	12	6	6	3
11-21	1	12	6	6	3
12-12	1	15	6		

To the three groups listed in Table 9 were added groups 4 and 5, consisting of six guinea pigs each, remaining from the original intradermal sensitization study. These animals did not receive any of the dust inhalation exposures. Three weeks after the last inhalation sensitization exposure and 2 months after intradermal challenge for groups 4 and 5, the following treatments were given:

Groups 1, 4 and controls: a 1-hour exposure to 15 mg/m^3 fluomine dust.

Groups 2, 3, 5 and controls: an intradermal injection of 0.05 ml of a 0.01% suspension of fluomine in peanut oil.

All animals in the inhalation challenge group exhibited eye and nose irritation. The severity of symptoms was equal in all groups, and none of the guinea pigs showed any signs of anaphylactic response after 3-4 hours. Three of the six guinea pigs in group 2 showed a sensitization response to intradermal injections of fluomine. These three exhibited a mean reaction score of 72. By 48 hours, the response had subdued to a mean reaction score of 41. No response to intradermal challenge was seen on the part of group 3 or controls. Contrastingly, in group 5, sensitization responses were found in four of the six guinea pigs at 24 hours and five of the six at 48 hours. The mean reaction scores at these time periods were 600 and 405, respectively. The conclusions of the study may be summarized in the following way:

I. Guinea pigs exposed 6 or 7 times to fluomine dust demonstrate a mild to moderate sensitization response to an intradermal challenge. However, three inhalation exposures do not elicit this response.

II. Guinea pigs do not show a sensitization or anaphylactic type response when challenged by an inhalation exposure of fluomine dust.

III. Intradermal challenge presented two months after termination of a fluomine intradermal sensitization study still elicits a strong reaction.

DISCUSSION

Single peroral doses of fluomine are toxic to both rats and mice at very low levels. The mouse appears to be more susceptible to the irritating properties of the compound, evidenced by the lower LD_{50} and the incidence of bloated, blood filled stomachs found in the mice but not seen in any of the rats.

Fluomine particles are highly irritating to the eyes of rabbits. The compound causes severe irritation to the conjunctivae resulting in chemosis and marked swelling. If the particles are not removed from the eye, they provide a source of constant irritation to the eye and surrounding membranes. A similar irritation is produced when the compound is in contact with broken or abraded skin. Little or no effect was noted on unbroken skin.

Single inhalation LC_{50} results indicate that fluomine is highly toxic by this route. Rats are more sensitive than mice but not nearly as sensitive as guinea pigs which died after a single two-hour inhalation exposure to 30 mg/m^3. The toxicity appears to be directly proportionate to the increase in animal size tested. This may be directly related to the different respiration rates, volumes, and the differences in the size of the pulmonary passageways of the three species.

Fluomine dust has a highly irritating effect on the entire respiratory systems of all species tested. Also, a systemic action is evidenced by the effect on livers and the necrosis of the lymphoid elements within the germinal centers of the spleen.

The dust of this compound is a potent sensitizer by the intradermal route to guinea pigs. Although an anaphylactic-type response was not elicited by the guinea pigs given an inhalation challenge, an intradermal injection showed that it is possible for the animals to produce antibodies if exposed to repeated inhalations of low concentrations of fluomine.

If the assumption that the larger nares and passageways allow for more and larger particles to reach the lower respiratory tract, then man could be more severely affected than any of the species tested. Presumptively, until metabolism is studied, man must be considered to be as vulnerable as the most sensitive guinea pig. Any prolonged inhalation, oral, or skin contact of this compound should be avoided. Eye protection should be provided anyone working in an atmosphere containing fluomine dust.

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OPEN FORUM

DR. MAC EWEN (University of California, Irvine): I'd like to ask a question of Capt. Harris. When you compared your results of tests made on unanesthetized animals with those previously conducted on anesthetized preparations, were there any significant differences between the two sets of results?

CAPT. HARRIS (6570 Aerospace Medical Research Laboratory): The differences were quite significant. In one of my slides, I showed a summary of the results obtained on anesthetized animals where we found significant differences in glucose extraction and myocardial contractility from control values whereas with the conscious animals, we didn't get any significant differences from normal at all.

DR. BACK (6570 Aerospace Medical Research Laboratory): I have to qualify Capt. Harris' statement because there's really no comparison between the two sets of data. The reason is that the doses used in the tests weren't anywhere close to being the same. Don't go away with the impression that the data obtained on anesthetized animals can be compared, one to one, on the unanesthetized because we used massive doses in the anesthetized allowed by the fact that the animal was anesthetized. If you expose an unanesthetized animal to a 20% concentration of $CBrF_3$, it will convulse. So, we didn't use doses anywhere comparable to 20% $CBrF_3$ in unanesthetized animals. The same is true of Halon 1211. You would not dare expose animals to those kinds of doses. The two kinds of test procedures complement each other. You use the open chest animal to make pharmacodynamic measurements at very high dose levels and they won't convulse because they are already anesthetized. If you want to find out whether your predicted safe levels are real, predicted from observed effect in an anesthetized animal, then you expose unanesthetized animals at the very low doses that Van Stee contemplated as being safe. That's what these animals were exposed to and we didn't get any change from normal. If you use unanesthetized animals at higher dose levels, some of them would convulse, in which case you couldn't use the data. So we can't compare the two experiments. We can't say that any changes were there because we didn't use a high enough dose level, maybe, to see one.

DR. CROCKER (University of California, Irvine): I have a question for Dr. Cavender which relates to the lesions that he demonstrated. To demonstrate one of those lesions you had, unfortunately, to show a picture of Dr. Dungworth's that was from a lesion resulting from exposure of a rat for 7 days to ozone. Were your ozone exposures 2 or 7 days?

DR. CAVENDER (Becton, Dickinson & Company): They were for 7 days, also. Two days and 7 days.

DR. CROCKER: Were you able to see such lesions as early as 2 days or did it require 7 days of exposure to see the lesion? Also, were those the specific ozone type lesions of the small airways or actually the respiratory ducts in the alveolar areas? My second question relates to the morphology of the lesion that you show in the guinea pig exposed to sulfuric acid mist. It had a sharp line of demarcation which did not look like an area of obstructed circulation, an infarcted area, but as though it might be anatomically related to a lobular area of the lung that might have been atelectatic. It looked as though there may have been obstruction of the airway to particular segment of the lung. Is it possible that an obstructive lesion was present that distributed the sulfuric acid in limited portions of the lung in which you couldn't recognize the bronchial obstruction as present?

DR. CAVENDER: First, the ozone question. The lesion produced by ozone was more pronounced at 48 hours than it was, actually, at 7 days. As reported by many workers, the edematous lesion occurs maximally at approximately 48 hours after initial exposure to 2 ppm ozone and adaptation will begin beyond that point, and there was very little regression of the lesion whereas at 1 ppm or less, there was some regression at 7 days. This is also similar to what Mr. Vernot reported this morning. In the case of the line of demarcation, these lungs were examined very carefully and there was no indication of any type of obstruction anywhere in any of the lobes, and the line of demarcation occurred in all lobes. This lesion has also been shown by Walt Tyler and his group out at Davis. In both cases, there is no apparent obstruction so that you get a lesion in part of the lobe and not in the other. It's my opinion that as you get further and further down the airways, they're not always straight or smooth bifurcations so that one stream divides equally into two streams, but somewhere

along the line, there's an unequal distribution and when that bronchus constricts, the airway that is most directly lined up is the one that gets the greatest air flow and perhaps the lesion occurs in that airway as a result of the uneven exposure.

DR. CROCKER: Could I ask a question about the biochemical lesion? Along with these morphologic observations, you did some enzyme analysis, I believe. What correlation between these, in terms of concentration and time dependence, was there?

DR. CAVENDER: We really didn't do a time dependent study on it. We didn't do all of the biochemistry at both time periods. We measured primarily G6PD and superoxide dismutase in these lungs. In all cases, the G6PD was elevated at 48 hours and remained elevated at 7 days. We've only made SOD measurements after 7 days exposure and the values were elevated in both cases. There was no difference in the elevation between ozone only or ozone and the combination group, and there was no statistical difference between those two exposure groups. But one of the interesting things that we did was to lavage some of these lungs and remove macrophages. The G6PD increase can be associated with the infiltration of the macrophages into the lung because when you lavage them out, the levels of G6PD on the lavaged lung tissue are normal for those lungs and comparable to controls. The SOD levels are not affected by lavaging the lungs. Perhaps the SOD is within the type 2 pneumonocytes as they occur in the lung.

DR. LEWIS (National Institute for Occupational Safety and Health): I'd like to direct my question to Mr. Vernot. When you discussed the interactions of ozone and NO_2, you stated and correctly so that there were reaction products. You postulated that among them was nitrogen tetroxide. Did you analyze for that? Now I don't recall whether Dr. Stokinger analyzed for it or not, do you? And did you look for increased nitrate concentrations? I recall that Dr. Campbell reported finding nitrate particulates in the chambers in which dogs were exposed to 25 to 26 ppm of NO_2 inferring that molecular oxygen can oxidize NO_2 to nitrate particulate.

MR. VERNOT (University of California, Irvine): I well remember Dr. Campbell's paper, and we saw evidence of that in our own chambers when we were performing long-term exposures to NO_2; the deposition of

what appeared to be a product of nitrogen dioxide. No, we didn't analyze for nitrogen pentoxide in the present study. What we did do was to take well recognized rate constants and calculate what the rate formation of the nitrogen pentoxide would be at the levels of ozone and nitrogen dioxide that we were working with. And we calculated that at around 2 ppm ozone and 20 ppm NO_2. A half life in our chambers was approximately a minute and a half. Now, we also inferred that the nitrogen pentoxide did not have a significant toxic effect on the lungs because when we were working at higher concentrations of ozone and nitrogen dioxide, we must have had concentrations of nitrogen pentoxide which were at least as high as that of the nitrogen dioxide. And we would have expected, if it had some toxic effect, to have seen added toxic effect in the mixture itself, and we didn't see it. So it's more inference than anything else.

DR. PIER (University of Texas): A question for Mr. Wohlslagel. In your work with the alumina exposures, did you characterize the alumina further in terms of particle size other than the maximum that you gave, and in terms of the forms, particularly as to whether it was alpha or gamma?

MR. WOHLSLAGEL (University of California, Irvine): No, the scope of our work didn't include particle size characterization although some information on the particle size of the dust used was obtained. The supplier informed us that the maximum size was about 1.4 microns and approximately 90% of the particles were under 0.7 micron. The alumina concentrations we were using, 500 mg/m^3 or more, in these exposures were far above the upper limits for any particle sizing instrument and would have required extensive dilution or extremely short time sampling for any of the other particle sizing techniques. As far as the form that the alumina is in, the information that we had from the Air Force stated that it was the gamma form that was produced during the firing of the missiles. Now, the Greek letter gamma formerly was used to designate a crystalline type that was formed during the burning of alumina in air. There are two types of nomenclature. This is the old nomenclature for that gamma form. The new nomenclature, brought forth by Alcoa, lumps the gamma form into transition forms of alumina that are formed during the thermal decomposition of hydrated forms of alumina. Now, there are three stages that they go through. You get the gamma, delta and then an alpha stage which is the anhydrous stable form of alumina. Now, in our studies, I believe that we had the alpha form which is the stable form and during the firing of the rockets, I believe that

most of the alumina form would be of the alpha form but much of the transition forms would be manufactured also during this process. So it would be a conglomeration of all different forms of alumina formed during the firing. I believe that we used mainly alpha.

DR. S. LEE (Environmental Protection Agency): Could you tell us something about the chemical profile or composition of the rocket motor fuel?

MR. WOHLSLAGEL: That information wasn't given to us, but I do know that there was elemental aluminum in the rocket fuel.

DR. LEE: What percent does hydrochloric acid occupy of the whole exhaust?

MR. WOHLSLAGEL: I believe it was something of the order of 16%. I don't have the figures with me. I can get them for you later.

DR. MAC EWEN: There is approximately 35% HCl gas by volume in the exhaust. You might wonder why Mr. Wohlslagel used such high concentrations of alumina dust in the exposures. That was to achieve approximately the same ratios of alumina and hydrogen fluoride and hydrogen chloride in the mixture. Would someone in the audience like to discuss the composition of the rocket exhaust?

CAPT. SUTAY (Air Force Rocket Propulsion Lab.): Most solid propellants contain 20% aluminum particles and the remaining 80% is compound HTPB and ammonium.

DR. LEE: Where does the hydrogen fluoride in the rocket exhaust come from?

CAPT. SUTAY: There won't be HF unless the propellant contains new high energy NF binders that we're doing work on now. The new NF high energy binders emit about 8 to 10% HF in the exhaust mixture.

MR. WANDS (National Academy of Sciences): What is the particle size of the alumina as it comes out of the rocket exhaust? I would expect that this would be a very fine particle, a fume, rather than anything as coarse as a bag of aluminum.

CAPT. SUTAY: There hasn't been much work done on characterization of the particle size distributions of the alumina particles. I agree that most of them were less than a micron. Melvin's report from the Naval Weapons Center at China Lake indicated a bimodal distribution; one around 20 microns and the other around 1 micron or less.

DR. MAC EWEN: Dr. Lerman has been doing some work with this same material at the University of California, Riverside, and he's going to present this data tomorrow but perhaps he would like to comment on his particle size distribution. He used the same material and we split the sample.

DR. LERMAN (University of California, Riverside): As far as particle size distribution is concerned, according to General Propulsion Lab in Pasadena, the normal range of the upper limit is 0.7 micron. The alumina which I obtained from Mr. Vernot was reported by the supplier to be gamma form with an upper size limit of 1.4 microns. In our study, we used a cyclone and a one stage impactor to remove particles above 0.7 micron. Tomorrow I will present the particle size distribution obtained using a Cascade impactor and a Bausch and Lomb particle counter. The crystalline form of the alumina used was the gamma. I analyzed this using x-ray diffraction. According to JPL, the rocket exhaust contains a mixture of alpha, beta and gamma forms of alumina since the exhaust temperature varies tremendously. It is very important to know what crystalline form is used since the gamma is the most hygroscopic. If there is any reaction between the hydrogen chloride and alumina, it is most likely to take place with this particular form. Hydrogen chloride produces some surface reaction with the gamma form down to a depth of about 50 angstroms. We did find some synergistic effect of the two compounds in plants and I think this is one of the factors involved.

DR. PETERING (University of Cincinnati): I'd like to ask Mr. Kinkead a couple of questions about fluomine. Did you find any cardiac lesions from fluomine at all? The other thing that intrigues me is do you know anything about the dissociation of this compound, possibly delivering cobalt in vivo?

MR. KINKEAD (University of California, Irvine): No, we didn't find any cardiac lesions. As far as the second question, we didn't examine the fate of the inhaled fluomine in the animal.

MR. WANDS: I have a question for Mr. Haun. Do I understand that you got decreased lung weights in the exposed animals?

MR. HAUN (University of California, Irvine): The RJ-4 exposed rats were smaller than the controls and they had smaller lungs.

MR. WANDS: Was the lung to body weight ratio also decreased after exposure? Is this an artifact or a function of the exposure? I think it's a little unusual to find decreased lung weights after exposure to a pulmonary irritant.

MR. HAUN: You have a good point. Apparently the pulmonary irritation I reported was not sufficient to cause edema and other changes which would cause increased lung weights compared with control weights. I shouldn't have referred to it as decreased lung weight since the animal was smaller also.

DR. CAVENDER: It is interesting to look at some of these special situations we've set up that we call a toxicological experiment. But in looking at rat lungs, for instance in a sulfuric acid study, these lungs were minisculely smaller than the control animals. This is, perhaps, due to the fact that they were cleaner. The acid in the environment tended to, perhaps, reduce the bacterial population they might be exposed to. So they were a little bit cleaner looking overall than the controls were and this can lead to some of the things you might have been thinking about.

DR. CAMPBELL (Environmental Protection Agency): This question is for Dr. Cavender. I think I misunderstood something you said about your experiment. I assume that you ran a multidose series of experiments for both the ozone and the sulfuric acid. Is that correct? Can you elaborate on what the lowest dose and earliest time was at which you saw detectable lesions in the rat or the guinea pig with sulfuric acid mist? Can you also eliminate the possibility that there was any ammonia in your chamber that might have affected the measurement of your chamber acid level?

DR. CAVENDER: There was no effect seen at any time in the rats at any level of sulfuric acid up to 100 mg/m^3. The toxicity data show that the LC_{50} is indeed about 800 mg/m^3. In the case of the guinea pigs, we saw minimal effects at 10 mg/m^3 in a 7-day exposure experiment. Those effects

were not present after 48 hours but they were definitely present after 7 days. The answer to the ammonia question is not quite as easy since we did not monitor ammonia continuously. We did make some measurements of ammonia levels and we got some fluctuation which followed the changing of the animal bedding. If you change bedding every other day, the ammonia level is a little higher on the second day, so this perhaps does affect the acidity. It's very difficult to say precisely how much effect it had but there was some ammonia there. We did not measure it all the time.

DR. CAMPBELL: Based on evidence from our laboratory, in looking at the ammonia situation, a very low animal loading and a very frequent chamber cleaning operation is necessary if you are exposing animals to such things as sulfuric acid and relying on the acidity measurement for chamber concentration. I have one further question since I assume that your first answer to me last time related to histological effects. Does that answer also include any effects with respect to macrophage recoveries in the guinea pigs or rats?

DR. CAVENDER: The increase in macrophages did not occur in sulfuric acid exposed animals. In fact if anything, the number of macrophages was reduced in those animals. This relates to what I said to Mr. Wands a few minutes ago. These animals looked extremely clean and extremely healthy. The deflated lungs looked smaller than those of controls and the actual weight was somewhat smaller than the controls. Low levels of sulfuric acid did not change macrophage counts very much. When you expose guinea pigs at a concentration where you start seeing the lesion then the infiltration of macrophages increases very rapidly.

DR. PASI (University of Zurich): I'm trying to understand your findings obtained at 7 days in the ozone treated group, or ozone and sulfuric acid treated group. My question pertains to the increase in SOD which you mentioned is probably located in Type 2 cells. Is this SOD increase a manifestation of the increasing number of Type 2 cells or is it an effect of ozone itself, let's say by superoxide radical formation, or are both phenomena involved in this SOD increase?

DR. CAVENDER: I cannot answer your question unequivocally. It looks as though the SOD levels in the animals that we've seen effects in

follows not from the ozone itself but from the macrophage infiltration. One way that macrophages kill bacteria is by bombarding them with superoxide, and when you get an increase of macrophages in the lung, making the superoxide Type 2 cells apparently produce the more SOD to neutralize it. That's not the only indication that we have more Type 2 cells in there. You can see that microscopically and with the scanning electron microscope as well.

MR. WANDS: I have a question for Mr. Kinkead. You made quite a point of the extreme insolubility of fluomine and the indications that you've had, particularly in the eye studies, that there was some kind of soluble material coming out that might be creating the continuing chemical damage. I wonder if you have any data on the purity of the fluomine, particularly I'm wondering if there might not have been some free ethylene diamine there which would be coming off as adsorbed off onto a solid surface and might be the source of much of the irritant action because that's fairly strong base and could very well do the kinds of things you were seeing through it in all of the tissues that you worked with.

MR. KINKEAD: We didn't look at the compound to see what was coming off. Dr. Kleineberg reported a pyrolysis study on this material yesterday and showed various breakdown or gas-off products. I don't remember exactly what he reported finding, but we do believe that the material is slightly soluble in body fluids because we were able to sensitize the guinea pigs with it.

MR. WANDS: Of course, one can get sensitization to nickel metal also. That's solubilized by the body fluids. They are very good solvents, or complexing agents of one kind or another.

MR. VERNOT: Mr. Wands, we can certainly resolve that question by measuring the pH of the saturated solution over fluomines and see how basic it is, and we will do it.

COL. LEE (National Aeronautics and Space Administration): Capt. Harris, did you notice any impact on the EKG's of the dogs before and after your operation?

CAPT. HARRIS (6570 Aerospace Medical Research Laboratory): No, we monitored the EKG while the operation was being performed and there were no observable changes during or after. Once in a while, we observed

a PVC but no more often than you would expect in a normal animal. Sometimes we had this from dogs before the implant and sometimes right after the surgery from the penetration of the ventricular wall with a scalpel blade in the implantation of the catheter but there is nothing consistent about it that I would say that it's something to be concerned about. Usually, all the tracings during exposure as well as during the surgery showed very normal EKG's.

COL. LEE: Did you notice any changes with time after this preparation?

CAPT. HARRIS: Time, again, is going to have to be a relative thing. Most of the animals we used were not retained very long. We had one dog that was held for 4 months, but most of them are only kept for about a month. During this period of time, at least, there are no major changes.

COL. LEE: Have you had occasion to investigate the effects of chloroform or trichloroethylene using this type of animal preparation?

CAPT. HARRIS: No, we haven't studied any compounds other than those I indicated this morning. But there are plans. When the studies on these compounds are finished, we will probably be using other aliphatic halides.

COL. LEE: Would it appear that this preparation would be applicable to study the toxicity of high density jet fuels?

CAPT. HARRIS: A lot depends with this particular type of preparation on the ability of a conscious animal, unrestrained and unsedated, to tolerate an exposure to something like that. As long as the level of the exposure compound is tolerable, there is no reason why not.

DR. WIESTER (Environmental Protection Agency): I would like to direct this question to Capt. Harris. I'm curious to know, when you prepare a dog or possibly a baboon in this way, how many times do you use the animal and do you use him for just one acute insult or do you use him repeatedly or do you use him for a graded dose? How do you justify this very involved and expensive preparation?

CAPT. HARRIS: The preparation, outside of manhours involved, is not as expensive as you might think. Most of the probes that are used in this are reusable and the animals can be used for more than one experiment. We have used a dog for as long as 4 months with an exposure every 10 days.

DR. WIESTER: Did these animals recover after each insult?

CAPT HARRIS: Almost immediately. The fluorocarbons disappeared from the blood virtually within 10 minutes of the termination of the experiment, except for the CH_2BrCl which persists for several hours because it is more lipid soluble.

MR. VERNOT: I have a question for Dr. Cavender. I wonder whether you might explain to me your reason for choosing 2 molar sulfuric acid for aerosolization in the chambers? Did you do any preliminary work which indicated that there might be some equilibrium between relative humidity and SO_3 or concentrated sulfuric acid to give this kind of concentration?

DR. CAVENDER: The selection of 2 molar acid was an arbitrary decision at the starting point. We did quite a bit of work as we got further into the study with generation from various acid concentrations. One of the most surprising things we found was that if we selected 1 micron particle as what we wanted in the chamber, and generated from either concentrated or very dilute sulfuric acid, the molarity of the acid mist particle was governed solely by the relative humidity. It really didn't make any difference what molarity of acid we used in particle generation. Our chambers were controlled at 60% RH with a fixed animal loading and at this specific RH the particles are 5 molar. In the chronic study, we generated the particles from 5 molar acid to minimize fluctuations that might occur.

SESSION IV

PROBLEM ISSUES IN ENVIRONMENTAL CARCINOGENESIS AND MUTAGENESIS

Chairman

Ralph C. Wands, M.S.
Director, Advisory Center on Toxicology
National Research Council
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Washington, D.C.

AMRL-TR-75-125

PAPER NO. 18

A NEW TECHNIQUE: SERIAL BONE MARROW BIOPSIES FOR MONITORING CHEMICALLY INDUCED CHROMOSOMAL CHANGES

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and
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INTRODUCTION

In the assessment of chromosomal mutagenesis by drugs and environmental chemicals it is important to ascertain not only whether damage has occurred but also whether the induced damage is of a type which can lead to a viable (persistent) aberration or whether the aberration induced will lead to cell lethality. When the assessment of chromosomal aberrations is performed in rat bone marrow cells, such cells are usually obtained from sacrificed animals. Thus with currently available techniques it is not easily possible to establish a baseline of spontaneous chromosomal aberrations and to follow, in the living animal, the chromosomal situation after chemical treatment. To overcome such difficulty we have, therefore, developed a new bioptical technique of obtaining rat bone marrow samples and have demonstrated that such samples are suitable for cytogenetic analysis.

ADVANTAGES OF THE NEW TECHNIQUE

The advantages of the new technique are the following: (a) the operator performing the biopsy does not require the assistance of a second person; (b) neither aseptical precautions nor surgical procedures are required; (c) the

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procedure is rapid so that for each animal only one-two minutes are required to obtain one bone marrow sample; (d) the procedure does not affect the health of the experimental animal so that each animal can be resubjected to the same procedure.

DESCRIPTION OF THE PROCEDURE

Experimental animals are anesthetized by inhalatory exposure to methoxyflurane and then immobilized in a supine position. . Immobilization is achieved and maintained by simply wrapping the animal in a towel so that the lower emerging extremities are available for biopsy (Figure 1). The bone marrow sample is gained from the distal cavity of one femur. To reach this cavity with the biopsy needle, the needle is inserted not through the metaphysis as has been recommended in other techniques (Narayana Rao, et al., 1967) but, to avoid femur fractures (Petersen, 1970), through the distal intercondyloid zone. The site of entry to such zone is, as shown in Figure 2, conveniently defined as that area of the proximal part of the patellar tendon located just above the upper border of the patella. As shown in Figure 3, biopsy is performed transcutaneously with the help of a spinal needle (20 x 1"). To insert the needle into the distal femoral cavity, the femur is held in an approximate vertical position between thumb and middle finger of one hand, while the index finger of the same hand is used to palpate the above described site of needle insertion. The other hand is then free to manipulate the needle, which is introduced, in the direction of the femur axis, through the unclean, unclipped and undisinfected fur. Following needle insertion, as shown in Figure 4, the bone marrow sample is collected through gentle and steady aspiration into a sterile 5 ml plastic syringe, the inside of which has been previously wetted with 0.1 ml of a 1:1000 heparin solution. It is usually not difficult to obtain at least 0.1 ml of bone marrow material, a volume that, under normal conditions, is sufficient for the preparation of slides containing 20-50 metaphases.

After biopsy, bone marrow samples are incubated and slides are prepared according to established methods (Cohen et al., 1971). The only noteworthy modification is in the timing of the administration of the antimitotic agent (demecolcine, colcemide). Since the experimental animals had to be available for further biopsies, this toxic antimitotic agent was not administered to the animals prior to bone marrow biopsy, but was added directly to the incubated bone marrow cell suspension.

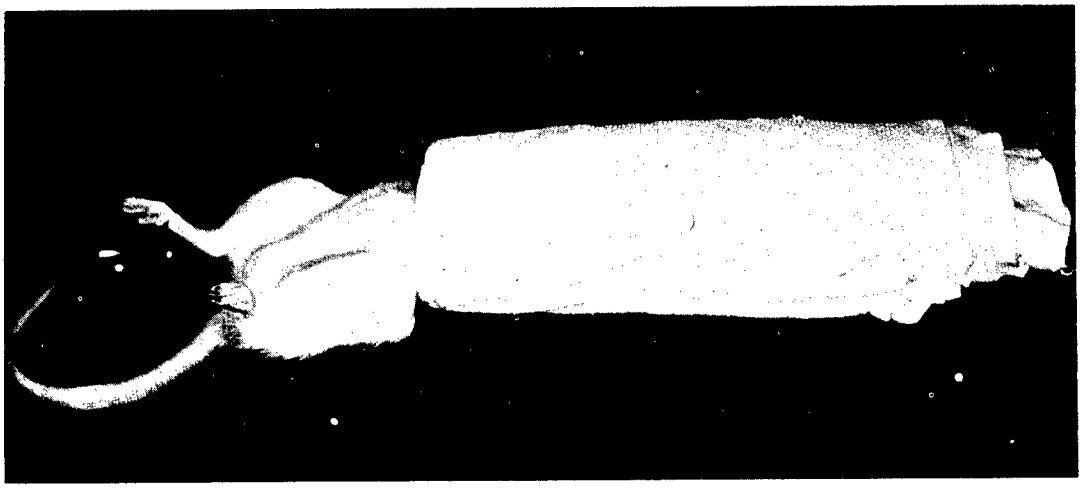

Figure 1. Immobilization of a rat for femoral bone marrow biopsy.

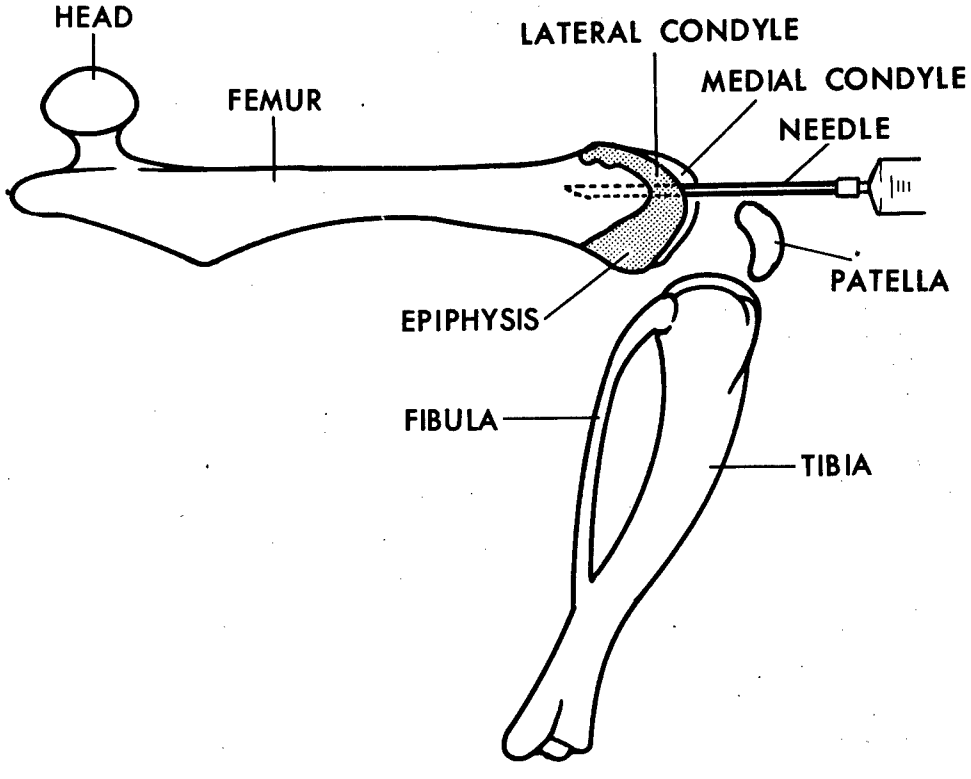

Figure 2. Site of femoral entry for transcutaneous bone marrow biopsy in the rat.

Figure 3. Needle manipulation for femoral bone marrow biopsy in the rat.

Figure 4. Rat bone marrow collection into a sryinge.

USE OF SERIAL BONE MARROW BIOPSIES FOR CYTOGENETIC ANALYSIS

To demonstrate that bone marrow samples obtained serially by means of the described bioptical technique are suitable for cytogenetic analysis, a mutagenetic study was performed, in which one randomized group of 10 Osborne-Mendel rats was used as control and treated with a single ip. administration of distilled H_2O (1 ml/kg B.W.). An analogous group of rats was given a single ip. injection of 0.3 mg/kg B.W. anhydrous triethylenemelamine (TEM). In both experimental groups the bioptical time schedule being identical, the first of a series of four biopsies was obtained 10 days before treatment. These pretreatment bioptical materials were used to determine the baseline frequency of spontaneous chromosomal aberrations. Additional biopsies were performed at 1, 7 and 30 days after treatment. Therefore, each animal was subjected to a total of four biopsies during a 40-day period, two biopsies being obtained from each femur in an alternate sequence. For each sample 20 metaphases were prepared, so that the total number of prepared metaphases amounted to 1600. Each of these metaphases was microlocated and microphotographed and each photomicrograph was enlarged, printed and scored for numerical and structural aberrations.

RESULTS OF THE MUTAGENIC STUDY

Results obtained from observations on chromosomal aberrations observed in control and treated animals are summarized in Table 2. For purposes of statistical evaluation all varieties of observed chromosomal discontinuities were grouped in one single category and defined as "breaks" (Cohen et al., 1971). Similarly all chromosomal aberrations representing the result of a healing attempt were grouped in another category defined as "rearrangements" (Cohen et al., 1971). An example of extensive cytogenetic damage observed after TEM-treatment is shown in Figure 5 and, for comparison, a normal rat bone marrow metaphase is shown in Figure 6.

Control frequencies of breaks and rearrangements observed at 1, 7 and 30 days after treatment with distilled water were not significantly different ($p \geq .01$) than baseline frequencies obtained in the same animals or in animals that were then subjected to treatment with TEM. One day after treatment with TEM, however, the frequency of both breaks and rearrangements was significantly higher than the correspondent baseline values of 3.0 and 0%, respectively. Thus, in spite of such a dramatic effect of TEM, frequencies of breaks and rearrangements returned, 7 and 30 days after treatment, to baseline values.

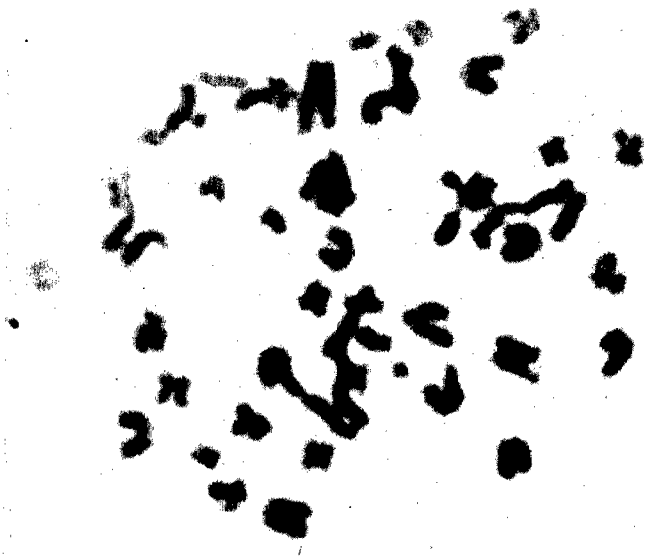

Figure 5. Rat bone marrow metaphase with chromosomal breaks and rearrangements one day after TEM-treatment.

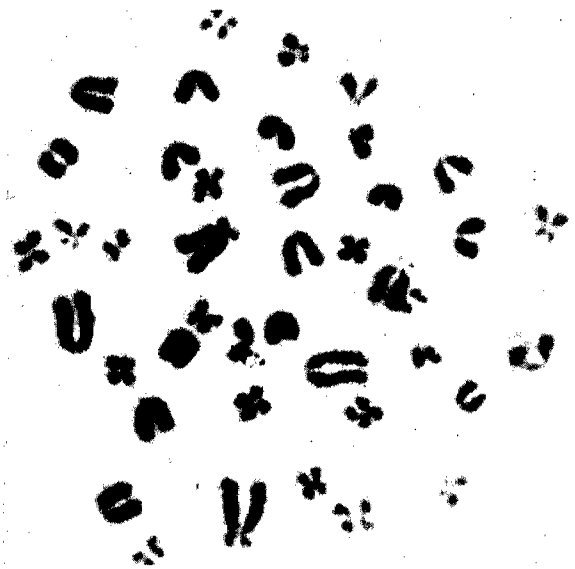

Figure 6. Normal rat bone marrow metaphase.

DISCUSSION

The present study describes a new bioptical transcutaneous technique, which allows the collection, from the same animal, of a series of bone marrow samples, in a rapid, noninvasive and reproducible manner. Moreover, the study demonstrates that this technique can be used for serial cytogenetic analysis. Further applications of the technique would also include the use of the technique for selecting, in a heterogeneous population of exper imental animals, those animals carrying a low spontaneous frequency of chromosomal aberrations. Such selected animals would obviously provide more homogeneous and sensitive levels of chromosomal aberrations that could be conveniently used for statistical computations of differences between control and treated groups. The technique could also be used to provide serial bone marrow samples for studies on long term hematological effects of toxic substances including mutagens, carcinogens and teratogens.

Techniques that provide serial bone marrow biopsies have been published before (Cameron et al., 1948; Narayana Rao et al., 1967; Petersen, 1970). However, such techniques have various disadvantages that are not present in the newly developed technique. For instance, one technique (Cameron et al., 1948) has the disadvantage that it requires asepsis and, in the perspective of routine mutagenicity screening studies on numerous animals, a rather lengthy surgical procedure. Another technique (Narayana Rao et al., 1967) offers the possibility of transcutaneous femoral bone marrow needle biopsy. However, such a technique also has certain disadvantages, namely (a) it requires the assistance of a second person and (b) since the femur metaphasis is used as site of entry for needle insertion, such technique is associated with an increased risk of femoral fractures (Petersen, 1967). Moreover, the just cited technique does not contain demonstrations that serial bone marrow samples were in fact obtained and used for serial hematological or cytogenetic analysis. Finally, a recent developed technique for bone marrow biopsy and cytogenetic preparation in the rat (Petersen, 1970) also required aseptical surgery and drilling of the femoral intercondyloid area.

Our study demonstrates that the technique itself did not introduce factors enhancing the spontaneous frequency of chromosomal aberrations in the animal to which it was applied. This was demonstrated by obtaining serial bone marrow biopsies in animals treated with distilled H_2O. In such animals the baseline frequency was not significantly different from those of the TEM-group prior to treatment with TEM (Table 2).

The technique, as shown in this study, can be applied to test the mutagenicity of chemicals. TEM was selected as a potent mutagen. Twenty-four hours after TEM-treatment there was a statistically significant increase of chromosomal aberrations (Tables 1 and 2). Such aberrations must be of a dominant lethal nature, since they were not present any longer 7 and 30 days after treatment, indicating that the bone marrow cells carrying such aberrations did probably not produce a viable progeny.

TABLE 1. CHROMOSOMAL ABERRATIONS IN BONE MARROW METAPHASES* OF TEM-TREATED RATS

	Breaks		Rearrangements	
Animal	N/Cells	% Cells	N/Cells	% Cells
10	4/20	20	14/20	70
34	7/20	35	8/20	40
22	4/20	20	1/20	5
18	2/20	10	9/20	65
30	8/20	60	7/20	35
1	7/20	35	3/20	15
40	5/20	25	9/20	65
32	9/20	45	8/20	40
2	7/20	35	1/20	5
5	6/20	20	2/20	10
Total	5/200	28.5	62/200	31

*Bone marrow samples were obtained through biopsy one day after TEM-treatment.

TABLE 2. CHROMOSOMAL ABERRATIONS IN SERIAL RAT BONE MARROW BIOPSIES

	Baseline[1]		Days after Treatment					
	C	T	C	T	C	T	C	T
Breaks	1.0[2]	3.0	3.5	28.5[4]	2.5	3.1	2.5	4.5
	(.7)[3]	(1.1)	(1.8)	(3.5)	(1.3)	(1.6)	(1.1)	(1.9)
Rearrangements	-	-	-	31.0[4]	-	-	-	-
	-	-	-	(6.8)	-	-	-	-

[1]Determination 10 days before treatment with distilled H_2O (control animals: C) or TEM (treated animals: T).
[2]Values are averages of percentage values from rats.
[3]Standard error in brackets.
[4]$p \leq .01$.

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AMRL-TR-75-125

PAPER NO. 19

1,4-DIOXANE: CORRELATION OF THE RESULTS OF CHRONIC INGESTION AND INHALATION STUDIES WITH ITS DOSE-DEPENDENT FATE IN RATS

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and
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INTRODUCTION

Dioxane (1,4-dioxane) is a colorless liquid used as a speciality solvent since about 1930. The principal toxic effects of dioxane have long been known to be centrilobular, hepatocellular and renal tubular epithelial degeneration and necrosis (DeNavasquex, 1935; Fairley et al., 1934; Schrenk and Yant, 1936; Kesten et al., 1939).

More recent reports by Argus et al. (1965) and Hoch-Ligeti et al. (1970) described nasal and hepatic carcinomas in rats ingesting water containing massive doses (up to 1.8% in the drinking water) of dioxane for over 13 months. Due to the massive doses which the rats received in these more recent studies, it is difficult to use the information to assess the hazard which may be incurred by individuals exposed to lower, more realistic levels of dioxane. Thus, a series of experiments has been conducted in our laboratory. This series has included a study of rats ingesting various dose levels of dioxane in the drinking water for 2 years (Kociba et al., 1974), a study of rats exposed to vapors of dioxane for 2 years (Torkelson et al., 1974), and a study of the pharmacokinetics and metabolism of various dose levels of dioxane (Young et al., 1975).

ORAL ADMINISTRATION STUDIES OF DIOXANE

Four groups of Sherman strain rats, each containing 60 males and 60 females, were maintained on drinking water containing 0, 1.0, 0.1 or 0.01% dioxane for up to 716 days. These concentrations of dioxane in the drinking water were calculated to provide approximate mean daily doses of dioxane of 1015 and 1599 mg/kg/day for males and females ingesting water containing 1.0% dioxane, 94 and 148 mg/kg/day for males and females ingesting water containing 0.1% dioxane, and 9.6 and 19 mg/kg/day for males and females ingesting water containing 0.01% dioxane (Table 1).

TABLE 1. CALCULATED DAILY DOSES OF 1,4-DIOXANE INGESTED BY RATS MAINTAINED ON DRINKING WATER CONTAINING DIOXANE

Concentration of Dioxane in Drinking Water	Sex	Calculated Mean Daily Dose of Dioxane* (mg/kg/day)
0 (Control)	M	0
1.0%	M	1015
0.1%	M	94
0.01%	M	9.6
0 (Control	F	0
1.0%	F	1599
0.1%	F	148
0.01%	F	19

* Based on data obtained from days 114-198.

The parameters monitored in this study included water consumption, clinical observations, body weights, hematologic determinations after 4, 6, 12, 18 and 24 months, survival rates and gross and histopathologic examination of all rats dying during the study or killed at the termination. Organ weights were recorded as part of the pathologic examination at the terminal necropsy. The alterations in these parameters considered related to treatment included increased mortality and decreased body weight gain in both males and females ingesting the high dose of 1.0% dioxane in the water.

Pathologic examination of tissues from rats maintained on drinking water containing 1.0 or 0.1% dioxane revealed evidence of renal tubular and hepatocellular degeneration and necrosis accompanied by regeneration (Table 2). In addition, rats receiving 1.0% dioxane in the drinking water had an increased incidence of hepatic tumors (hepatocellular carcinomas in 6 males and 4 females) and nasal carcinomas (1 male and 2 females). These hepatic and nasal tumors are considered related to the ingestion of 1% dioxane in the drinking water (Table 3). Rats which had received 0.1 or 0.01% dioxane in the drinking water showed no tumor formation considered to be related to treatment.

TABLE 2. HEPATIC AND RENAL LESIONS CONSIDERED RELATED TO TREATMENT WITH 1,4-DIOXANE

Dosage Level	Hepatic Lesions	Renal Lesions
1% (1015 mg/kg/day - M) (1599 mg/kg/day - F)	Hepatocellular Degeneration and Necrosis Hepatocellular Regenerative Hyperplastic Nodules Cholangiolar Hyperplasia Hepatic Neoplasms	Renal Tubular Epithelial Degeneration and Necrosis Renal Tubular Regeneration
0.1% (94 mg/kg/day - M) (148 mg/kg/day - F)	Hepatocellular Degeneration and Necrosis Hepatocellular Regeneration (Present to a lesser degree)	Renal Tubular Epithelial Degeneration and Necrosis Renal Tubular Regeneration (Both present to lesser degree)
0.01% (9.6 mg/kg/day - M) (19 mg/kg/day - F)	No lesions considered related to treatment	No lesions considered related to treatment

TABLE 3. INCIDENCE OF HEPATIC AND NASAL TUMORS IN RATS MAINTAINED ON DRINKING WATER CONTAINING 1,4-DIOXANE FOR 2 YEARS

Level of Dioxane in Drinking Water	Effective Number of Rats (Alive at 12 Months)	Number of Tumor Bearing Rats	Number of Rats With: Hepatic Tumors (All Types)	Hepatocellular Carcinomas	Nasal Carcinomas
0	106	31	2	1	0
1%	66	21	12[a]	10[b]	3[c]
0.1%	106	28	1	1	0
0.01%	110	34	0	0	0

[a] $p = 0.00022$
[b] $p = 0.00033$
[c] $p = 0.05491$

INHALATION STUDIES OF DIOXANE

A group of 288 male and 288 female rats of the Wistar strain was exposed to atmospheres containing 111 ppm dioxane seven hours/day, 5 days/-week for 2 years. A group of 192 male and 192 female rats served as controls. Parameters monitored included appearance, body weight, mortality, hematologic determinations after 16 and 23 months, and patholgoic examination of rats dying during the course of the study or killed at the termination. Organ weights and clinical chemistry determinations (blood urea nitrogen, serum alkaline phosphatase, serum glutamic pyruvic transaminase and total serum protein) were included as part of the pathologic examination at the termination of the study. Results of the study revealed no treatment-related alterations in appearance, mortality, body weight gain, hematology, terminal organ weights or clinical chemistry determinations. Pathologic examination of tissues revealed no lesions that could be attributed to exposure to dioxane. No hepatic or nasal tumors were observed in any of the rats and the incidence of all observed tumors appeared to be unrelated to exposure to dioxane.

Making several assumptions, it is calculated that these rats inhaled approximately 105 mg of dioxane/kg/day, a dose level similar to that which produced liver and kidney injury in the ingestion study with dioxane. Since the inhalation exposure was conducted 5 days/week rather than 7 days/week as in the ingestion study, the inhalation study provided a smaller overall dose as well as recovery period of 2 days per week; the extent of absorption may have been lower from the lungs of the inhalation rats than from the gut of the rats of the ingestion study.

PHARMACOKINETIC STUDIES OF DIOXANE AFTER ORAL OR INTRAVENOUS DOSES

Pharmacokinetic studies were conducted in male Sprague-Dawley rats housed in all glass, Roth-type metabolism cages. The dose was administered as a water solution using uniformly labeled ^{14}C-1,4-dioxane as the tracer. Oral doses were administered from a syringe and feeding needle, and samples of blood were collected from the retro-orbital sinus. Intravenous doses were administered and samples of blood collected from a cannula in the jugular vein. Radioactivity was determined by liquid scintillation counting of aliquots of blood plasma and urine. The concentration of dioxane in plasma and urine was determined by gas chromatography-mass spectrometry.

The concentration of radioactivity, expressed as μg equivalents/g of plasma, is shown in Figure 1 for rats given single oral doses of 2, 10, 100 or 1000 mg/kg. After the lowest doses given, 2 and 10 mg/kg, the radioactivity was eliminated from the plasma in an apparently log-linear manner with a half-life of about 1.5 hour. Higher doses, 100 and 1000 mg/kg, were eliminated more slowly with broad peaks which increased in duration with increasing dose. Since, after the broad peaks, the elimination was again apparently log-linear with a half-life comparable to the lower dose, it was concluded that high doses of dioxane had saturated either metabolic or excretory mechanisms.

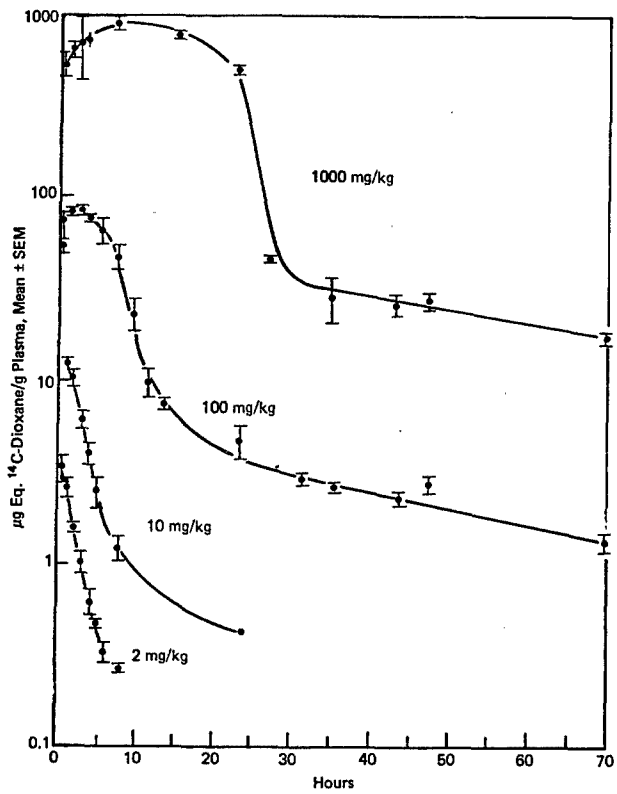

Figure 1. Concentration of radioactivity in plasma of rats after single oral doses of ^{14}C-dioxane.

The urinary excretion of radioactivity as a function of time and dose is shown as a semilog plot in Figure 2. During the first 8 hours, approximately 90% of a 10 mg/kg dose was excreted; this was in contrast to only 55% of a 100 mg/kg dose and only 13% of a 1000 mg/kg dose. Thereafter, as was seen in the plasma curves, the urinary excretion was apparently linear after the lowest dose and became increasingly nonlinear after higher doses.

The nonlinear plasma and urinary elimination curves for radioactivity demonstrate that at doses above 10 mg/kg the elimination of dioxane and/or metabolites of dioxane approaches saturation, although the mechanism cannot be determined from analysis of radioactivity alone.

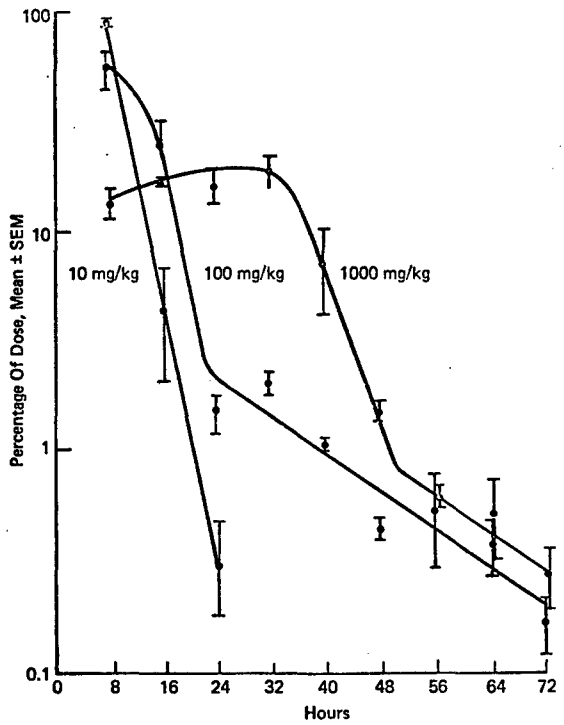

Figure 2. Excretion of radioactivity in the urine of rats given single oral doses of ^{14}C-dioxane.

The concentration of unchanged dioxane in plasma was determined as a function of time for rats given various intravenous doses of dioxane, Figure 3. After a brief distribution phase, the concentration of dioxane in plasma declined with an apparent half-life which increased with increasing dose. The apparent half-life increased from about 1 hour after a 3 mg/kg dose to over 14 hours after a 1000 mg/kg dose. Looking at the plasma curves in Figure 3 in a longer time span, Figure 4, the linear portions of the curves are, indeed, only apparent, and they are the initial part of curves which are really nonlinear throughout. It is noteworthy that, regardless of the dosage, the rate of elimination of dioxane from the plasma is identical only in the same concentration range. For example, the slopes of the plasma curves after each dosage are about equal when the concentration reaches 1 μg/ml;

but at a particular time, such as 5 hours, the slopes are dependent on the dose level. Therefore, the rate of elimination of dioxane is proportional to the amount left in the body, but, at doses above about 10 mg/kg, the elimination of dioxane from the plasma and the body occurs at a rate slower than that predicted for a first order process. Consequences of repetitive administration of a compound which demonstrates a capacity-limited elimination include an unusually large increase in the toxicity starting at the dose level where saturation effects become evident.

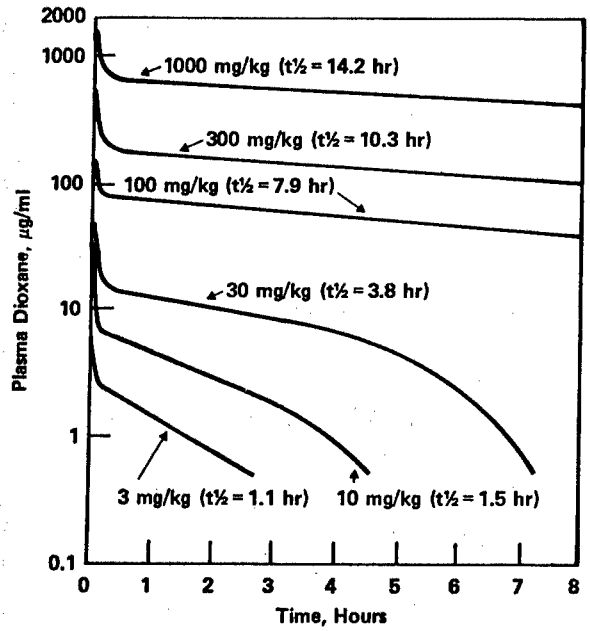

Figure 3. Concentration of unchanged dioxane in plasma of rats after various single intravenous doses.

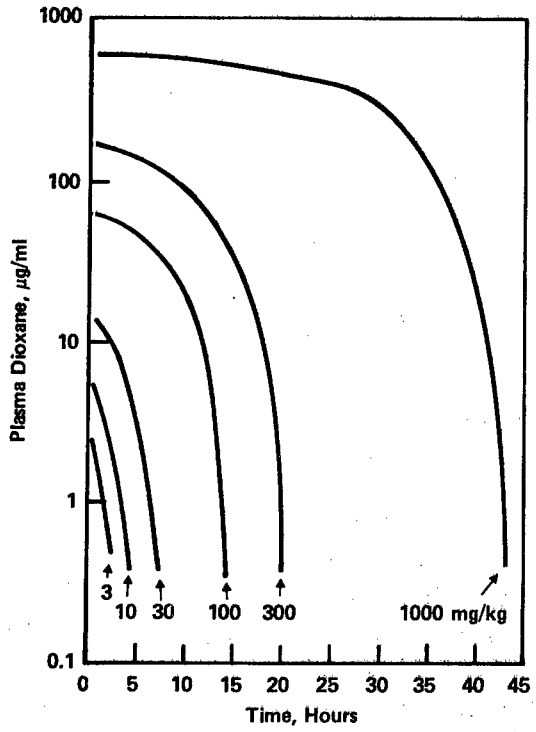

Figure 4. Concentration of unchanged dioxane in plasma of rats after various single intravenous doses.

A single oral dose of 10 mg/kg was eliminated rapidly; repetitive ingestion of this dose level (in the drinking water for 2 years) caused no discernible ill effects. However, single oral doses of 100 and 1000 mg/kg were

eliminated progressively more slowly; repetitive ingestion of these dose levels (in the drinking water for 2 years) caused renal and hepatic damage as well as tumors at the highest dose.

An evaluation of these data indicates the importance of considering possible metabolic differences in the disposition of various doses of the same compound in the body. This is especially important when evaluating those toxicologic effects that may be observed at an abnormally high dose level that is associated with an abnormal metabolic disposition within the body. The pharmacokinetic profile of dioxane together with toxicological studies support the conclusion that the toxicity of dioxane occurs only when doses are given which are sufficient to saturate processes for detoxification and elimination.

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AMRL-TR-75-125

PAPER NO. 20

CONCERNS OF INDUSTRY RELATED TO CARCINOGENIC HAZARDS

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The title of my talk implies that I am to present views and/or concerns of industry in toto about carcinogenic hazards. Before proceeding, however, I must state that I am making this presentation because Dr. Perry Gehring of our laboratory could not be here. The presentation constitutes the views and concerns of both of us as individuals and are not necessarily those of industry.

I assume you are all familiar with the various experimental methods employed to determine whether a chemical may be a carcinogen for human beings. A more difficult problem, and one which evokes a wide array of emotions, is the evaluation of the actual hazard incurred by exposure to a chemical shown to be carcinogenic in some test species, when administered at some dose by a certain route or by epidemiological evidence which almost always is devoid of definitive exposure data. So the operative word in the subject title is HAZARD, which can be defined in this context as THE PROBABILITY THAT CANCER WILL RESULT FROM EXPOSURE TO A CHEMICAL AT CERTAIN CONCENTRATIONS AND UNDER PARTICULAR CONDITIONS.

There are a number of investigators who maintain that the evaluation of a carcinogenic hazard should be different from the evaluation of other toxic chemical responses. Disregarding the principles of dose-response and biological thresholds, they believe that the effect of a carcinogen is all or none, that a single molecule is sufficient to be the ultimate agent of harm.

Note, I have not equated dose-response and threshold. Traditionally, dose-response curves have been developed empirically to describe the frequency of definitive events that occur within a certain limited range of doses. Dose-response data for most biological activity follow normal distribution curves. Assuming that normal distribution remains true below, as well as above, the narrow range of observable events dictates that some individuals will die no matter how small the dose and some will live no matter how large the dose. That is to say the normal distribution curve approaches zero and 100% asymptotically.

Although pharmacologists, toxicologists, epidemiologists and other biologists, by experience, have chosen to ignore these tails in assessing the therapeutic efficacy and/or hazard of most chemicals, society is now asking that these tails not be ignored for carcinogenicity, mutagenicity, and teratogenicity. Other 'icities' have escaped such attention because they have less emotionalism associated with them. Unfortunately, it is impossible to prove that something will not happen. To relieve this irritating dilemma, the statistician has stepped in and applied his stochastic statistical process, and I emphasize "stochastic" which is defined as guess work, to replace the stochastic process of the biologists. A simplistic scenario is that for a faster runner to pass a slower one, he needs to traverse 1/2 the distance between the two, then 1/2 that distance and so on. Mathematically, one can show that the faster runner will never catch up and pass the slower one. Definitive observation leads us to reject this mathematical simulation. Unfortunately, such definitive observation is nonexistent for carcinogenicity and I believe will remain so for a long time.

There are convincing theoretical arguments that a dose must exist below which a carcinogen is ineffective, due to the presence of multiple interfering substances and other factors as described by Dinman (1972). Controversy will continue regarding these two views. The important point is that a dose-response relationship has been demonstrated experimentally, with a decrease in tumor incidence and an increase in the latent period observed with a decrease in dose. So that even if one molecule were to have the potential for inducing cancer, the latent period may be increased way beyond life expectancy. Furthermore, acceptance of a dose-response relationship and threshold as well as utilization of them are essential in the evaluation of the hazard of chemical carcinogenesis. It is all well and good to demand that carcinogens be eliminated from our environment, but there is simply no way that this can be accomplished.

Industrial experience with vinyl chloride proves a practical example of a dose-response relationship for carcinogens. Angiosarcomas of the liver have been linked to occupational exposures to VCM. The concentrations to which afflicted workers were exposed are not known. Subjective estimation based on odor thresholds, central nervous system effects and the prevalence of other disease states, scleroderma and acroosteolysis, indicate the exposures were high.

In 30 years experience with VCM production and handling at The Dow Chemical Company, epidemiological studies of 594 employees revealed no angiosarcomas or other liver tumors, nor any cases of acroosteolysis. Based on animal toxicity studies, the levels of VCM in the Dow work operations had been controlled and sequentially reduced, with the levels generally below 50 ppm since 1961. Exposure levels of these 594 employees have been documented, and the studies show further that over the 30-year period no discernible medical abnormalities were associated with intermittent exposures as high as 200 ppm. Now we cannot claim to have protected the workmen against cancer to a risk of one in a million. Or even claim that the basis for limiting exposures was the cancer hazard, but at least the record serves to illustrate that for all practical purposes a dose-response relationship is operative in this case. I am curious as to why some scientists ignore or depreciate the value of these data and rely entirely on data collected from studies with smaller numbers of rats or mice to assess the hazard to man of low level exposures to vinyl chloride.

I am aware of the deficiencies in the data base; however, it has appeared to me that there has been a predisposition to bring to light positive data and ignore negative. Even more curious to me is why in animal experiments proclaimed to be elegantly conducted little, if any, attention has been paid to pathology other than tumors. It is well recognized by most that one of the most valuable tools in assessing the hazard of an untoward effect is relating the dose-response for it to those of other untoward effects. Even between species, these relationships tend to remain constant although the dose needed to cause them may be different. Evidently, such historically important relationships for assessing hazard have become out of vogue. My point is that mortality, yes mortality, in mice and rats exposed to 50 ppm and above vinyl chloride has exceeded that in controls. Are we to assume that pathology other than tumor development did not occur? Perhaps not. But if it did it should be reported together with the tumor incidence, so that the animal

data can be more properly evaluated in assessing the hazard to man. I make a plea that "tunnel vision" for carcinogenicity not negate our visibility of other "icities" which are so valuable for assessing tumorigenic hazard and indeed in themselves may constitute the real hazard.

To preempt unintentioned interpretation of what I have just said, I do not proclaim that animal data for carcinogenic activity have no utility for assessing the hazard in man. Rather, I consider properly designed animal studies to be the basic tools for evaluating carcinogenicity. I am sure that improvements can be made in the present-day design of such studies and as that occurs, the predictability of animal data will be enhanced. Even in our present "state of the art" animal tumor data have shown surprisingly good correlation with known chemical carcinogens in man. A number of known human carcinogens have been shown to cause cancer in laboratory animals: diethylstilbesterol, vinyl chloride, benzidine, beta-naphthylamine, 4-amino-biphenyl, 4-nitrobiphenyl, bis(chloromethyl)ether, asbestos, chromates and nickel dust (Wands and Broome, 1974).

However, the solution is by no means simple because for the 1000 or more chemicals shown to be carcinogenic in animals, there is no evidence of their carcinogenicity in man. Does this mean that animal models are too sensitive? I do not believe it does. Rather this is largely a testimonial to the inappropriateness of deeming carcinogenic, chemicals given in large doses by artificial routes to animals. A beautiful example is the 1,4-dioxane story Dr. Kociba, of our laboratory, just presented.

Probably the greatest contribution to enhancing the predictability of animal data are pertinent pharmacokinetic/metabolism studies. Similar pharmacokinetic and/or metabolic profiles in animals and man increase the reliability of using the data collected from animals to assess the hazard in man.

Equally important is the evidence being obtained in our laboratory and others showing dose-dependent pharmacokinetic/metabolism profiles for many compounds. For these chemicals the pathways for their disposition following small doses are saturable. Doses which exceed these metabolic and physiological thresholds cause prolonged retention and/or formation of different proportions of metabolites. Some examples include benzene, benzopyrene, aniline, nitrobenzene, aspirin, vinyl chloride, ethylene glycol, bromobenzene, and 1,4-dioxane.

One must interpret with caution data collected from animals given saturating doses of chemicals. Indeed, an a priori assumption precludes the use of such data to predict statistically what may occur at lower doses. That is, even the stochastic, guess work statistical projections of such data become invalid.

Before leaving my concern for the scientific aspects of carcinogenicity, I would like to say a couple of things about epidemiological studies since they constitute a major aspect of the evaluation of cancer hazards. As with other approaches, problems exist in evaluating epidemiological data. Initially, a long latent period generally is required for tumor induction to occur making correlation with exposure to a specific chemical difficult. This also renders difficult assessment of today's hazard because often we are reaping today's harvest because of the poorer hygienic practices of yesteryear and the resulting higher exposure levels. This, in turn, I feel has caused our regulatory agencies to sometimes overreact. Another caution to be exercised is drawing conclusions from comparisons between recently obtained epidemiological data and data collected in the past. Often concern about a parameter increases dramatically the intensity of the search to find it, thus distorting the meaningfulness of a temporally associated increase in its incidence.

Approximately 1500 chemicals have been classified as suspected carcinogens or neoplastigens in the Toxic Substances List for 1975 tabulated by NIOSH, ranging from bis(chloromethyl) ether to lactose. So even with previous experiences upon which to draw, the decision as to which chemicals demand the most critical attention is a difficult one. It is impossible to deal simultaneously with the large number of industrial chemicals implicated as carcinogens. Each company, agency and institution needs to establish priorities. Items which must be considered in developing priorities are quantities produced, animal data suggesting carcinogenicity, similarity to known carcinogens, potential for human exposure and so on. Having established priorities, they must be communicated. Even more important is the communication of studies underway or being initiated. Then, and only then, can intensive efforts be made to elucidate the real hazard without diluting resources to the point of ineffectiveness. Barriers among government, industry, and academia must be minimized to prevent unneeded redundancy; more cooperation in utilization of talents and facilities must be pursued.

It should be clear that in the evaluation of cancer hazards, as with all biological phenomena, we are dealing with possibilities rather than absolutes, and so it follows that we are also dealing with risks. I think it is appropriate to explore the amount of risk that an individual is willing to assume when confronted with a hazard. The degree of risk assumed willingly seems to be not so much a function of the severity of the hazard, but rather whether the risk is taken voluntarily or is imposed by outside forces. According to an expert in the study of risk taking the public appears willing to accept voluntary risks roughly 1000 times greater than involuntary risks; and "as one would expect, we are loathe to let others do unto us what we happily do to ourselves" (Starr, 1970).

If the trend toward refusal to accept any so-called 'externally inflicted' risks continues, where is it likely to lead us in the future? The ever increasing sophistication and sensitivity of analytical methods will continue to reveal the presence of minute traces of 'toxic' chemicals in our food and environment, including the detection in the industrial atmosphere of low levels of chemical carcinogens, to which it formerly may have been believed there were no exposures. In fact, the advances in this area probably have had the greatest single impact on awareness and subsequent action regarding evaluation of hazards of chemical exposures.

The fact is obvious that the general unacceptability of the presence of a carcinogen, whether forbidden by law in food or strictly regulated in the work environment, is going to create even more complicated problems when, in fact, more and more of these chemicals are detected in lesser and lesser amounts.

The unwillingness to accept risks by demanding that chemicals and drugs be 'completely safe' could have some repercussions that would have direct impact on the consumer. For example, a decline of new developments by the chemical and drug industry may well result because of the increased expense of developing those materials and investigating their safety, only to be met in the end with opposition and rejection generated by so-called 'public interest' groups. Drug companies, for instance, are anticipating no further development of oral contraceptives because of the increased demand that use of these materials result in no side effects and are therefore '100% safe.' The inherent risk of pregnancy and childbirth obviously is being overlooked.

Unnecessarily restrictive standards for the chemicals used in industry could result in decisions that would have as significant an impact on the economy and the consumer as would unrealistic requirements for '100% safe' consumer products. Of the top 50 chemicals in industrial production in 1973, ranked according to pounds used, (C&E News, 52, p. 11, 5/6/74) 8 were listed by NIOSH as carcinogenic or neoplastigenic in animals. These were acetaldehyde, ammonia, ethanol, ethylene glycol, benzene, formaldehyde, phenol, and vinyl chloride. I do not believe even the worst alarmists would suggest that all these materials be eliminated from the industrial environment. But, if prohibitively low limits for air levels made it economically unfeasible to continue production involving any one of these chemicals, serious repercussions would be heard. All of these chemicals are important, high volume intermediates or products per se.

In many cases, industry has not done a very good job in communicating the rapid escalation of costs necessary to make incremental reductions in exposure levels. I am hoping my colleagues, the engineers, will be able to do a better job on this point than we are doing in dealing with the tails of the dose-response curves. If they do, it should be clear that continuing demands for 'zero risk' would not only have serious economic consequences, but would ultimately result in the presence of greater hazards, because of the shortage or elimination of materials that actually improve the quality of our lives. Further overcommitment in the areas that actually present little hazard would be a drain on the resources available for combating the real threats to the health and well being of society. Sorely needed in our scientific community and in society is the adoption of a "RULE OF REASON" which, most simply stated, recognizes that there is some risk involved in any activity of man, and society must accept some risk for the benefits it receives.

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AMRL-TR-75-125

PAPER NO. 21

ANIMAL TESTING FOR CHEMICAL CARCINOGENESIS. WHY, WHERE, WHEN AND HOW DO WE START?

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Last year at this conference it was my privilege to present a paper on animal carcinogenesis prepared by Joan Broome and myself. In that paper we reviewed the literature on known human chemical carcinogens and the associated animal data. Our findings were that the animal data were predictive of all human cases, although only limited animal data were available on arsenical cancer. We also pointed out that the prediction was not necessarily precise as to site, or type of cancer. Of course, given the limitations of epidemiology the predictability of animal data for human dose relationships is not easily resolved. Our conclusions were that present animal experimental procedures were predictive and that we had numerous suspect chemicals and, therefore, we should get on with the job of evaluating materials and start preventing chemical carcinogenesis in humans.

That paper and its challenge were successful in at least one way. It resulted in Dr. Crocker handing the challenge right back to me saying in effect, "O.K., wiseguy, tell us where to start." So here I am back again and there you are waiting for me to say something new and brilliant about an old problem. How do we set priorities for efficient utilization of limited facilities, personnel, and funds?

I'm not really a wise-guy in that colloquial sense. If I were, I would tell you to start a new carcinogenic evaluation each week on whatever was the "carcinogen of the week." In the last five weeks we have had five carcinogenic alarms run through our scientific community. They were about trichloroethylene, hexamethylphosphoramide, lead chromate, nitrosamines, and peroxyacetic acid.

Since I'm not really a "wise man" either, I'm not going to try to answer the question. I think it is presumptuous for one person to attempt to do so. Instead, I'm going to give you some concepts that I've picked up over the years as a basis for a discussion among all of us here. Perhaps by that method we can make some progress. You are cordially invited, and indeed expected, to discuss, debate, or downright argue with me and with each other.

Before I begin I must remind you that what I say today will be my own ideas and concepts and they do not represent those of the National Academy of Sciences nor any of its components, nor do I speak for ACGIH and its TLV committee.

To begin with, we have, through the courtesy of NIOSH, a list of 1500 compounds for which there is some evidence in the literature for their carcinogenicity. We also have from NCI a list of 6700 compounds which have been tested for carcinogenic action including those in the NIOSH list. There is a third list, also from NCI, of several hundred compounds for which testing is underway or planned. That's an impressive sum of knowledge but compare it with the total number of known compounds and with the number of new ones being synthesized each year. Clearly, we are facing a problem of great magnitude that is getting no better fast. The question is not only, "Where do we begin?" It is also, "How do we begin?" Perhaps, we should first ask,"Why begin?" For that I would say our purpose in addressing the question of priorities is not for the conduct of basic research, but rather to focus on achieving maximum efficiency in generating data applicable to regulatory controls which will prevent human cancer.

I propose that we develop first a set of criteria to rank those compounds to which the largest number of people are exposed in the largest concentrations. I propose that we next evaluate each compound in order of its rank in terms of what is known about the conditions of exposure, that is, duration and quantity, and what is known, or suspected, about its toxicity, including carcinogenicity. With a large measure of expert judgment going into that evaluation we would then have a priority ranking for testing. I'll return to that question of judgment in a moment.

I would then propose that we devote a major fraction of our resources, perhaps 80%, to two kinds of testing. One set of tests for screening to determine the qualitative issue of whether or not cancer is produced. The other set of tests to quantify the carcinogenic action, i.e., how many tumors are produced, how rapidly, and at what concentrations. I'll come back later to some details of these testing procedures. I would reserve a small fraction of our resources, perhaps 10%, for qualitative and quantitative evaluation of those compounds which do not fit the original mold to give them a high priority ranking, but for which there is something unique, or some new discovery about their chemistry, toxicity, or human exposure conditions, so that good scientific judgment says these need to be tested now. The remaining small fraction of resources, about 10%, I would reserve for evaluation of materials about which there is a great deal of social or political concern and for which there are little or no data.

Let's go back to this issue of judging the chemistry and toxicology of a compound in order to rank its priority for testing. The National Cancer Institute has gone through this exercise during the last year or two in establishing priorities for its list of 400 or 500 compounds to be screened for carcinogenicity in the next few years. To the best of my knowledge their procedure has not been published, but I understand it has the following steps in it. First, they collected a large list of compounds that someone for some reason felt should be tested. Next, all of the available information on each compound was organized. This included its chemistry, its proposed use and volume of use, its toxicity, and why it was thought necessary to test its carcinogenicity. Then a sizeable group of scientists representing many disciplines went over the information and grouped the compounds for their priority on the basis of the scientific evidence. The final selection was then made by a relatively small group of scientists responsible for program administration. As far as I know, there is no way of learning precisely what factors and weightings went into these decisions. Perhaps some of you in the audience can add to our understanding on this point during the discussion period.

Another group that has tried to evaluate the significance of data on the toxicity and carcinogenic activity of chemicals is the Threshold Limit Values Committee of the American Conference of Governmental Industrial Hygienists. They have a subcommittee on industrial carcinogens which has been trying to develop criteria for evaluating animal data for its importance in establishing TLV's. Their procedures have been written down but are not yet final, so they have received very little publicity. I think a brief description of these tentative procedures might be helpful to our discussion.

The TLV Committee has two broad categories, human carcinogens and suspect carcinogens. Human carcinogens are those with solid epidemiologic data, exclusive of single case histories, or have demonstrated high carcinogenic potency in animals. This latter criterion is germane to our discussion today. For a compound to be classed as a "High Potency" carcinogen it must have (1) produced cancer at concentrations below 1 mg/m^3 in 6 months of repeated daily exposures or by a single intratracheal dose of particulate matter of 1 mg or less per 100 ml of the animal's respiratory minute volume; or (2) elicited skin cancer in 20 weeks of twice weekly skin paintings at 0.1 mg/kg; or (3) by 6 months ingestion of 0.01 mg/kg/day. Alternatively, a high potency compound will cause tumors by all 3 routes of administration - inhalation, skin, and ingestion; or produce tumors in 3 or more species, one a nonrodent.

The subcommittee has also tentatively described Experimental Animal Carcinogens of Low Potency. These only produce tumors by inhalation at 10 or more mg/m^3 daily for 2 years; or 75 weeks of twice weekly skin paintings at more than 10 mg/kg; or by ingestion of more than 1 mg/kg/day. I repeat, these criteria for high and low potency carcinogens are tentative and may be revised next week when the subcommittee meets. My point in presenting them here is to generate discussion of whether this kind of a classification could be useful in prioritizing compounds for further study before determining what regulatory controls, if any, are needed.

Now let's take a look at the kinds of preliminary data that might justify the expense and effort of a thorough carcinogenic evaluation. It seems obvious that if one has evidence classifying the compound as a human carcinogen or one of high potency, little more experimentation can be needed except possibly to establish dose-response relationships which would get a high priority. It is equally clear that Low Potency compounds have a low priority for additional work unless there is a very high rating for exposure and use at concentrations approaching the cut-off criteria.

Compounds of Medium Potency will challenge the skills of those making the judgment on whether or not to spend upwards of $100,000 on estimating the potential of a compound to cause human cancer. In this group greater emphasis will have to be placed on auxiliary data. These would include similarity of structure or of metabolism to those of known human carcinogens.

Structure-activity correlations are not in themselves sufficient to give a high priority. Compounds which are cross-linkers or which form free radicals are likely candidates. Other classes which are of concern include aromatic amines, azo compounds, N-nitrosos, polycyclic aromatics, three-membered heterocyclics.

Other kinds of data that can be helpful in these decisions are those from the various Short-Term Bioassays. These include transformation of cells in either tissue cultures or as monolayers grown in the presence of the test compound. Transformation of the cells is presumptive evidence of carcinogenic potential. If the transformed cells are inoculated into animals they will produce tumors. These tests can and should include a run in which enzyme inducers are in the cell cultures in order to pick up those compounds whose carcinogenic activity arises from a metabolite.

The microbial assay for mutagenic activity developed by Ames looks particularly promising as a screening tool for compounds that should go into animal carcinogenic tests. Positive mutation activity in one or more of Ames' five microbial strains gives an 85% positive correlation with known animal carcinogens. There is also a good negative correlation from the approximately 500 compounds that have been reported. This procedure is not effective for compounds having hormonal mechanisms of carcinogenesis or for metals such as arsenic, nickel or chromium. It is good for the free-radical formers and cross-linkers.

For a brief survey of these procedures and for guidance on protocols for chemical carcinogenicity in animals, I refer you to a publication from the National Academy of Sciences, "Principles for Evaluating Chemicals in the Environment," ISBN 0-309-02248-7.

In conclusion, let me answer the questions: why, when, where, and how do we start?

We start because we have known occupational carcinogenic problems with insufficient information for proper control and we strongly suspect many more such problems which are unknown and getting steadily more numerous. We start now to quantify our known problems and to identify our unknown problems.

There are available some procedures to help us determine where to start; that is, for setting priorities. We have available some easy screening procedures and our old, laborious carcinogenic testing procedures.

Thus, in a very simplistic way, I have set up a strawman for this scientific audience to tear down. Before you attack, let me ask one more vital question: Who starts? Obviously, we all do, but let's not everybody start on the first one, or the first ten. Let's get some coordination, otherwise we are in danger of unnecessary duplication which will waste our scarce supplies of manpower, facilities, and funds.

AMRL-TR-75-125

PAPER NO. 22

USE OF COMPARATIVE METABOLISM STUDIES IN EVALUATING CARCINOGENIC RISK IN MAN

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INTRODUCTION

There are several aspects of the problem of evaluating the risk that potential environmental carcinogens present to human populations. One aspect is trying to understand and establish "safe" or "no-effect" levels of a carcinogen for human populations of a few score to hundreds of millions. The more immediate problem, however, is the attempt to establish whether the compound is a human carcinogen. As so clearly presented by our session chairman today at last year's environmental toxicology conference, in almost every instance a compound now known to be a human carcinogen was first demonstrated to be carcinogenic in man and only subsequently shown to produce tumors experimentally in animals (Wands and Broome, 1974). There must be ways to improve the record of the experimental toxicologist in predicting the human carcinogenic risk of environmental compounds; perhaps focusing part of our attention on comparative metabolism of compounds in man and in animals both sensitive and resistant to the carcinogenicity of the compound can provide more accurate predictions. There are at least two experimental situations on record that illustrate the potential assistance comparative metabolism studies might provide -- the N-nitrosamine story and the aflatoxin story.

N-NITROSAMINES

The N-nitrosamines have been the object of much experimentation. They provide an abundant source for structure-activity relationship studies, and, as a class, they produce malignant tumors in a wide variety of tissues and species (Magee and Barnes, 1967; Druckrey et al., 1967, Shank, 1975a).

Until recently, it was difficult to see how these compounds, which are limited primarily to laboratory use, would ever be widescale environmental problems. A series of studies by several investigators (Ender et al., 1964; Sakshaug et al., 1965; Sander, 1967, 1971; Mysliwy et al., 1975) have shown that the common food additive, sodium nitrite ($Na\ NO_2$), apparently vital in preventing the formation of the deadly botulinum toxin (Christiansen et al., 1973) in certain processed foods can, under restricted conditions, nitrosate secondary and tertiary amines and amides, and several of the nitrosated products are known carcinogens for animals.

A recent study on the carcinogenicity of a purified rodent diet containing sodium nitrite and morpholine illustrates the need to predict human susceptibility to nitrosamine carcinogenesis. The study was designed to determine if tumors could result from feeding animals diets which contained low levels of sodium nitrite and a secondary amine; morpholine was chosen because it was an allowed incidental food contaminant, because N-nitrosomorpholine could be accurately determined in foods and gastric juice, and because N-nitrosomorpholine is known to be a carcinogen (Shank and Newberne, 1975). Rats and hamsters were fed for lifetimes through two generation chemically-defined diets which contained various levels of sodium nitrite and morpholine. Table 1 summarizes the results of the feeding study, showing that diets to which was added morpholine and only 5 ppm sodium nitrite (and which assayed up to 80% less nitrite after diet preparation due to the instability of nitrite in aqueous mixtures) were still able to induce hepatocellular carcinomas and liver and lung angiosarcomas; such low nitrite levels are thought to approach human exposures. Such studies press for an answer to the question, "are nitrosamines carcinogenic for man?"

Perhaps suggesting the use of comparative metabolism studies to aid in extrapolating from the rat to the human may seem naive; after all, many carcinogens are metabolized to a variety of products; it may be difficult to decide which pathway(s) should be followed in comparative studies to be certain the data are relevant to carcinogenesis and not only necrosis, enzyme inhibition, or detoxication.

TABLE 1. TUMOR INCIDENCE AMONG RATS* FED DIETS CONTAINING VARIOUS LEVELS OF SODIUM NITRITE AND MORPHOLINE

Dietary Concentration, ppm**			Liver Tumor	Lung Tumor
$NaNO_2$	Morpho.	NNM***	Incidence, %	Incidence
1000	1000	0	97	23
1000	50	0	59	6
1000	5	0	28	8
1000	0	0	1	0
0	0	0	0	0
1000	1000	0	97	23
50	1000	0	3	1
5	1000	0	2	1
0	1000	0	3	2
1000	1000	0	97	23
50	50	0	2	1
5	5	0	2	2
0	0	50	93	20
0	0	5	58	9

* 94-172 animals per group.
** Concentration of added material; nitrite was not stable in diet preparation.
*** NNM = N-nitrosomorpholine.
From: Shank and Newberne (1975).

N-nitrosamines can be metabolized to alkylating agents. An attractive hypothesis in chemical carcinogenesis is that these compounds can lead to the alkylation of DNA inducing nonlethal changes in template fidelity and loss of regulatory processes in the cell, and that these changes can be passed down to daughter cells which ultimately form tumors.

In the early studies a most interesting pattern relating tumor production to metabolism was developing. N-nitrosamines required metabolic activation by the target organ and the proximate carcinogen alkylated DNA at the N-7 position. Where the enzyme was not present, alkylation did not occur and tumors did not develop. This relationship is illustrated in Table 2 where oxidation of the carcinogen to carbon dioxide is taken as a measure of metabolic activity and activation to an alkylating agent.

TABLE 2. ASSOCIATION BETWEEN METABOLISM OF NITROSAMINES AND TUMOR PRODUCTION IN VARIOUS ORGANS

		$^{14}CO_2$ from ^{14}C-DMN/DEN (% ^{14}C converted/g/hr)		
Compound	Animal	Liver	Resp. Tract	Sm. Intest.
DMN	Rat	22 (+)	0.12 (-)	0.11 (-)
	Hamster	36 (+)	0.2 (-)	0.05 (-)
DEN	Rat	7.9 (+)	1.3 (-)	0.6 (-)
	Hamster	8.9 (+)	12.4 (+)	0.9 (-)

(±) = tumor response
From: Bartsch et al. (1975).

A great many studies were carried out to further test this relationship. In several instances there was promise, such as the demonstration that human liver slices were almost as effective in activating dimethylnitrosamine as were rat liver slices (Table 3). The association, however, did not remain unscathed. Although Wilhelm and Ludlum (1966) showed that methylation of the N-7 position of messenger RNA guanine inactivated guanine in translation of the genetic code, polyribonucleotides containing 7-methylguanylic acid serve as normal templates for RNA polymerase (Ludlum, 1970). As seen in Figure 1, Swann and Magee (1968, 1971) demonstrated disagreement between alkylation at the N-7 position of guanine and tumor production. A single administration of dimethylnitrosamine produced extensive liver DNA and RNA methylation but no liver tumors; chronic exposure or single doses to newborn rats does produce liver tumors. Methyl

methanesulfonate methylates kidney nucleic acids as well as dimethylnitrosamine but doesn't induce kidney tumors.

TABLE 3. DMN METABOLISM BY RAT AND HUMAN TISSUES

Tissue	O_2 Uptake (μl O_2/g/hr)	$^{14}CO_2$ Production (% ^{14}C Added)	Methylation of Nucleic Acids (% G as 7-MeG) in vitro	in vivo
Rat liver slices	760	5.2	0.19	1
Human liver slices	240	3.0	0.13	-
Rat kidney slices	2560	0.5	0.01	0.1

From: Montesano and Magee (1970).

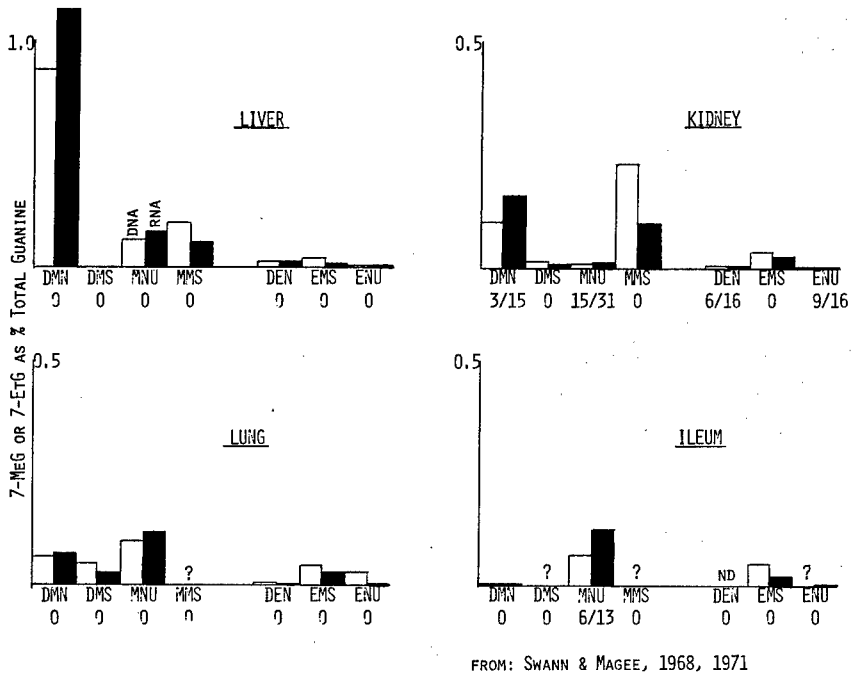


Figure 1. Association between alkylation of nucleic acids at N-7-guanine and tumor incidence in rat organs produced by various N-nitroso compounds

DMN = dimethylnitrosamine
DMS = dimethylsulfate
MNU = methylnitrosourea
MMS = methyl methanesulfonate
DEN = diethylnitrosamine
EMS = ethyl methane sulfonate
ENU = ethylnitrosourea

The N-7 position of guanine is the most favored site on DNA for alkylation (Brookes and Lawley, 1960) and for the past fifteen years has been the focus of the attention of the researchers working with alkylating carcinogens. The intensive effort to study the alkylation of the N-7 position of guanine until recently has precluded detailed investigations of sites quantitatively minor but perhaps more relevant to the action of the proximate carcinogen.

Loveless (1969) at the Chester Beatty Research Institute has focused on a secondary site on DNA, subject to alkylation to a considerable lesser extent, but, indeed, perhaps more relevant to carcinogenesis. And it must be remembered that to construct a meaningful relation between comparative metabolism studies and tumor production, metabolism relevant to carcinogenesis is the keystone. Loveless (1969) pointed out that the usual method of preparing nucleic acid hydrolysates destroyed O-6-alkylguanine residues, the O-6-alkylation is much more likely than 7-methylguanine to disrupt base pairing, that incubation of nitrosomethylurea, nitrosoethylurea and ethylmethanesulfonate, strong carcinogens, with deoxyguanosine resulted in O-6-alkylation, but methyl methanesulfonate and dimethylsulfate (possibly weak carcinogens) did not.

Support for the significance of O-6-alkylguanine quickly came from Lawley's group, also at the Chester Beatty (Lawley and Thatcher, 1970; Lawley and Shah, 1972). Table 4 compares the methylation at both the N-7 and O-6 positions of guanine and the 3-position of adenine in rat liver DNA by the strong hepatocarcinogen, dimethylnitrosamine, and the non-hepatocarcinogen, methyl methanesulfonate. The results show an association between methylation and carcinogenicity only with O-6-methylguanine (O'Connor et al., 1973). Similar associations have been demonstrated by Craddock (1973), and others. Goth and Rajewsky (1974), Kleihues and Margison (1974) and Margison and Kleihues (1975) present convincing evidence that not only is alkylation at the O-6 position of guanine apparently important to carcinogenesis but so also is the rate at which that particular base is removed from the macromolecule, that is, DNA repair of O-6-alkylguanine bases. Gerchman and Ludlum (1973) have shown that the presence of O-6-methylguanine in a copolymer of cytidylic and O-6-methylguanylic acid used as a template for RNA polymerase leads to misincorporation of UMP or AMP into the product copolymer and suggest formation of this alkylated base is an important mutagenic event.

TABLE 4. SPECIFIC METHYLATION OF RAT LIVER DNA BY DIMETHYLNITROSAMINE AND METHYL METHANESULFONATE

Alkylated Base	DMN	MMS
7-methylguanine	+++	+++
3-methyladenine	+	+
O-6-methylguanine	+	-

From: O'Connor et al. (1973).

The next question to answer seems to be: do carcinogenic N-nitrosamines produce O-6-alkylguanine moieties in human target organ DNA followed by a greatly reduced rate of repair? Would not such evidence give the toxicologist more confidence in predicting the carcinogenic potential of a compound for human populations?

Based on the animal data now available, it seems warranted to conduct epidemiological studies in selected populations to relate nitrosamine exposure to human cancers. Indeed, such studies are in progress under the support of the International Agency for Research Against Cancer. Such studies hold promise in being successful in demonstrating that an environmental contaminant is a human carcinogen. The logic presented here has been tested in the field and is nicely illustrated by the aflatoxin story.

AFLATOXINS

Aflatoxins are a group of fungal metabolites produced by many strains of the ubiquitous mold Aspergillus flavus. Aflatoxin B_1 is the most potent hepatocarcinogen known for laboratory animals and is found in the human food supply in virtually every country where analyses have been performed. The aflatoxin story has been reviewed several times (Goldblatt, 1969; Wogan, 1973; Shank, 1975).

The first studies on aflatoxin metabolism indicated that several hydroxylations were occurring, as shown in Figure 2. Aflatoxin M_1 was first discovered in milk from cows given aflatoxin B_1 - contaminated feed (Allcroft and Carnaghan, 1963). It has been found in urine from children

who had eaten contaminated peanut butter, but could not be detected in human milk under similar circumstances (Campbell et al., 1970). The acute toxicity of M_1 is comparable to that of B_1 (Purchase, 1967; Pong and Wogan, 1971) but the carcinogenic potency of M_1 is considerably less than that of the parent compound (Sinnhuber et al., 1970; Wogan and Paglialunga, 1974), and therefore this metabolic route is probably not relevant to the carcinogenicity of B_1.

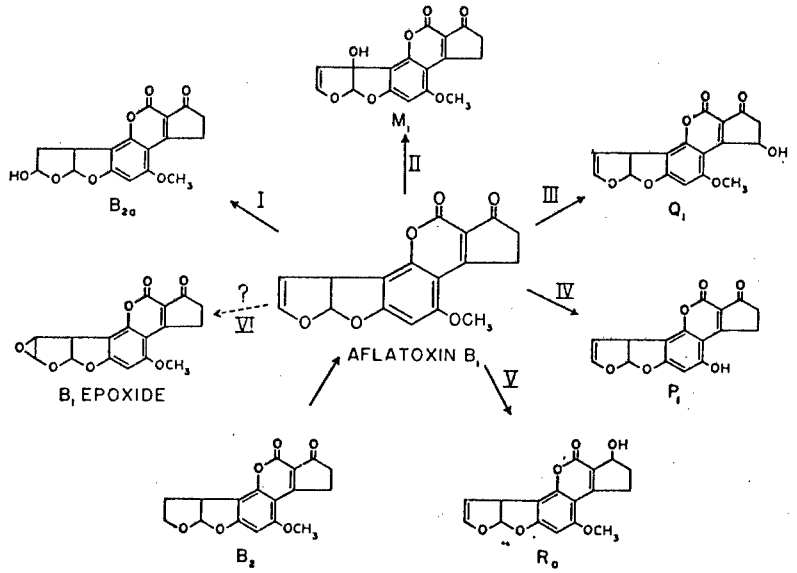


Figure 2. Structures of aflatoxin B_1, M_1, Q_1, P_1, R_0, B_2, B_{2a}, and B_1 epoxide.

In the monkey and rat, oxidative demethylation of aflatoxin B_1 to P_1 is a major pathway, but serves as a simple detoxication mechanism, for the phenolic compound is rapidly conjugated and excreted in the urine (Wogan et al., 1967; Dalezios and Wogan, 1972) and is at least twenty times less acutely toxic than the B_1 compound (Buchi et al., 1973).

Three additional aflatoxin metabolites, Q_1, B_{2a}, and R_0 (aflatoxicol), have been recognized in mammalian systems, but little significance has been ascribed to them regarding activation of aflatoxin B_1 to the ultimate carcinogen.

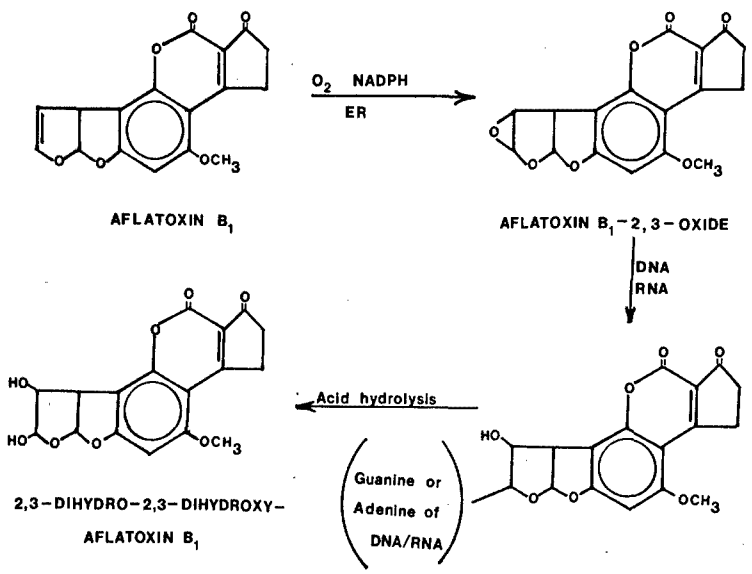
Table 5 summarizes the comparative toxicity, carcinogenicity and metabolism of aflatoxin B_1 for twelve species. An attempt has been made to rank the species according to how little aflatoxin B_1 is required to induced liver tumors, but it must be emphasized that detailed dose-response studies have been done only in the rat and rainbow trout. The association of aflatoxins with human liver cancer was the result of a series of epidemiological studies. Patterson (1973) at the Central Veterinary Laboratory in Weybridge, England, where the aflatoxin story began in 1960, pointed to the interesting association between the rate at which a given species could metabolize a high dose of aflatoxin B_1 and the kind of liver lesion (acute and chronic) that was typical of aflatoxin in that species. Those species which metabolized one LD_{50} dose in less than 12 minutes ("fast") and those that metabolized the dose in a few hours ("medium") are absent from the top of the list in Table 5, that is the group highly sensitive to the carcinogenicity of aflatoxin B_1. This last group is characterized by a slow rate of B_1 metabolism at high doses, and the parent compound therefore persists longer in the liver. Placing man in the slow group is based on observations that children in the Philippines excrete aflatoxin M_1 in urine more than 24 hours after ingestion of B_1 (Campbell et al., 1970), and children in Thailand dying with Reye's syndrome have readily detectable amounts of B_1 in their livers and bile, even 2 to 3 days after not eating (Shank et al., 1971a).

As already mentioned, the oxidation of aflatoxin B_1 to M_1, P_1, Q_1, B_{2a}, or R_0 seems to have little relevance to the carcinogenic potential of the parent compound. Recently Garner, in the Millers' laboratory, provided evidence that, similar to the polycyclic aromatic hydrocarbons, the double bond in the terminal furan ring of aflatoxin B_1 is subject to epoxidation (first proposed by Schoental in 1970) (Garner et al., 1972; Garner, 1973). The support for the epoxide, aflatoxin B_1-2, 3-oxide, as the ultimate carcinogen, binding to nucleic acids, is given by Swenson et al. (1973, 1974). The epoxide (Figure 3) apparently forms a glycoside-like bond with nitrogen or oxygen or both in nucleoside residues, probably at the N-7 or O-6 positions of guanine. Acid hydrolysis of the nucleic acid-aflatoxin adduct yields the 2, 3-dihydro-2, 3-dihydroxyaflatoxin B_1; this diol has been found in nucleic acid hydrolysates of aflatoxin incubations containing rat, hamster, and human liver microsomes.

TABLE 5. COMPARATIVE TOXICITY, CARCINOGENICITY AND METABOLISM OF AFLATOXIN B_1

Species	(mg/kg BW) LD_{50}	Tumors	Metabolic Rate	Recognized Metabolites M_1	P_1	Q_1	B_{2a}	R_0	Di-OH
Trout	0.81	++	?	?	?	?	?	?	?
Rat	5.5-17.9	++	Slow	+	+	+	?	-	+
Monkey	2.2-7.8	+	Slow	+	+	+	-	-	?
Man	?	+*	Slow	+	+	+	-	-	+
Duckling	0.34-0.56	+	Fast	+	-	-	+	+	?
Mouse	Ca. 9.0	+	Med.	+	+	+	+	-	?
Sheep	2.0	(+)	Med.	+	-	?	-	-	?
G. Pig	1.4-2.0	(+)	Fast	+	?	?	+	-	?
Pig	0.62	?	Med.	+	-	?	-	-	?
Chick	6.5-16.5	?	Med.	+	-	?	+	+	?
Hamster	10.2	?	?	?	?	?	?	?	+
Rabbit	0.3-0.5	?	Fast	+	+	?	+	+	?

* epidemiologic association
? = not determined
- = none detected
Summarized from reports and reviews by Newberne and Butler (1969), Campbell et al. (1970), Shank et al. (1971b, 72a, 72b), Patterson (1973), Swenson et al. (1973, 1974), Krieger et al. (1975), and Roebuck (1975).



From: Swenson et al., B.B.R.C. 53. 1260 (1973)

Figure 3. Source of 2,3-dihydro-2,3-dihydroxyaflatoxin B_1 in aflatoxin metabolism studies.

The structure-activity relationship studies on the aflatoxins have indicated that all modifications of B_1 (except the epoxide) reduce acute toxicity and carcinogenicity; the furofuran moiety and the double bond in the 2,3-position are essential for carcinogenicity (Wogan et al., 1971). In support of the epoxide being the ultimate carcinogen is the fact that the dihydro-form of aflatoxin B_1, aflatoxin B_2, because it lacks the olefinic 2,3-double bond, cannot be epoxidized and therefore should not be carcinogenic; indeed, aflatoxin B_2 is at least 150 times less potent a carcinogen in the rat compared to B_1 (Wogan et al., 1971). In addition, aflatoxin B_2 is about four times less acutely toxic than B_1 in the duckling (Carnaghan et al., 1963).

It has recently been shown by Dr. Bill Roebuck and Mr. Wayne Siegel in Dr. Wogan's laboratory at MIT (Roebuck, 1975) that duckling liver can oxidize up to 8% B_2 to B_1; this pathway could not be demonstrated in rat and human liver in Roebuck and Siegel's in vitro system. What needs to be tested now is, is B_2 carcinogenic to the duck to the extent that B_2 is oxidized to the 2,3-epoxide via a B_1 intermediate? More comparative studies are needed to see which species do and do not metabolize aflatoxin B_1 to the epoxide.

And now, the ultimate question: is man like the rat (B_1 epoxidizer), sensitive to B_1 carcinogenicity, or the mouse (untested for epoxidation), more resistant to B_1 tumor induction? The animal data are sufficiently strong to justify epidemiological studies to determine whether aflatoxin is a human carcinogen. Such studies have been made.

Field association studies for aflatoxins and human liver cancer have been carried out in Uganda (Alpert et al., 1971), in the Philippines (Campbell and Salamat, 1971), Swaziland (Keen and Martin, 1971), Kenya (Peers and Linsell, 1973), Thailand (Swank et al., 1972a-e) and Mozambique (Van Rensburg et al., 1974). In the studies done in Kenya, Thailand, and Mozambique, the actual consumption of aflatoxins was measured in carefully defined populations and correlated with the incidence of primary liver cancer in those same populations. These studies are the subject of a recent review on the epidemilogy of aflatoxin carcinogenesis (Shank, 1975).

Each individual study in these three countries offers strong support in relating aflatoxin consumption to human liver cancer. If the data from the three studies are pooled, one can obtain regression lines, separately for males and females, with high correlation coefficients (Figure 4). The regression line for males is $y = 18.08 \log x - 13.51$ with a correlation coefficient of 0.90 for four degrees of freedom ($0.02 > P > 0.01$), and for females, $y = 8.61 x - 6.73$ with a correlation coefficient of 0.87 for two degrees of freedom ($0.05 > P > 0.02$). These approximations are valid only for the data used to derive them and should not be used to extrapolate to the y-intercept to determine the level of aflatoxin consumption that would not produce tumors, for there is no guarantee that the lines remain linear for lower, or higher, exposures. Also, the distinction must always be made between an incidence of "zero" and an incidence that is less than one per given population size. This may be the first clear case where an environmental compound has been recognized as a carcinogen, first in animal studies and then followed by a deliberate effort to measure the human risk, and comparative metabolism studies have played a significant role in this association.

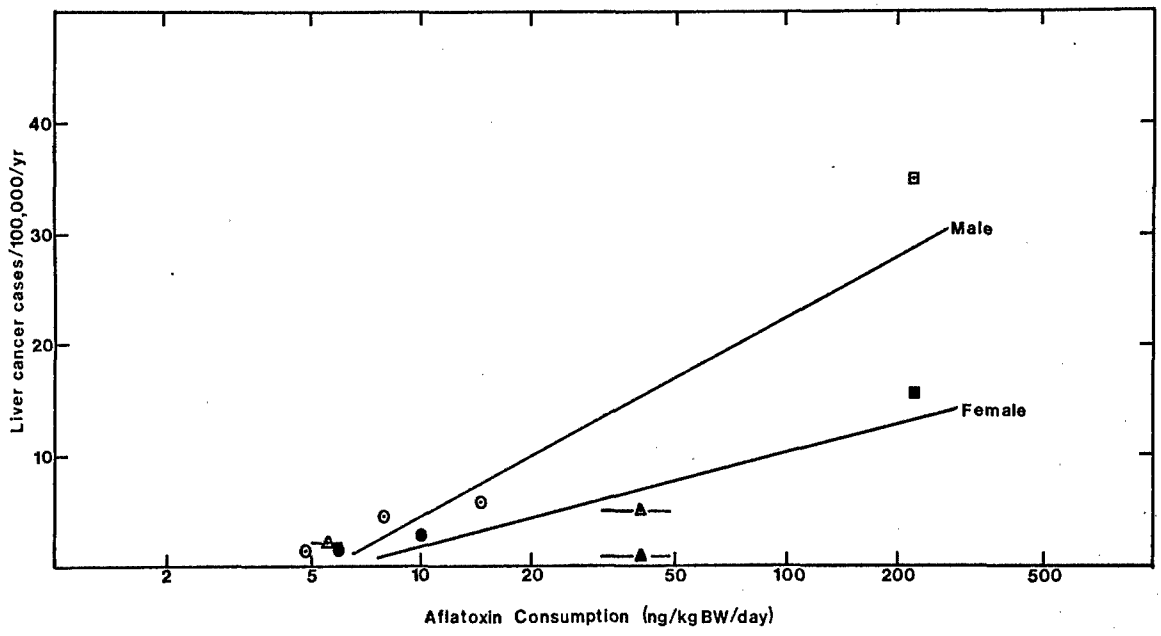


Figure 4. Regression lines relating aflatoxin consumption and human liver cancer in three countries.

What prompted these three large-scale, expensive, international studies on the threat of one compound causing an uncommon disease? First, by 1965 it was recognized that there was human exposure to this most powerful carcinogen. Second, there were also at that time readily definable "cancer gardens" where unusually large numbers of human liver cancers were seen in social structures in which epidemiological studies could be conducted. Third, the six animal species examined in carcinogenicity tests by that time had responded with liver tumors. And, fourth, it was the view at that time, at least among those who pursued the field studies, that aflatoxin B_1 required metabolic activation to the ultimate carcinogen, and there was no reason to believe from the animal data that the human would not be an "activator" similar to other species so tested. Comparative metabolism studies were helpful in deciding on the seriousness of the aflatoxin risk to human health.

Hopefully, in the future, more laboratory studies on carcinogenicity will go beyond the two-year feeding tests and include comparative metabolism studies to complement the exposure results.

Returning to the question, "is man as sensitive to the carcinogenic potential of the aflatoxin B_1 as the rat or as resistant as the mouse?" let's compare doses of aflatoxin that cause tumors in rat and doses that apparently cause tumors in man. Wogan et al. (1974) in dose-response studies fed rats purified diets containing as little as 1 μg aflatoxin B_1/kg diet and observed 10% incidence of liver carcinomas, and one-tenth this level produces liver tumors in trout (Halver, 1969). Expressed in the same terms as the values for human aflatoxin consumption, the young adult rats eating 60 ng/kg body weight/day develop a tumor incidence of 10,000/100,000 over their lifespan; this compares to a human consumption of approximately 50 ng/kg body weight/day associated with a tumor incidence of 6/100,000 people per year, not per lifespan. Man, then, is probably not as sensitive to aflatoxin carcinogenicity as the rat, but sensitive enough to still develop tumors. In Swenson's studies on rat and human liver in aflatoxin oxidation, the rat appears to form the 2,3-oxide about 5 times more effectively than the human (Swenson et al., 1973).

The Toxic Hazards Research Unit of the Aerospace Medical Research Laboratories at Wright-Patterson Air Force Base is currently conducting studies to determine quantitatively the carcinogenicity of hydrazine, monomethylhydrazine, and unsymmetrical dimethylhydrazine under conditions

appropriate to practical human exposure. These inhalation carcinogenesis studies are investigating the sensitivity of several species, recognizing marked interspecies differences in response to these compounds.

These carcinogenicity studies are being supported by investigations on comparative metabolism relevant to the compounds' carcinogenic potential. The results of the comparative metabolic studies will be correlated with the results of the comparative carcinogenicity studies currently in progress here at the Toxic Hazards Research Unit. It is hoped that this approach will be helpful in improving the toxicologist's record in predicting human carcinogenic risk of environmental compounds.

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OPEN FORUM

MR. WANDS (National Academy of Sciences): I'd like to address this question to Dr. Pasi. How do you avoid infections in the bones as you do this septic technique of drawing the bone marrow samples?

DR. PASI (University of Zurich): In comparison to the lymphocyte culture technique in which lymphocytes are cultured for 3 days, the incubation period for bone marrow cells is much shorter, one hour and a half. We have found that it is not necessary to add an antibiotic to the culture medium. We didn't have any problems.

MR. WANDS: I'm amazed that sampling through all that dirty fur and skin directly into the bone marrow you don't get an abscess or infection in the bone.

DR. PASI: The rat is not affected either locally or systemically as far as we can ascertain. It is well known that rats are unusually resistant to infections. It would be interesting obviously to find out what the reasons are for this unusual resistance but this was not in the scope of the study.

DR. HILADO (University of San Francisco): I'd like to direct this question to Dr. Shank. In your talk, you mentioned that there were studies done in several countries. What happened to the data from the Philippines?

DR. SHANK (University of California, Irvine): The Philippine data has been published. That study was done by Engle, Campbell and Saloma from Virginia Polytechnic Institute. The major body of data was published in a book called Mycotoxicosis and Human Liver Cancer which was the proceedings of a symposium held in 1970 in Pretoria, South Africa. Most of the data are published in that book.

DR. HILADO: Did the data correlate with yours?

DR. SHANK: I did not put their data in my table because they did not measure consumption of aflatoxin but they measured aflatoxin in food purchased from farms, markets and schools. They didn't actually measure

how much aflatoxin was being consumed, so there was no way of comparing it with the other studies. Also they did not, themselves, measure cancer incidence in any specific population but took uncorrected cancer registry data. Their study and the one conducted in Swaziland did not produce the kind of data that could easily be equated. We don't know how much aflatoxin was being consumed in those countries.

DR. HILADO: Is the liver tumor which you were reporting on the same type which was produced by vinyl chloride?

DR. SHANK: No, these are strictly hepatocellular carcinomas.

MR. HAUN (University of California, Irvine): I read with interest Bill Dykeman's Cummings Memorial Lecture in which he spoke about aldrin and dieldrin cases and he said, "The fact was given too little consideration that effects in mice, in general, are not without reservation predictive of other mammalian species including other rodents as well as man." Dr. Rowe, would you like to comment or give impressions on that remark?

DR. ROWE (Dow Chemical Company): I guess I can only say I wish I knew. As you well know, mice are extremely susceptible to many things. I think some of the answers may come when we obtain comparative metabolic studies to demonstrate differences between the metabolic capabilities of the mouse and other species.

DR. KOCIBA (Dow Chemical Company): You referred specifically to the hepatic tumors that were induced with dieldrin in the mouse. This again gets into the problem area of definition of hyperplastic nodules versus hepatomas versus hepatocellular carcinomas. In a rodent, especially the mouse, it is very difficult to get complete agreement as to where you draw the line between hyperplastic response and a neoplastic response in a mouse liver that has been metabolizing increased amounts of the compound. There's no doubt the rodent liver has tremendous regenerative capacities, as a matter of fact some people claim you can remove 80% of the rodent liver and it will rapidly regenerate to its original condition and size. Therefore, when you are dealing with a compound that has an effect on the liver, you run a definite risk of inducing hyperplasia and probably other changes in the liver which are going to be called neoplastic by some. There are discussions going on among pathologists right now as to what are good definitive criteria that can be used

uniformly by all research laboratories that are operating in this area so that we can get some consistency regarding classification and interpretation of the mouse liver changes.

DR. HAYS (U. S. Department of Agriculture): I'd like just to comment briefly on Dr. Shank's discussion of aflatoxin to say that there's another approach to this problem of what to do with agricultural commodities that have been contaminated with aflatoxin. In the USDA laboratory in New Orleans and also in the laboratory in Peoria, Illinois, a significant break through has been made in being able to detoxify aflatoxin in highly contaminated agricultural commodities. Treatment with gaseous ammonia can reduce aflatoxin levels as high at 1000 ppb down to 1 or 2 ppb. We think that is a very significant break through. I'd like to ask Mr. Wands, in your reference to the number of species, were you saying that it should be carcinogenic in three species or that you use three species in your test?

MR. WANDS: If a material has been found to produce tumors in three different species, one of which is not a rodent, then the TLV committee would classify it as one of high carcinogenic potency.

DR. JACOBSON (National Institute for Occupational Safety and Health): I'd like to address my remarks to Dr. Rowe and make five points. One is the question of whether or not there is a safe dose of a carcinogen, primarily in relation to the Delaney Amendment. Toxicologists for many years get together at their various meetings and agree that there is a safe dose. I wish a good bit of the energy spent in agreeing that there is a safe dose were spent on finding out how to determine a safe dose. I believe the principles here are very deficient. One possibility, of course, Dr. Rowe has alluded to in that instead of talking about a completely safe dose, talk about a dose that has such a long latency period that it is, for all practical purposes, safe. The analysis of this approach by Jones in a recent issue of Food and Cosmetics Toxicology has cast a little doubt as to whether this will be effective, at least with urethane. Dr. Rowe, you also made a criticism of the judgement of carcinogenicity based on results reporting at a very high dose level. It seems to me that the use of a high dose level is primarily an attempt to compensate for the inability to use a sufficient number of animals. In the data that Dr. Shank presented on aflatoxin, and I would reiterate that aflatoxin is one of the most potent, if not the most potent, experimental carcinogen known, and yet at very low doses in humans, it caused cancer incidence of

several per 100,000. Considering statistical problems, it would probably require several hundred thousand animals to reproduce such an incidence in animals. And this is clearly not feasible. Dr. Kociba has shown with dioxane that the use of a high dose is probably misleading. What is the alternative? I don't think we're now ready to use several thousand animals at each dose level as an alternative. My fourth point, Dr. Rowe, is that you referred to NIOSH as labelling about 1400 materials as carcinogenic. I believe you've misinterpreted what NIOSH has said. What NIOSH said was that there is evidence in each case that the 1400 chemicals on the list are carcinogenic. NIOSH did not say this evidence is good. The evidence was compiled without regard to validity, with a request for information that would help in the evaluation of the carcinogenicity of each one of these materials. The last point I'd like to make concerns your remarks about regulatory activity. I don't know whether the degree of regulatory activity by government is too little or too much. I would certainly challenge any suggestion that there should be no such regulatory activity. I'm citing the fact that several work sites have used carcinogens under unhygienic conditions with apparently full knowledge of the risks that they were posing to their workers. I believe that they had full knowledge. Yet, they nevertheless proceeded with these unhygienic conditions. The consequences are fairly evident. There is a high incidence of cancer in those plants.

DR. ROWE: I'll start with the last one first, if I may, Dr. Jacobson. I have no qualms about the fact that we have to have regulation. My plea is only that we don't go overboard and that we use a little common sense in coming to decisions. I'm sure that we can never be absolutely certain that we have a noncarcinogenic dosage. I don't know how we can achieve that certainty. But we have to be a little bit cognizant of the practicalities of working with these things in a time frame. When you look at the ROI (return on investment) of trying to achieve zero, you plot the achievement against what you have and the cost, and you find that you can achieve a big increment of that goal for a relatively small cost, both in terms of money and human effort. On the other hand, when you move further down that curve, you find that your costs for each increment of improvement are going to be extremely high. My plea is, let's not say we have to reach zero exposure in every situation. Let's be temperate in how we behave in that respect. In considering your remark about animal numbers, it is a statement that has been made many times over many years. You have to use a lot of animals or you use high doses because you can't use the tens of thousands of animals that the statisticians tell you are

necessary. I don't mind the use of high doses if you are certain that you're not creating an entirely artificial situation. If you change your metabolic pathways or you force materials into different body compartments and you change metabolic products, then I don't think you're really testing the material you set out to test and to prevent exposure to man to those kinds of dosages. So I think, again, there's a strong reason why we need to go into more detailed study of perhaps a smaller number of animals dealing primarily in the field of pharmacokinetics and metabolic products as they are dose dependent. Does that answer all your questions?

DR. JACOBSON: It does not respond to the question of how to set a safe dose, but the question may well have been rhetorical. I don't know the answer to it.

DR. BACK (Aerospace Medical Research Laboratory): I will try answering Dr. Jacobson's question about how you set a safe dose for a carcinogen. I think you do it the same way you do for an occupational exposure that causes any other kind of a toxic response. There are dose response curves for most toxic effects and we have to realize that there are good dose response curves. If you can produce a dose effect curve for a compound which is shown to be an oncogenic agent, you can make a good estimate of what would be a safe level. If you can't get a good dose response curve, you certainly can't do that. But I think we're finding more and more that most carcinogenic agents can be shown to be dose dependent. If ACGIH and official agencies can set a limit for a hepatotoxin, they certainly ought to be able to set one for a carcinogen. I don't think this is voodoo. If it is, then why are we doing research at all? Mr. Wands, what is the TLV committee calling a carcinogen or a tumorigen in the scheme for potency classification? Any tumor? Are you defining tumor as a lump or as a carcinogen meaning as a compound which causes a metastatic lesion or one that can be metastatic? Are you just talking about papillomas and adenomas or are you talking about compounds which cause true carcinomas in three species?

MR. WANDS: We are talking the latter.

DR. BACK: What if a compound causes adenomas or papillomas in three species?

MR. WANDS: We would not consider that evidence for carcinogenicity. We mean frank cancers. I would include in that definition leukemias and sarcomas as well. That brings me to a question I would like to ask Dr. Kociba.

Is there agreement amongst all pathologists that there are such things as benign tumors, or are benign tumors considered to be merely precursors of truly cancerous lesions as some of the legal types in Washington would like to have us believe?

DR. KOCIBA: Well, I'm sure you could receive several different responses to that question depending on who you asked. The use of the terms benign, malignant, carcinoma, sarcoma, are abused quite a bit. Clinically speaking, there are some benign tumors that histologically have a cellular composition that would indicate that they probably are not going to really cause any impairment of the ultimate health of the individual. There are other types of benign growths that, by token of their location, may cause an undesirable outcome. So you have to be careful as to whether you're talking about the clinical interpretation of benign and malignant or if you're talking about the morphologic diagnosis. As we use this terminology in conducting a carcinogenic bioassay, we mean the morphologic categories of benign and malignant. I believe there are benign tumors that will stay benign for an indefinite period of time which in some cases will exceed the remaining life span of the animal. There are some benign lesions that have the capability to expand into an advanced morphologic state which could be interpreted by some as malignant.

MR. WANDS: Is hyperplasia a precursor? You spoke specifically of that this afternoon.

DR. KOCIBA: There's no doubt hyperplasia can be one of the accompanying lesions. If you have tissue response to a continuing irritant, you can see hyperplasia that precedes or accompanies neoplasia. You cannot unequivocally draw the conclusion that hyperplasia will precede the neoplasia if given enough time.

MR. WANDS: Are these benign lesions reversible at any place in time if the causative agent is removed?

DR. KOCIBA: There's no doubt that hyperplasia is reversible. Part of the definition of hyperplasia is that it is a reversible lesion. If you are asking the question as to whether a benign tumor is reversible or not, I don't think I would want to give an answer to that because I think each case would have to be evaluated on the basis of its own specific points. What was the

causative agent? What is the stage that it is at? What tissue are you talking about? Are you talking about a tissue of the body that has a relatively high turnover rate? Or are you talking about a tissue of the body that has a relatively low turnover rate? All these factors come into play when you are assessing the potential for reversibility. There are other pathologists in the audience and I solicit their comments on this subject.

DR. THABET (Automated Medical Services of Ohio, Inc.): Our usage in these terms in human pathology is the same as yours. Our point of takeoff, however, between benignity and malignancy is termed dysplasia, and we're using this in cytology. The dividing line between hyperplasia and dysplasia as a morphologic point is definitely reversible and not recognizable. Therein lies the problem with light microscopy. There are benign tumors in humans. There are also some so-called benign lesions that act in a malignant form in that they are life threatening to the animal or the human in which they occur. They are histologically benign but progressive and life threatening. We also use these terms for tumors in human pathology with the same degree of difficulty.

DR. CROCKER (University of California, Irvine): Mr. Wands, you said in classifying a chemical as a carcinogen that the demonstration of a carcinogenic effect in at least one species other than a rodent was required. What are the acceptable and convenient nonrodent species in which to do carcinogenic studies?

MR. WANDS: None of them are convenient. The TLV committee has not selected a specific nonrodent species but we would like to see data from something besides the conventional mouse and rat as a basis for classifying a chemical as a high potency carcinogen.

DR. HODGE (University of California, San Francisco): I'd like to suggest that we could make one major step forward if we could abandon the use of absolutes. Let me give you a couple of examples. Do you remember what Dr. Dixon said in his keynote address? He was talking about finding some change in the animal which then would be interpreted for the human. He said, "Positive changes apply to man unless proved otherwise." I submit that as an example of an absolute. Have you heard the statement,"No benefit justifies the risk of cancer." That's an absolute. Have you heard the statement that,"No additive shall be deemed to be safe." That's an absolute. As

long as we talk in absolutes, we never are going to reach a common ground for a decision as to what number we can accept as a risk. The job for the toxicologist and the clinician is to define the risk. The job of selecting an acceptable or negligible risk is a social political one, not one for the toxicologist and the clinician. Selecting a number or probability of cancer is not our job. The toxicologist can define the risk. That is not an absolute.

MR. WANDS: The concept of acceptable risk in setting exposure standards for carcinogens is being spoken of with a great deal more frequency, not only outside of regulatory agencies but also within them. Regulatory agencies are beginning to recognize the difficult problem that arises from the use of absolutes. This concept of acceptable risk has been developed in some detail at two workshops that were held over the past three years under the direction of Norton Nelson. The first workshop resulted in a NAS-NRC publication entitled "Principles for Evaluating Chemicals in the Environment." This one publication deals mostly with concepts of risk assessment and how to assess it. It does touch briefly on socio-political benefits but not very deeply and, as a result, another workshop was held in February of this year under the direction of Terry Davies. The proceedings have just come off the press and are a companion to the first book. The workshop dealt primarily with benefit assessment and the political and social issues of balancing the risks and the benefits.

DR. LEWIS (National Institute for Occupational Safety and Health): My question, I'm afraid, is philosophical and therefore will not have a finite answer. It relates to the problem that faces all of us who are involved in the protection of worker health. How far can society or government extend themselves to regulate and prevent health hazards? As Dr. Rowe pointed out, man is much more apt to expose himself to a voluntary risk, whether it be smoking or alcohol, than an industrial exposure. Can we confine his chemical environment to such a degree that we can exclude bronchiogenic carcinomas that may arise from cigarette smoking? And there's a basic philosophical difference between ACGIH and the NIOSH in terms of protection. NIOSH's legal mandate as I interpret it is to protect the health of everyone working. The TLV's established by ACGIH are predicated on the basis that we cannot protect everyone. Some people may be affected by chemicals at the TLV level and have to leave that working environment even though the industry is in legal compliance with the TLV or the Federal Air Standard. We even go further to say that we cannot pretend to think that we can protect each and

every worker from a health hazard when he is working. These are the types of problems that we have to grapple with when we establish TLV's and particularly when we establish them for carcinogens.

MR. WANDS: I think you've defined very well the dilemma that faces NIOSH and OSHA, more specifically, since they are the regulatory authority. That OSHA Act specifically states that every worker shall be protected, whereas the TLV committee attempts to set limits to protect the average worker, recognizing the high variability of the human species. When you have to protect every individual regardless of their personal sensitivity, some times the best way to do this is not to hire them or to transfer them to some other job where their exposure becomes zero.

DR. BACK: I have one question for Dr. Shank. Has anybody tried to correlate the metabolic path of any chemicals with the production of malignant tumors as opposed to nonmalignant tumors?

DR. SHANK: Most studies are focused on the production of malignant tumors or frank cancers. All of the data that I've presented here were on malignant tumors even if the slide only said tumor. In the case of liver tumors, it would mean hepatocellular carcinomas and angiosarcomas. I couldn't list all of the various cancer forms, but where I had a plus marked for a tumor, it was a frank cancer. Several research groups in Europe and the U.S. are trying to correlate the rate of alkylation of DMN and its persistence in these various species and organs.

DR. BACK: That answers one part of the question. You said that all the work has been done on compounds which cause malignancies in animals but none on nonmalignancies. I assume that if, as some people say, some benign tumors may well turn into malignant tumors later on, we ought to see some of the same things happening metabolically in benign tumor formation. Is that correct?

DR. SHANK: Yes.

DR. BACK: But nobody's done that.

MR. WANDS: I'd like to ask a question of Dr. Hays related to his announcement of USDA's discovery of a means of inactivating aflatoxins by treatment with ammonia. Is the action of ammonia related to its basicity? If so, could we bypass the problems of aflatoxin in corn by eating grits?

DR. HAYS: Yes. The ammoniation process opens the lactone ring and decarboxylates it so that it cannot reform. We believe we have a good process with which we can salvage a vast amount of our agricultural commodities to be used as animal feed. Probably it will never be possible to use the ammonia treated food for human consumption unless we change the Food, Drug and Cosmetics Act which would permit its alteration.

DR. BRUCE (Environmental Protection Agency): Dr. Shank, this afternoon in your presentation you talked about the methylation of RNA and DNA and the reaction with aflatoxin. You stated that some of these groups were labile to hydrolysis. Was this the reason you could not locate where methylation occurred on the RNA or DNA molecule?

DR. SHANK: The reason no one had found this particular epoxide metabolite after 10 years of study was that everyone who was doing the studies, myself included, extracted whatever biological preparation we had with a variety of organic solvents which precipitated the protein and nucleic acids. Therefore, we lost all of the metabolites that were bound to the nucleic acid. Garner and Swenson and the Miller's showed that if you took DNA itself and hydrolyzed it mildly with hydrochloric acid, you could break this aflatoxin oxide RNA or DNA bond and get the diol. With the new technique, there was no difficulty in locating what positions of RNA and DNA were methylated.

SESSION V

ENVIRONMENTAL RESEARCH

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AMRL-TR-75-125

PAPER NO. 23

THE PHYTOTOXICITY OF MISSILE EXHAUST PRODUCTS: SHORT TERM EXPOSURES OF PLANTS TO HCl, HF, AND Al_2O_3

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INTRODUCTION

Hydrogen Chloride Effects on Vegetation

Hydrogen chloride gas is considered to be a threat as an air pollutant only in isolated cases. Elevated levels of the gas and related vegetation injury have been detected in the vicinity of specific types of industry involving the use of chlorine and in areas where chlorine containing waste or fuels are burned.

Haselhoff and Lindau (1903) reported severe injury to vegetation in the vicinity of soda factories in England and Germany. They demonstrated a wide range of phytotoxic responses of various plant species to HCl gas. Seedlings of Viburnum and larch were killed after less than two days exposure to 5-20 ppm of HCl. On the other hand, HCl concentrations as high as 1,000 ppm for one hour were required to produce bleached lesions on the leaves of fir, beech, and oak; and 2,000 ppm HCl one hour daily for 80 days to cause necrosis on the margins of maple, birch and pear leaves. Lacasse (1968) observed HCl-type injury symptoms on vegetation within a half mile from a location on which incineration of polyvinyl chlorides took place. Shriner and Lacasse (1969) exposed 28-day old tomato plants to 5 ppm HCl gas for two hours. The test plants developed interveinal bronzing followed by necrosis within 72 hours after exposure.

The relative sensitivity of twelve tree species to HCl gas was studied by Means and Lacasse (1969). Coniferous and broadleaf seedlings, two to five years old, were exposed to concentrations of 3 to 43 ppm of HCl gas for 4 hours. The most sensitive of the broadleaf species was Liriodendron tulipfera, which showed visible injury at 3 ppm. Pinus strobus was the most sensitive of the coniferous species, showing visible injury at 8 ppm. Thuja occidentalis was not injured at 43 ppm. Lind and London (1971) exposed mature flowering marigold plants to high concentrations of HCl gas for five-minute periods. Groups of plants which were exposed to 95 ppm showed little or no visible damage. Temporary wilting and bleached leaf spots were the responses of plants exposed to 300 ppm. Exposure to 2071 ppm of HCl gas resulted in severe wilting, marginal and interveinal leaf necrosis, stem collapse, and death of plants. Masuch et al. (1973) reported slight necrosis and chlorosis on Spinacia oleracea L. leaves exposed to HCl concentration of 1.6 mg/m^3 for 208 hr in two weeks. The effects of HCl gas on chloride accumulation and yield of common food plants were studied by Huelsenberg (1974). Carrots exposed 45 days after germination showed a 32.2-49.7% decrease in yield after 29 hours exposure to 0.5 mg HCl/m^3. When exposure was started 96 days after germination, there was only a 5.3% decrease in yield. Winter rape showed only leaf discoloration; radishes no damage at all. Tomato, cucumber and bush bean plants showed leaf damage and an increase in the content of leaf chlorides. The reduction in yield was severe in tomatoes but slight in cucumber plants.

Hydrogen Fluoride Effects on Vegetation

Fluoride injury to vegetation commonly results from gradual accumulation of the pollutant in the leaf tissues. Since fluorides in concentration as low as 0.1 ppb are toxic to some plants (Thomas, 1961) most investigators exposed plants to low concentrations of HF over long periods of time. Thomas and Hendricks (1956) regarded plants which were injured by continuous exposure to 5 ppb or less fluoride for 7-9 days as sensitive. Relatively limited studies have been carried out on the effects on plants of high concentration-short term HF exposures. Daines et al. (1952) observed severe foliar necrosis on peach trees exposed to 12 ppm HF for 3 hours and on tomato plants with 1.5 ppm for 2 hours. According to Yamazoe (1962) rice and barley plants were killed by 2000 ppm HF, and yields were significantly reduced by 25 ppm for 1 hour. Six citrus varieties and six ornamental

species of economic importance were exposed by MacLean et al. (1968) to high concentrations of HF gas for short durations. Postfumigation observations showed that the phytotoxic responses differed markedly from those of low concentration-long duration exposures. Hydrogen fluoride exposures of 0.5 to 10 ppm for periods of from 0.5 to 8 hours induced tip, marginal, and intercostal chlorosis and necrosis, and abscission of leaves of most plants tested. The duration of exposure had a greater influence on abscission of young citrus leaves than did HF concentration. Severely injured leaves contained quantities of fluoride that, if accumulated over longer periods, would induce little or no visible damage.

Particulate Air Pollutants Effects on Vegetation

Unlike gaseous air pollutants, many of which are readily recognized as the sources of injury to various types of vegetation, particulate air pollutants and their effects on vegetation represent an area where relatively little is known and investigated. Bohne (1963) reported a marked reduction of growth in poplar trees located about one mile from a cement plant. Anderson (1914) observed in New York that cherry fruit set was reduced on the side of the tree nearest a cement plant. He demonstrated that the dust on the stigma prevented pollen germination. Reduction in CO_2 uptake was reported in bean leaves exposed to kiln dusts of particle size less than 10 micron at rates of 0.5-3.8 g/m^2-day for 2-3 days (Darley, 1966). The interference with the carbon dioxide exchange was significant only in the presence of free moisture on the leaf surface. Lerman and Darley (1972, 1975) demonstrated limited clogging of stomatal openings on bean leaves which were heavily dusted with dry cement dust. Adding moisture in the form of dew to the dusted leaves resulted in breakdown of the protective layer on the upper leaf surface. Particles containing fluoride appear to be much less injurious to vegetation than gaseous fluorides (Pack et al., 1959). The possible indirect effect of magnesium oxide on vegetation was reported by Sievers (1924). He noted poor growth in the vicinity of a magnesite processing plant in Washington. Berge (1966) dusted experimental plots with iron oxide at the rate of 0.15 mg/cm^2-day over 1-10 day intervals through the growing season for six years. No harmful effects of the dust were detected on cereal grains or turnips grown on these plots. Because of the dearth of experimental results, the tenor of many reports is directed to the question of whether particles in fact have deleterious effects on plants rather than discussions of the actual extent of plant injury.

The studies reported in this paper were designed primarily to determine the concentration of missile exhaust products (HCl, HF, and Al_2O_3) required to induce injury to eight species of ornamental plants when they were exposed for short periods.

MATERIALS AND METHODS

Plant Material

The eight plant species listed in Table 1 were selected for the study because of their wide geographical distribution, broad adaptability to different soils and climates and thus could serve as potential indicators of polluted air. Seeds of selected varieties from the eight species were obtained from a retail seed supply house and were planted in a peat moss-sand mixture. After germination, the young seedlings were transplanted into four-inch plastic pots containing UC-Type II soil mix (Matkin and Chandler, 1972). The plants were fertilized weekly with 50 cc of half strength Hoagland solution, starting the second week after transplanting. Day temperatures were regulated by evaporative coolers equipped with activated charcoal filters. Daily temperature maxima were usually between 26-32 C, but occasionally periods of extreme heat caused temperatures to exceed this range. Night temperatures ranged between 15 and 21 C.

TABLE 1. LIST OF PLANT SPECIES AND VARIETIES FOR THE PHYTOTOXICITY STUDIES.

Aster (Callistephus chinensis Early Bird White var.)
Calendula (Calendula officinalis Orange Gem var.)
Cornflower (Centaurea cyanus Jubilee Gem var.)
Cosmos (Cosmos bipinnatus Sensation Early Mammoth var.)
Marigold, American (Tagetes erecta Senator Dirksen var.)
Marigold, French (Tagetes patula Dwarf Double French Goldie var.)
Nasturtium (Tropaeolum majus Double Dwarf Golden Jewel var.)
Zinnia (Zinnia angustifolia Lilliput or Pompon White Gem var.)

Exposure Chambers

A plexiglas chamber measuring 0.75 m^2 by 1.0 m high was used to expose the plants to hydrogen chloride gas. The basic design of the air handling system was similar to that described by Heck et al. (1968). Activated charcoal filtered air entered the chamber through 1.5-inch diameter PVC pipe. A blower on the exhaust side maintained a negative pressure of 0.4 inch of water in the exposure chamber at an airflow of approximately 40 cfm (two changes of chamber air per minute). An additional plexiglas chamber was modified to accommodate the exposure of plants to both gaseous and particulate pollutants. The blower on the exhaust side was replaced with a modified high-volume air sampler. In order to be able to maintain a negative pressure of not greater than 0.5 inch of water in the chamber an additional high capacity blower was installed on the inlet pipe (Figures 1 and 2). An air flow of 40 cfm (two changes of chamber air every 1 minute) was maintained by adjusting the speed of the blower motors and monitored by measuring the pressure drop across a calibrated orifice in the inlet pipe. The concentration of carbon dioxide in the chamber was 360 ± 5 ppm as measured with a Beckman IR 215 infrared analyzer.

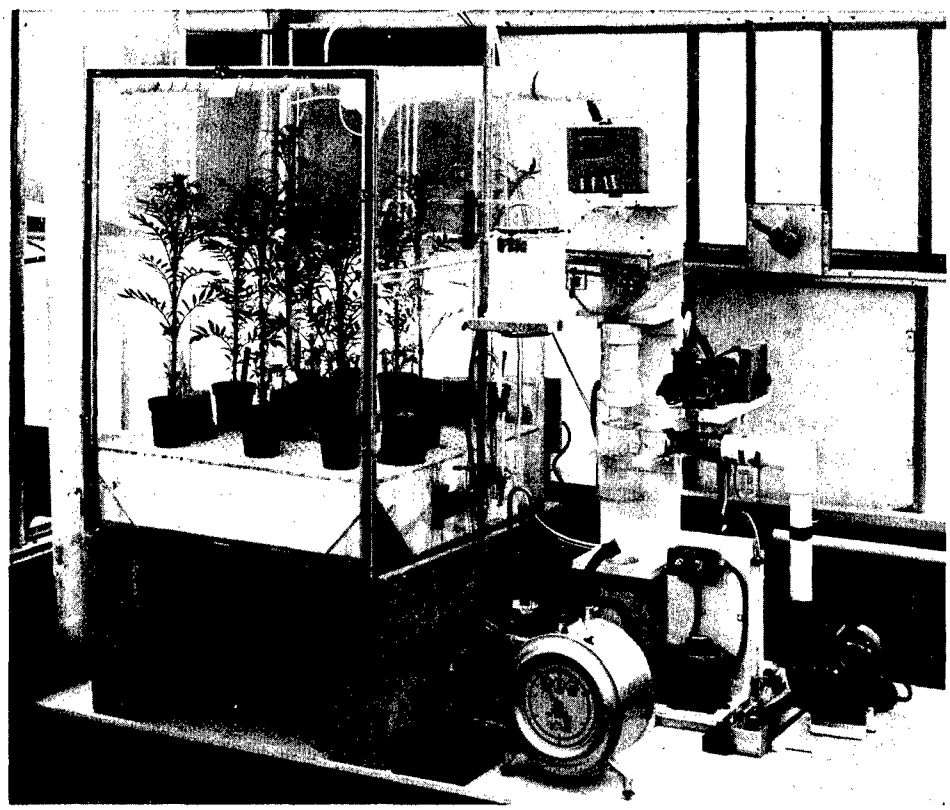

Figure 1. Plant exposure chamber for gas/aerosol studies.

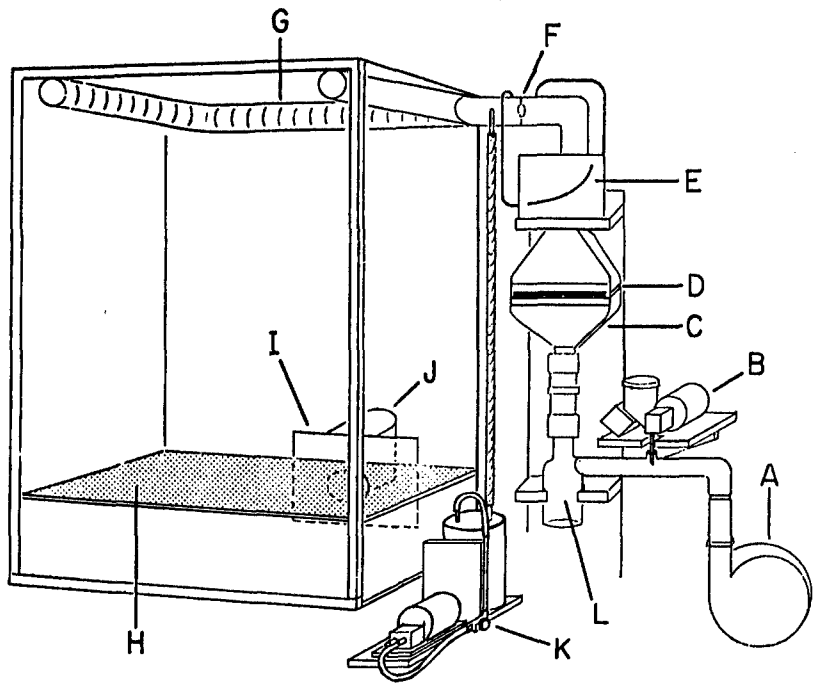

Figure 2. Plant exposure chamber for gas/aerosol studies. A. blower; B. vibrated hopper; C. impactor housing; D. slotted impactor; E. incline manometer; F. orifice meter; G. distribution tube; H. perforated base; I. 8 x 10" filter holder; J. high volume exhaust motor; K. HCl/HF gas generator; L. glass cyclone.

Equipment for Generating and Dispensing HCl and HF Gases

Hydrogen chloride and hydrogen fluoride gases have been generated by the method described by Hill et al. (1959) for generating HF gas. Air saturated with water vapor was bubbled through an aqueous solution of HCl or HF. The desired concentrations of the gases at the exhaust tube of the generator were obtained by controlling the air flow and the temperature of the HCl or HF solutions.

Equipment for Dispensing Aluminum Oxide Particles

The alumina particles were introduced at the inlet tube of the chamber by means of a feeder assembly which was adapted from a Bahco Microparticle Classifier. This vibrated-hopper, brush-fed system has the ability to meter dust at very low rates. The rate of alumina feed was controlled by adjusting the intensity of the vibration with a rheostat. To avoid clogging or aggregation of the alumina it was necessary to employ a flow of nitrogen through the appropriate port of the feeder assembly. The particles leaving the hopper were led by the air stream into a modified glass cyclone and then through a single stage slotted impactor which allowed only particles below a given size to pass.

Monitoring of HCl Gas

Periodic sampling of chamber air was used to monitor the concentrations of HCl. Volumes of chamber air ranging from ten to twenty liters were bubbled through a dilute solution of nitric acid. A wet test meter was placed in the line between the pump and the bubbler to record the total air sampled. Chloride in the solution was determined with an Aminco automatic chloride titrator. A Microcoulometric Titrator (Dohrmann Instruments) was used to study the rate of equilibration and removal of HCl in the plant exposure chambers.

Monitoring of HF Gas

A series of tests was conducted to evaluate two methods for collecting HF gas from the exposure chambers. In the first method a given amount of air containing HF gas was bubbled through TISAB (Total Ionic Strength Adjustment Buffer). In the second method chamber air was drawn through dry filters. The filters were prepared as described by Huygen (1963). Whatman no. 1 (Diam. 47 mm) filter papers were treated with a solution of 20% potassium hydroxide and 10% glycerol in water and then dried at 110 C. After air sampling, the fluoride was eluted from the filters with TISAB solution. The solutions were analyzed for fluoride with an Orion Model 94-09 fluoride ion electrode.

Physical and Chemical Properties of the Aluminum Oxide (Al_2O_3) Particles

According to preliminary studies by Jet Propulsion Laboratory, the majority of the alumina particles in the missile exhaust are of the gamma form with diameters of less than one micron. A sample of alumina with upper particle size limit of 1.4 micron was obtained from a commercial source (Research Organic Inorganic Chemical Corporation, Belleville, New Jersey). The sample was analyzed with X-rays for positive identification and for the assurance of its being relatively free of contamination. The X-ray diffraction patterns are presented in Figure 3.

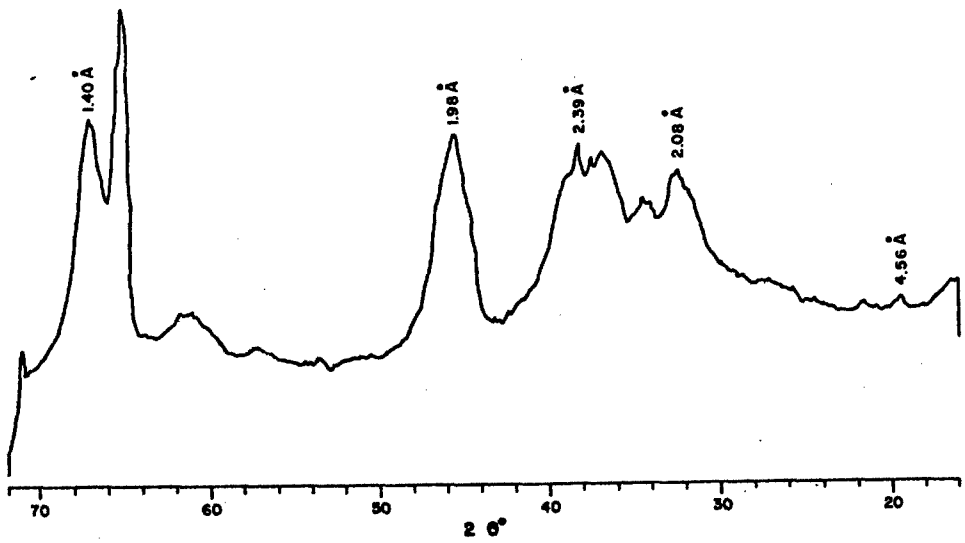

Figure 3. X-ray powder diffractogram of aluminum oxide (gamma form).

The particle size distribution of the alumina was determined in the exposure chamber by using a Bauch & Lomb 40-1 particle counter and Weathermeasure High Volume cascade impactor. The results are presented in Figure 4. The calibration of the particle counter was carried out using monodisperse polystyrene latex (PSL) aerosols, obtained by nebulizing aqueous suspensions of uniform latex spheres (Dow Chemical Co., Indianapolis, Indiana). The calibration curve giving the counter output (mv) as a function of the particle diameter is shown in Figure 5. The refractive index of PSL

is 1.6, whereas that of gamma aluminum oxide is 1.7. The collection efficiency curves and particle cut-off sizes for the cascade impactor were adjusted for the true density of aluminum oxide (3.5 g/cm^3). It should be pointed out that a scanning electron micrograph (Figure 6) of the alumina revealed particles with a sponge-like surface and many agglomerates which presumably means a smaller density value.

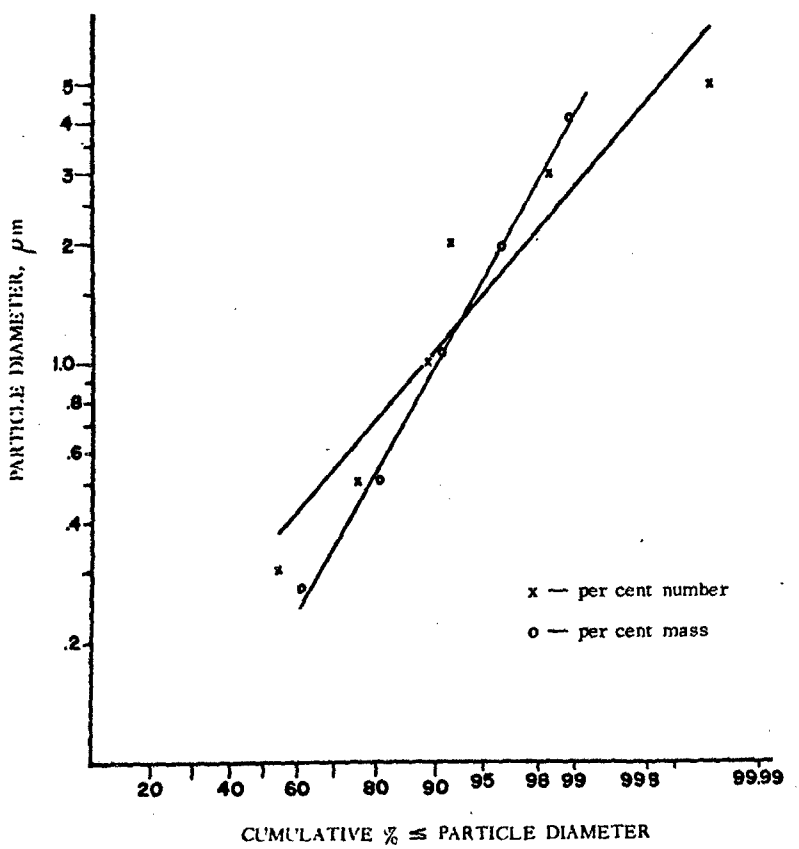

Figure 4. Particle size distribution of aluminum oxide determined with a hi-volume cascade impactor and optical particle counter.

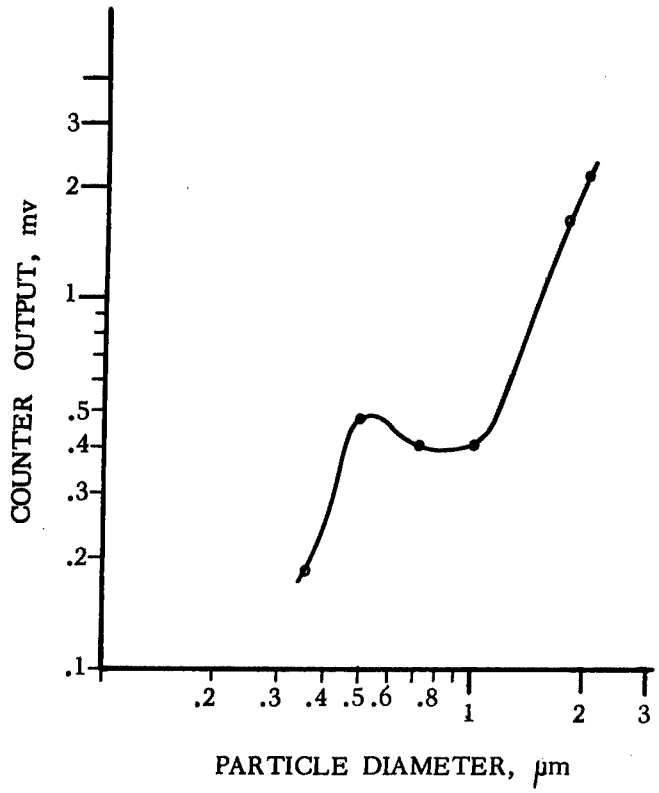

Figure 5. Response of the Bauch & Lomb 40-1 counter to PSL particles of 1.6 refractive index.

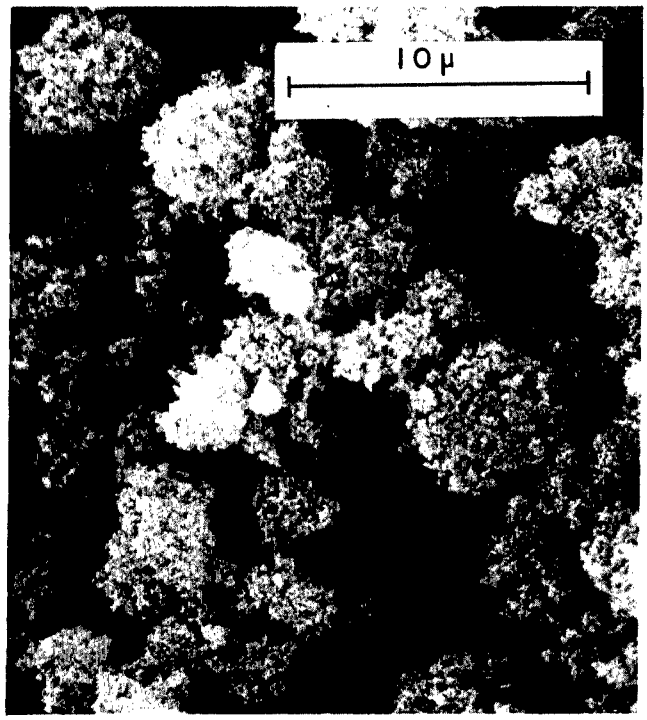

Figure 6. Scanning electron micrograph of impacted aluminum oxide particles (gamma form), X400.

Monitoring of Aluminum Oxide Particles

Particle concentrations in the chamber were determined by drawing chamber air through a 47 mm 0.2 μ pore size Nuclepore membrane filter. A wet test meter was placed in the line between the pump and the filter holder to record the total volume of air sampled. The membranes loaded with particles were desiccated for a few hours and then weighed.

Exposure Procedures

Groups of ten plants of several ages from each species were exposed to various concentrations of the pollutants for a period of twenty minutes each. An equal number of control plants was placed in an identical exposure chamber without the pollutants. The ranges of temperature and relative humidity in the chambers at the time of exposure were usually 24-35 C and 50 to 70%, respectively, but on a few occasions temperatures exceeded this range. The natural light intensities in the exposure chamber were as a general rule greater than $3.0X10^5$ ergs/cm^2-sec.

Evaluation of Phytotoxic Responses

At the end of the exposure period, the plants were returned to the greenhouse and were maintained there for 24 to 48 hours before injury was evaluated. The phytotoxic responses were classified according to the number of plants affected, number of injured leaves per plant, type of leaf (young, old) and surface affected.

The degree of injury on each plant was evaluated on an arbitrary one to ten scale. The evaluation was based on the number of injured leaves per plant, an estimated percentage of foliar surface affected, and the overall appearance of the plant. These arbitrary numerical values for each treatment were averaged and were expressed in percent as relative injury. Injury symptoms were described, and photographic records were kept.

RESULTS AND DISCUSSION

Evaluation of Exposure Facilities

Performance of the exposure chamber was measured under actual operating conditions. The principal concern was for an even distribution of the toxicant throughout the chamber and for the time period required to reach the desired toxicant concentration. A Microcoulometric Titrator was used to record the rate of equilibration after starting the HCl generator, and to measure the decay rate after stopping the dispensing system. As shown in Figure 7, curve A, in an empty chamber five minutes were required for the gas to reach an equilibrium at the twelve mg HCl/m^3 level, whereas seven minutes were required to reach a lower level when the chamber was loaded with ten 30-day old marigold plants (curve B). The output of the HCl generator with and without plants was the same. At lower concentrations of HCl gas the effect of the plants on the rate of equilibration and on the final level of HCl was more pronounced (curves C and D). Consequently, the sampling for HCl during the exposure of plants always began ten minutes after starting the HCl generator. The plant exposure period of twenty minutes included the time period required to reach the desired HCl concentration. The Microcoulometric Titrator was also used to verify the even distribution of the toxicant throughout the chamber. Variations in HCl concentration at plant level measured at ten different locations did not exceed five percent. The distribution of the gas was determined with several levels of HCl. Rate and uniformity of particle deposit in the chamber were determined by exposing glass slides at ten different locations on the chamber floor. Statistical analysis of mass distribution of the settled alumina particles indicated that there were no significant differences among the ten locations at the 0.05% level (Table 2).

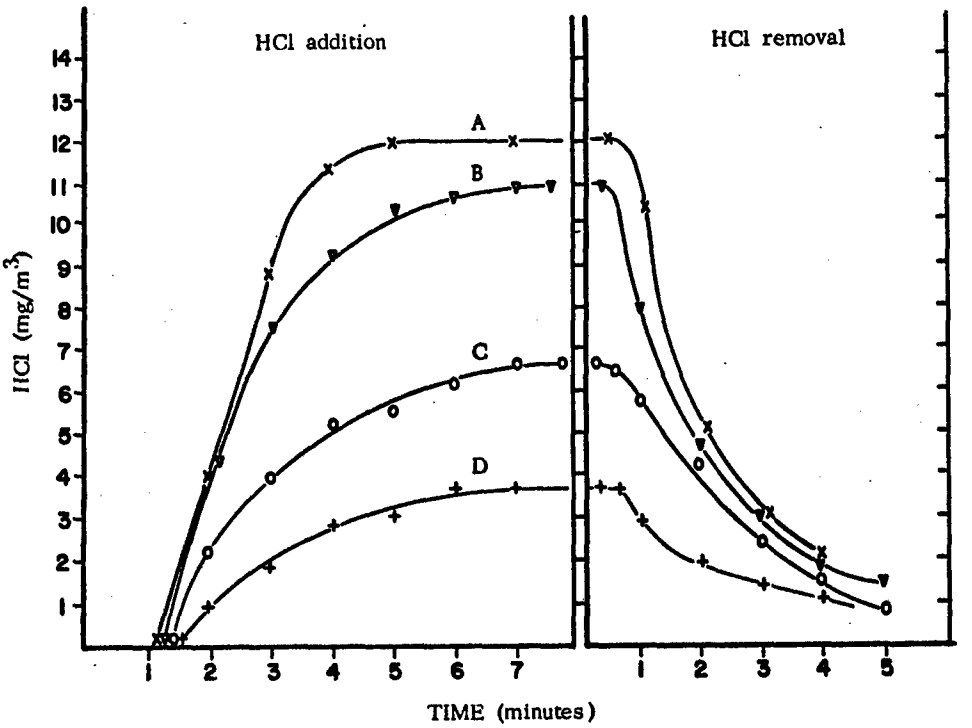

Figure 7. Rate of equilibration of HCl in plant exposure chamber. Input rates were equal in studies A-B and C-D. Thirty day old marigold plants were placed in the chamber in B and D.

TABLE 2. MASS DISTRIBUTION OF SETTLED Al_2O_3 PARTICLES IN PLANT EXPOSURE CHAMBER.

Observation Number	LOCATION 1	2	3	4	5	6	7	8	9	10
1	.14	.27	.22	.31	.20	.21	-	.30	.23	.18
2	.31	.26	.32	.35	.25	.38	-	.29	.45	.34
3	.36	.27	.30	.31	.28	.29	.39	.29	.43	.28
4	.27	.34	.30	.26	.23	.30	.32	.22	.25	.20
5	.21	.34	.28	.26	.26	.28	.33	.37	.42	.36
6	.31	.35	.23	.24	.27	.21	.28	.28	.34	.20
Means	.267	.305	.275	.288	.248	.278	.330	.292	.353	.260

All weights are in milligrams.

F = 2.08, not significant at .05 level.

Phytotoxic Responses to HCl

Injury symptom expression was similar on several of the species, but the kind and degree of injury frequently varied from species to species, and within the same species with different levels of HCl gas. A summary of injury symptom expression as a function of HCl concentration is presented in Table 3. Figures 8 and 9 show typical injury symptoms on nasturtium and marigold leaves. The percentage of plants affected by various concentrations of HCl varied with species and age (Figure 10). Each point represents a group of ten plants. Symbols indicate the percentage of plants affected by exposure to HCl. The multiple correlation between HCl concentration, plant injury, and plant age was determined for each species. Significant coefficient values were found for six out of eight species (Table 4). Correlations for Centaurea and American marigold were not significant. The correlations were rather complex; a straight line was not sufficient to characterize the relationship between HCl concentration, plant injury, and plant age. The regression equations (Table 4) are of the polynomial type; several of them are of the second degree or quadratic equations.

TABLE 3. EXPRESSION OF INJURY SYMPTOMS ON EIGHT PLANT SPECIES EXPOSED TO HCl GAS AT CONCENTRATIONS RANGING FROM 1-35 mg/m^3 FOR 20 MINUTES. PLANTS WERE EVALUATED 24 HOURS AFTER EXPOSURE.

HCl Concentration	Aster	Calendula	Centaurea	Cosmos
21-35 mg/m^3	Temporary wilting, extensive interveinal bronzing on lower leaf surface, necrosis of young tissue.	Temporary wilting, lower surface bronzing, discoloration, necrosis. The younger the leaf, the more distal the damage.	Extensive necrosis, rolling, speckling, temporary wilting, discoloration.	Extensive necrosis, extensive rolling, flower discoloration, tipburn of sepals.
10-20 mg/m^3	Interveinal bronzing on lower surface, trace of necrosis.	Bronzing of lower leaf surface, interveinal necrosis, marginal discoloration.	Discoloration along the leaf margins, rolling.	Tipburn, tip rolling.
1.5-9 mg/m^3	Trace of necrotic spots on young leaves.	Traces of lower surface bronzing.		Tipburn.

HCl Concentration	Marigold, Dwarf	Marigold, Sen. Dirksen	Nasturtium	Zinnia
21-35 mg/m^3	Severe necrosis of almost all leaves, rolling.	Severe necrosis, extensive rolling, tipburn of sepals on flowers.	Interveinal bleached lesions, on younger leaves in addition, marginal bleaching and rolling.	Bronzing on basal leaf portions, extensive necrosis and rolling on rest of leaf. Occasional petal necrotic spots.
10-20 mg/m^3	Discoloration, necrosis of mid-aged leaves, some rolling.	Interveinal discoloration of mid-aged leaves, some rolling.	Discoloration, necrotic speckling, rolling.	Speckling, interveinal bronzing.
1.5-9 mg/m^3	Traces of necrosis or discoloration.	Traces of necrosis or discoloration.	Traces of discoloration.	Trace of lower surface bronzing.

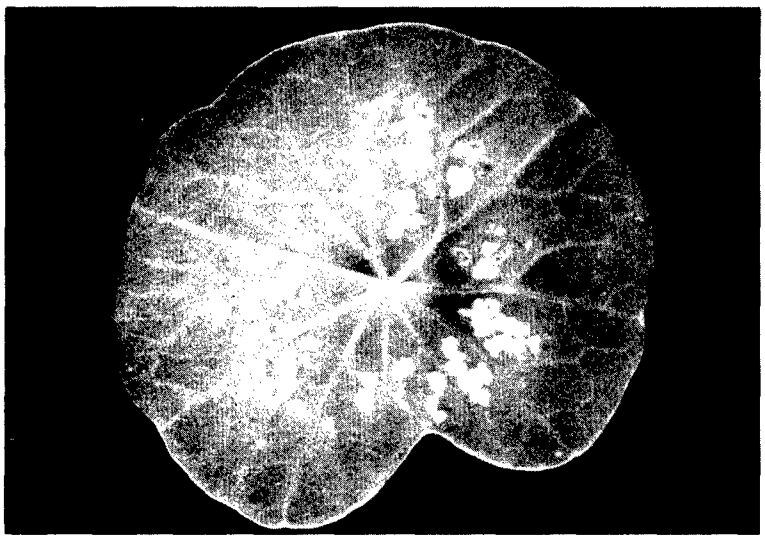

Figure 8. Hydrogen chloride injury on nasturtium leaf exposed to 19.5 mg/m^3 HCl for 20 minutes.

Figure 9. Hydrogen chloride injury on marigold leaves exposed to 20.2 mg/m^3 for 20 minutes.

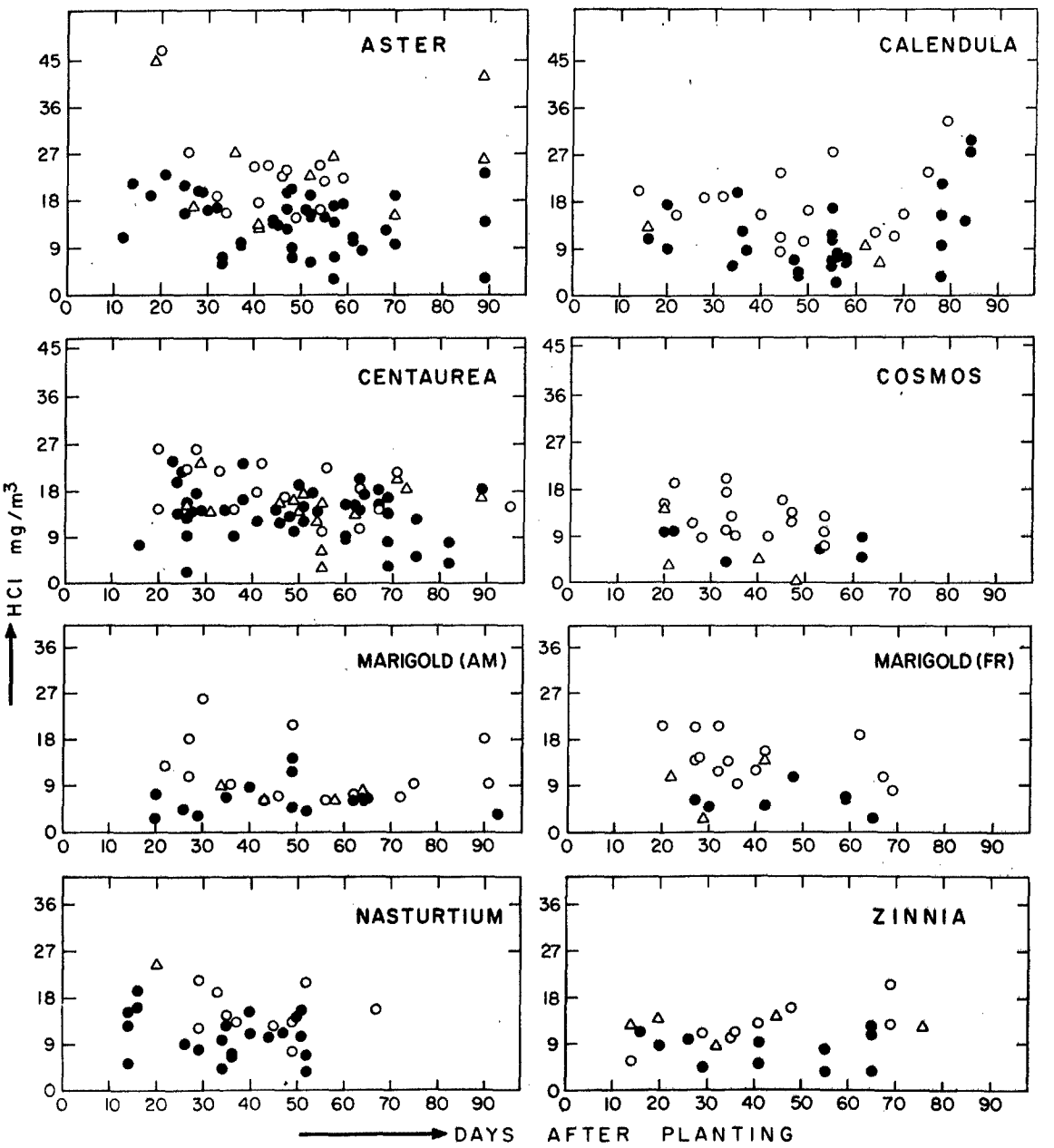

Figure 10. Phytotoxic responses of eight plant species expressed as percentage of affected plants to twenty minute exposures of various concentrations of HCl gas as a function of plant's age. Percentage of affected plants: ●, 0-20%, Δ, 21-50%; o, more than 50%.

TABLE 4. CORRELATION AND REGRESSION ANALYSIS FOR VALUES OF HCl CONCENTRATION (X_1), AGE OF PLANTS (X_2), AND PLANT INJURY (Y).

	Linear Regression For Two Variables Y and X_1		Regression for Three Variables Y, X_1, X_2	
Plant Species	Correlation Coefficient	Regression Equation	Correlation Coefficient	Regression Equation
Aster	0.50	$\hat{Y}=-4.97+0.50X_1$	0.54	$\hat{Y}=2.96+X_1^2(0.0027X_2-0.000026X_2^2)$
Calendula	0.56	$\hat{Y}=-6.79+1.04X_1$	0.61	$\hat{Y}=-127.90+X_1(9.01-0.092X_2)+X_2(3.64-0.026X_2)$
Centaurea	0.51	$\hat{Y}=-11.18+1.16X_1$	--	no significant function
Cosmos	0.52	$\hat{Y}=-6.00+2.46X_1$	0.80	$\hat{Y}=15.10+X_1(-52.77+3.22X_1+3.20X_2-0.039X_2^2)+X_1^2X_2(-0.18+0.0022X_2)$
Marigold (Amer.)	0.64	$\hat{Y}=-7.74+1.86X_1$	--	no significant function
Marigold (Fr.)	0.78	$\hat{Y}=-18.61+3.27X_1$	0.71	$\hat{Y}=64.07+X_2(-1.58+0.13X_1)$
Nasturtium	0.43	$\hat{Y}=-7.28+1.10X_1$	0.54	$\hat{Y}=4.22+0.0040X_1^2X_2$
Zinnia	0.64	$\hat{Y}=-5.20+1.00X_1$	0.59	$\hat{Y}=51.53+X_2(-1.46+0.11X_1)$

Correlation between HCl and relative percent plant injury regardless of plant age was also determined for each species (Figure 11 and Table 4). Each point in Figure 11 represents an average value for ten plants. The correlation coefficient varied according to plant species from a low of +0.43 to a high of +0.78. The slope values indicate the response of various plant species to a unit change in HCl concentration. Comparison of the relative sensitivity of eight species of ornamental plants is presented in Table 5. The linear regression equations in Table 4 were used to calculate the mg HCl/m^3 required to cause ten percent relative injury shown in Table 5. Cosmos and French marigolds appeared to be the most sensitive to HCl. Plant injury estimated at the ten percent level was caused by twenty minute exposure to 6.5 and 8.8 mg HCl/m^3, respectively. Asters were the most resistant to HCl gas; a concentration greater than 29.9 was required to cause ten percent injury.

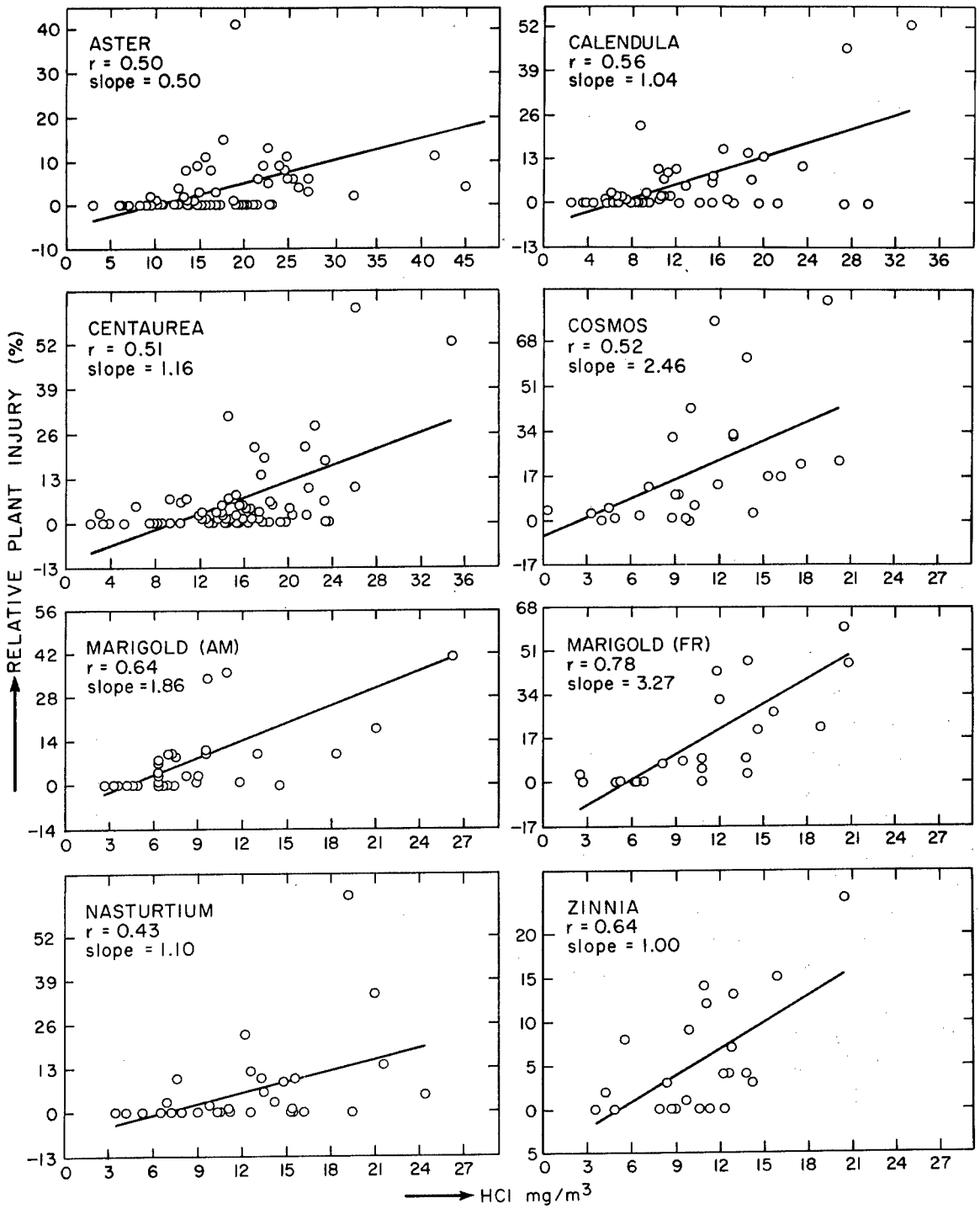

Figure 11. Phytotoxic responses of eight plant species expressed as percent injury to twenty minutes exposure of several concentrations of HCl gas.

TABLE 5. RELATIVE SENSITIVITY OF EIGHT SPECIES OF ORNAMENTAL PLANTS TO HCl GAS.

Species	Concentration of HCl Gas mg/m^3 in a 20 Minute Exposure Required to Cause Ten Percent Relative Injury*
Cosmos	6.5
Marigold (Fr.)	8.8
Marigold (Am.)	9.5
Zinnia	15.3
Nasturtium	15.7
Calendula	16.1
Centaurea	18.3
Aster	29.9

*As calculated from the linear regression equations in Table 5.

Since the ornamental plants used in this study exhibited a range of phytotoxic responses to HCl they could serve as indicators for phytotoxic levels of the gas in the vicinity of potential polluters. Further investigations are needed to evaluate the effects of HCl gas with variations of light, temperature, relative humidity, nutrients, and nutrient levels in the greenhouse and under field conditions.

Phytotoxic Responses to HF

The well established phytotoxic nature of HF gas was demonstrated in this set of experiments. Injury symptoms in the form of marginal and tip necrosis with a reddish-brown discoloration was typical but in many cases symptom expression was similar to that caused by HCl gas. A summary of injury symptom expression and incidence of symptoms induced on eight species of ornamental plants by ten minute exposure to various concentrations of HF gas are presented in Tables 6 and 7. Figures 12 and 13 show typical injury symptoms on marigold and cosmos plants.

TABLE 6. EXPRESSION OF INJURY SYMPTOMS ON EIGHT PLANT SPECIES EXPOSED TO HF GAS AT CONCENTRATIONS RANGING FROM 0.7-6.0 mg/m^3 FOR 10 MINUTES. PLANTS WERE EVALUATED 24 HOURS AFTER EXPOSURE.

HF Concentrations	Aster	Calendula	Centaurea	Cosmos
2-6 mg/m^3	Temporary wilting, necrosis, extensive interveinal bronzing on lower leaf surface.	Temporary wilting, marginal discoloration and necrosis with rolling, bronzing on lower leaf surfaces.	Temporary wilting, extensive necrosis and rolling.	Extensive necrosis and rolling.
1-2 mg/m^3	Bronzing on lower surface of young and old leaves.	Interveinal necrotic spots, marginal discoloration and bronzing of lower leaf surface.	Trace necrotic spots along leaf margins. Bronzing on lower surface of leaves.	Tipburn, tiprolling. Necrosis from tips back. Leaves affected droop and begin to roll.
0.7-1.0 mg/m^3	Trace necrotic spots and bronzing on lower surface of older leaves.	Trace necrotic spots on upper surface of younger leaves.	Trace necrotic spots on upper surface of leaves.	Tipburn, and a reddish-brown discoloration with rolling.
	Marigold, Dwarf	Marigold, Sen. Dirksen	Nasturtium	Zinnia
2-6 mg/m^3	Extensive necrosis along margins and tips of leaves with rolling of injured tissue.	Severe necrosis and discoloration along margins and tips of leaves, rolling.	Necrosis and discoloration of both surfaces of leaves, with rolling and temporary wilt at highest concentrations.	Necrosis, bronzing and rolling of leaf margins.
1-2 mg/m^3	Marginal and tip necrosis with a reddish-brown discoloration. On upper surface of youngest full grown leaves large, scattered intercostal lesions.	Interveinal discoloration and necrosis on upper leaf surface.	Interveinal bleached lesions on both surfaces and marginal necrosis and rolling. Some silvering in lesions on lower surface of leaf.	Necrotic spots on upper surface of leaves and extensive interveinal bronzing on lower surface of leaves.
0.7-1.0 mg/m^3	Trace necrotic spots and discoloration on upper surfaces of leaves.	Trace necrotic spots on both surfaces, discoloration on lower surfaces of leaves.		Necrotic spots on upper surfaces of older leaves. Trace bronzing on lower surfaces of leaves.

TABLE 7. INCIDENCE OF SYMPTOMS INDUCED ON EIGHT SPECIES OF ORNAMENTAL PLANTS BY TEN MINUTE EXPOSURE TO HYDROGEN FLUORIDE GAS.

	Hydrogen Fluoride Concentration, mg/m^3				
Species	0-.5	.6-1.0	1.1-1.5	1.6-2.0	>2.0
Aster	-	76%	20%	-	100%
Calendula	-	57%	70%	40%	100%
Centaurea	-	55%	48%	-	100%
Marigold (Fr.)	-	33%	-	-	55%
Marigold (Am.)	17%	48%	48%	100%	-
Nasturtium	-	60%	-	-	93%
Zinnia	-	55%	80%	-	80%
Cosmos	-	70%	95%	60%	-

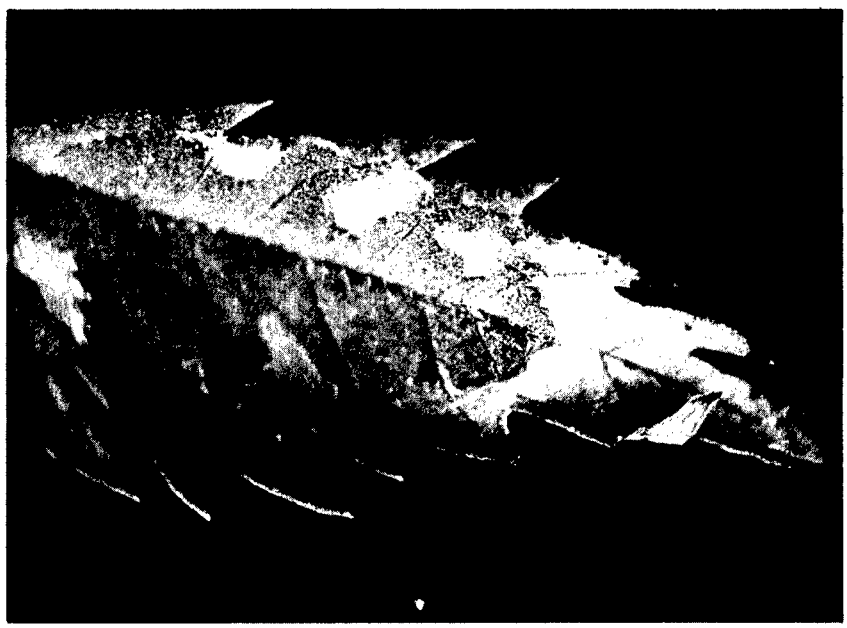

Figure 12. Hydrogen fluoride injury on marigold leaf exposed to 1.16 mg/m^3 HF for ten minutes.

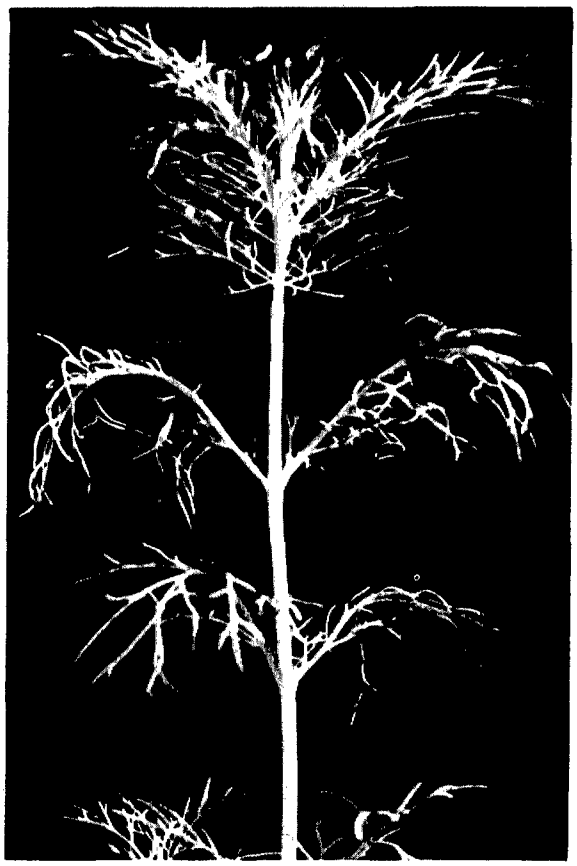

Figure 13. Hydrogen fluoride injury on cosmos plant exposed to 1.08 mg/m^3 HF for 10 minutes.

Phytotoxic responses of Ornamental Plants to Aluminum Oxide Particles, HCl Gas, and to Their Combination

Nasturtium and marigold plants were grown in a greenhouse. Groups of ten plants of various age levels from each species were exposed to several concentrations of the pollutants for a period of twenty minutes each.

Four groups of plants were studied at each concentration. Each group was exposed once to one of the pollutants or to their combination. A fourth group served as a control. The ratio of alumina to HCl in the combination treatments was 1:1 on a weight per volume basis.

Twenty-four hours after exposure the plants were evaluated and injury symptoms were recorded. Preliminary observations indicated no visible injury even on those plants which were exposed to relatively high (75 mg/m^3) concentrations of alumina. The magnitude of injury was higher in those plants which were exposed to the combination of alumina and HCl compared to the degree of plant injury caused by HCl treatments at the same concentrations. But the addition of alumina to HCl did not lower the threshold levels of HCl required to initiate damage symptoms. The synergistic effects of alumina to the phytotoxicity of HCl gas are shown in Figures 14 and 15. The phytotoxic responses were evaluated as percent leaves affected or as relative plant injury. Values from either method were positively correlated with HCl or HCl-Al_2O_3 concentrations. Figures 16 and 17 show alumina and polystyrene latex (PSL) particles on the surface of marigold and nasturtium leaves as were observed with scanning electron microscope.

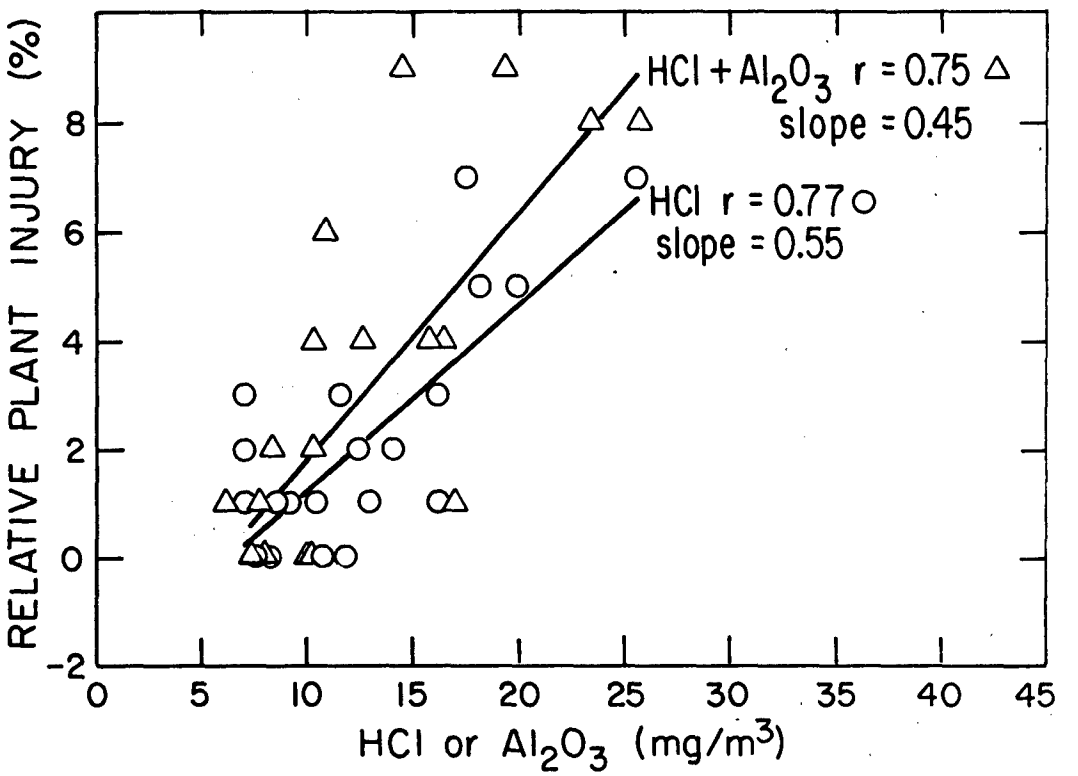

Figure 14. Relative plant injury induced by HCl gas and HCl-Al_2O_3 gas-particulate combination on American marigolds.

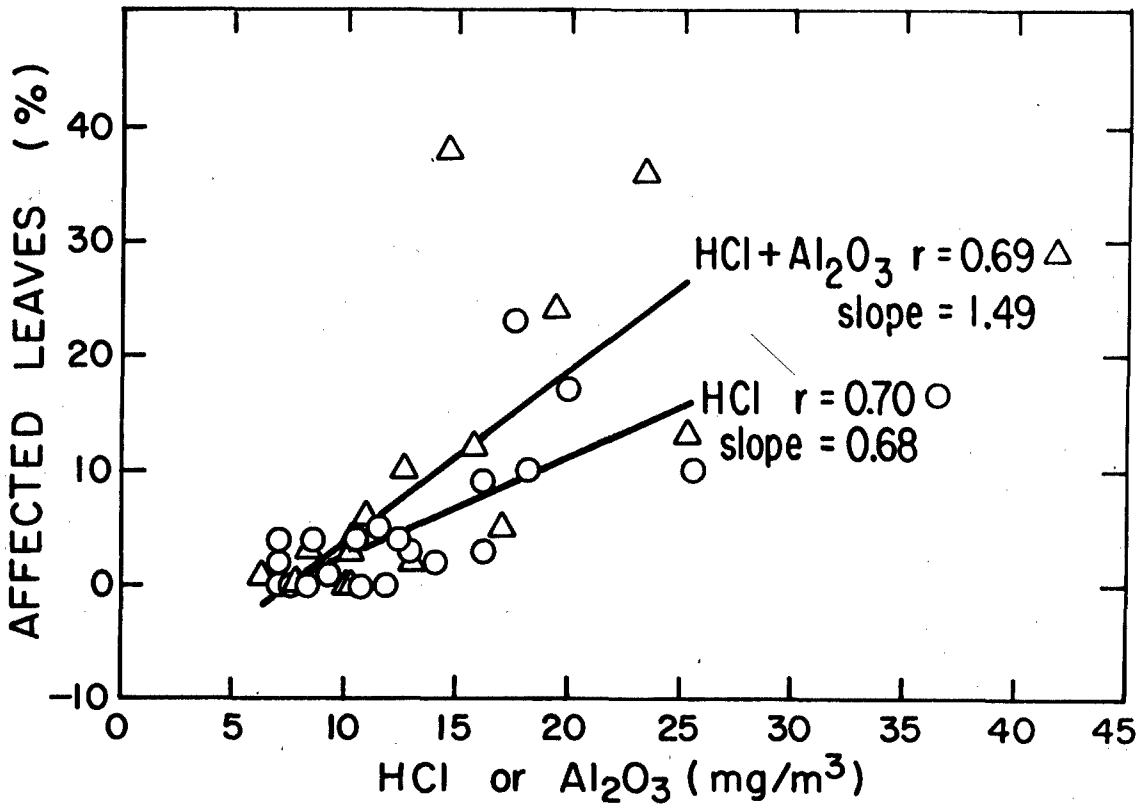

Figure 15. Incidence of leaf injury induced by HCl gas and HCl-Al_2O_3 gas-particulate combination on American marigolds.

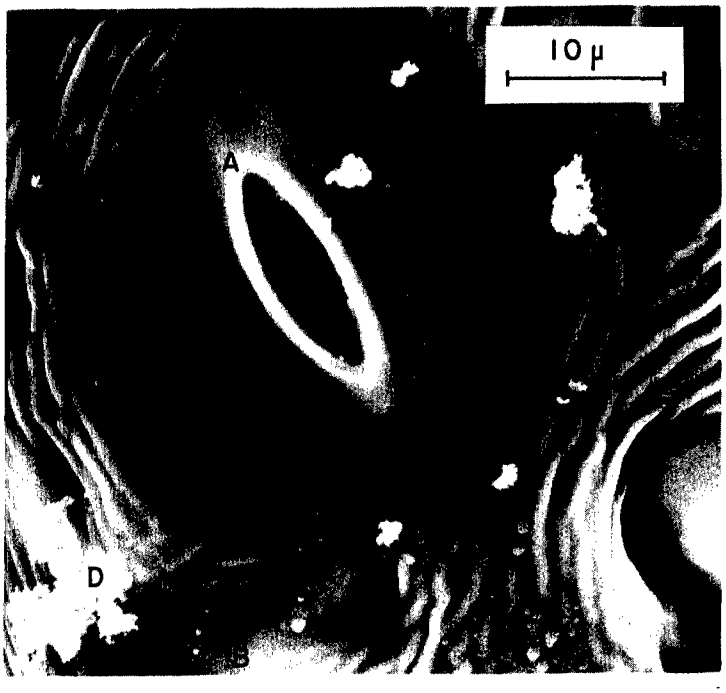

Figure 16. Scanning electron micrograph of the upper leaf surface of marigold plant exposed to PSL beads and alumina particles. A. stomate; B. PSL 0.357 μm; C. PSL 0.721 μm; D. agglomerate of alumina particles, X200.

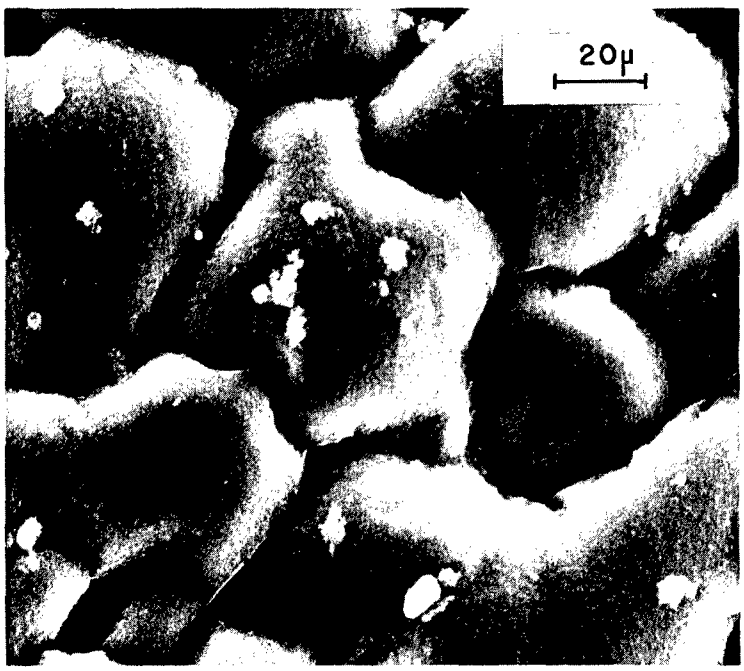

Figure 17. Scanning electron micrograph of the upper leaf surface of nasturtium plant exposed to alumina particles, X600.

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AMRL-TR-75-125

PAPER NO. 24

THE TOXICITY TO FISH OF THE JET FUEL JP-9, ITS COMPONENTS RJ-4, RJ-5 AND METHYLCYCLOHEXANE (MCH)

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and
Robert C. Cooper

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Berkeley, California

INTRODUCTION

This paper presents the results of fish toxicity studies on the jet fuel JP-9 and its components RJ-4, RJ-5 and MCH. Fish toxicity was measured in batch and continuous flow systems using both warm water fish (Jordanella floridae and Notemigonus chrysoleneas) and cold water fish (Salmo gairdneri). One can visualize that there are several ways in which a fuel may be introduced into the aquatic environment and come in contact with aquatic biota. Thus, a fuel spill may result in the formation of floating or sunken pools of pure fuel as well as emulsions of fuel droplets and fuel components in true solution. Surface runoff from areas exposed to fuel may also contain these three physical forms of fuel components. Waste discharges from fuel processing and manufacturing may also contain this variety of physical forms of fuel. Because of these possibilities toxicity studies were conducted on static pools of fuel, fuel emulsions and aqueous fuel solutions. In addition to measurements of fish toxicity as assessed by deaths of adult fish, experiments were conducted on the effect of fuel components on egg hatchability and fry development. The accumulation of fuel components in fish bodies and its relationship to toxic response was examined. The voiding of fuel components from fish placed in fuel-free water was also studied.

As a part of these studies developmental work was conducted on the extraction, concentration and gas chromatographic analysis of fuel components in water and fish flesh. It was also necessary to devise modifications of dosing and dilution apparatus for conducting continuous bioassays on volatile and poorly water-soluble compounds.

MATERIALS AND METHODS

Fuels and Fuel Components

The studies reported herein concentrate on the jet fuel JP-9 which has three major components: RJ-4 (a mixture of isomers of tetrahydromethylcyclopentadiene dimer), RJ-5 (a mixture of isomers of tetrahydronorbornadiene dimer - also known as Shelldyne H) and MCH (methylcyclohexane). The JP-9 and its components RJ-4 and RJ-5 were supplied by the Aerospace Medical Research Laboratory, USAF. MCH was technical grade supplied by J. T. Baker Company. Physical properties of the fuel components are presented in Table 1. The JP-9 fuel supplied varied in composition between batches. The first batch, on which static bioassays were conducted contained weight percentages of 40% MCH, 15% RJ-4 and 45% RJ-5. The second batch on which continuous flow bioassays were conducted contained weight percentages of 27% MCH, 16.2% RJ-4 and 56.8% RJ-5.

TABLE 1. PHYSICAL PROPERTIES OF FUELS AND FUEL COMPONENTS

Physical Property	RJ-4	RJ-5	MCH	JP-4
Boiling Point, °C	221.6	272.2	100-102	--
Vapor Pressure at 70 F, mm Hg	0.354	0.025	--	--
Density, g/mliter	0.925	1.0813	0.772	0.746

Limited studies are reported on the fuel JP-4 which is a complex mixture of aliphatic and aromatic hydrocarbons. The batch supplied contained weight percentages of 0.3% benzene, 0.2% toluene and 0.8% mixed xylenes.

Fuel and Fuel Component Concentrations in Water

Fuel and fuel component concentrations in water were determined by extraction into pentane, addition of a suitable internal standard (n-heptane for MCH, n-undecane for RJ-4 and n-tetradecane for RJ-5), concentration approximately 300-fold by rotary evaporation then gas chromatographic (GC) analysis using a Fisher Model 4800 with dual FID and 20 ft stainless steel columns of 10% SE 30 on 80/100 Chrom W. Operating conditions were: detector temperature, 280 C; injector temperature, 230 C; column temperature 60 C for MCH, 150 C for RJ-4, 180 C for RJ-5; carrier gas (N_2) rotameter setting, 2.5 for MCH, 3.0 for RJ-4, 5.0 for RJ-5.

Fuel Component Concentration in Fish Flesh

Fuel component concentration in fish flesh was determined on fish stored in pentane at 17 C. Fish were blended in pentane in either a chilled Waring Blender or a Virtis 45 Homogenizer. Blended extracts were combined and passed through anhydrous Na_2SO_4 and fluorosil columns prior to gas chromatographic analysis. A surface rinse for 2-5 sec in pentane was used for fuel analysis in trout but not in flagfish.

Flagfish (Jordanella floridae)

Flagfish (Jordanella floridae) were obtained from a commerical aquarium and maintained in the laboratory in 15-gal and 26-gal capacity aquaria. For breeding purposes one male and five females were placed in each aquarium. A dark green substrate consisting of yarn wrapped around stainless steel wire cloth received fish eggs. Eggs were collected daily and hatched in separate containers.

Golden Shiners (Notemigonus chrysoleueas)

Golden Shiners (Notemigonus chrysoleneas) were obtained from a commerical fish hatchery and were acclimated to dechlorinated Richmond Field Station tap water for one month in 300-gal capacity holding tanks prior to experimental work.

Rainbow Trout (Salmo gairdneri)

Rainbow trout (Salmo gairdneri) were obtained from the American River Fish Hatchery of the California State Department of Fish and Game. Before use in bioassays the fish were acclimated for one month to dechlorinated Richmond Field Station tap water.

Static Bioassays

Static bioassays were conducted in 20 liter capacity wide-mouth glass jugs filled to 15 liter and provided with the minimum amount of aeration with filtered air to maintain the dissolved oxygen concentration above 4 mg/liter throughout the bioassay. The fish weight/dilution water ratios specified in Standard Methods (1970) were observed.

Continuous Flow Bioassay Exposure Tanks

Continuous flow bioassay exposure tanks (Figure 1) were of stainless steel construction, 4 ft long x 1 ft wide x 1 ft deep and were filled with removable size 40-010 mesh stainless steel screens that enabled compartmentalization of the tanks (Figure 1). An adjustable and removable standpipe allowed maintenance of a variable water depth that was usually 8 inches giving an exposure tank capacity of 80 liters. Nominal hydraulic residence time in the assay tanks was 6 hr for all experiments.

Egg Cups

Egg cups for egg hatchability studies were 4 oz glass jars with their bottoms replaced with a 40-010 mesh stainless steel screen.

Fry Chambers

Fry chambers were glass chambers (12 in. long, 6 in. wide and 12 in. deep) with 40-010 mesh stainless steel screens at each end to allow free circulation of water. The chambers were designed to fit into the fish exposure tanks.

Figure 1. Continuous flow bioassay tanks.

Emulsified Fuels

Emulsified fuels and fuel components for static bioassays were prepared by blending 10 ml fuel with 990 ml tap water for 1 minute in a stainless steel Waring Blender. The emulsion so produced was transferred to a separatory funnel and allowed to separate for 1 hr. The middle portion was taken as the sample for static bioassay of "emulsified fuels."

Nonemulsified Fuels and Fuel Components

Nonemulsified fuels and fuel components were examined in static bioassays by exposing fish to dilution water in contact with a floating or bottom pool of fuel. In these assays the test fish were introduced into the assay vessels before the fuel was added so that direct contact between fish and floating fuel was avoided. In these assays fuel concentration was expressed as μliter fuel/liter assay vessel contents.

Fuel Dissolution and Dilution Apparatus

Fuel dissolution and dilution apparatus tap water, dechlorinated by passage through a 25-gal bed of activated carbon fed two constant head tanks which in turn supplied two fuel contacting chambers each consisting of 12 liter Florence flasks that contained 1.2 liter each of pure fuel or fuel component and were stirred with magnetic mixers. The water contacted with fuel fed to a separation carboy and thence to a final head tank (Figure 2). In the experiments on Rainbow Trout, synthetic wool was placed in the fuel separation chamber to prevent carryover of fuel droplets which was evident in the earlier studies on Flagfish. From this final head tank the water "saturated" with fuel passed to a proportional diluter (Esvelt and Conners, 1971; Mount and Brungs, 1969) that could be set to deliver dilutions of fuel "saturated" water over the entire range from 0% to 100% (Figure 3).

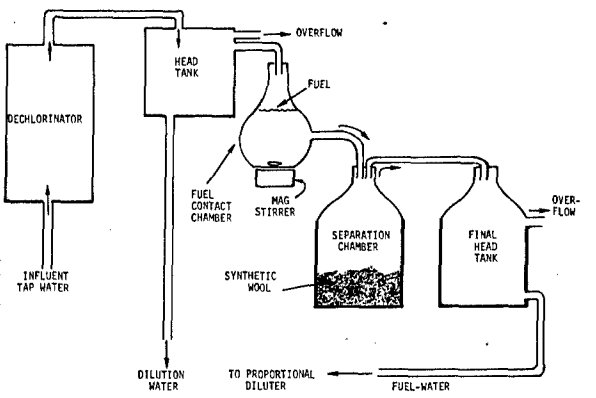

Figure 2. Diagram of dosing apparatus for "soluble" fuel components in continuous flow bioassay.

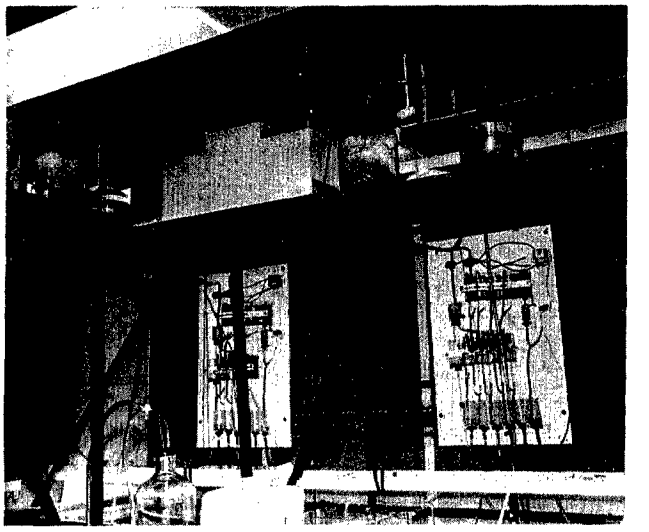

Figure 3. Fuel contacting and dilution apparatus.

LC_{50} Values

LC_{50} values were determined by both the Standard Methods (1970) technique and by the Reed-Muench interquartile range method reported by Woolf (1968) which allowed computation of 95% confidence limits. The two techniques gave virtually identical results.

EXPERIMENTAL RESULTS

Static Bioassay of Fuel Emulsions

The results of static bioassays of emulsified fuels using the Golden Shiner as a test organism are presented in Table 2 and Figure 4. No evidence can be found for any fuel or fuel component that would support the hypothesis that water hardness in the range 25-100 mg $CaCO_3$/liter had any effect on toxicity. In replicate 96-hr bioassays with no fuel renewal there were apparent large variations in the LC_{50} values obtained for MCH and for the fuel JP-4 which was also examined in these studies. Thus the 96-hr LC_{50} values for the bioassay with no fuel renewal for MCH were 81 and 53 μliter/liter; for JP-4 values of 49, 16 and 18 μliter/liter were obtained. These differences, however, were only statistically significant for JP-4 at the 95% confidence level and were attributed to the emulsification technique where minute differences in the standard settling period can cause significant changes in the nominal fuel concentration.

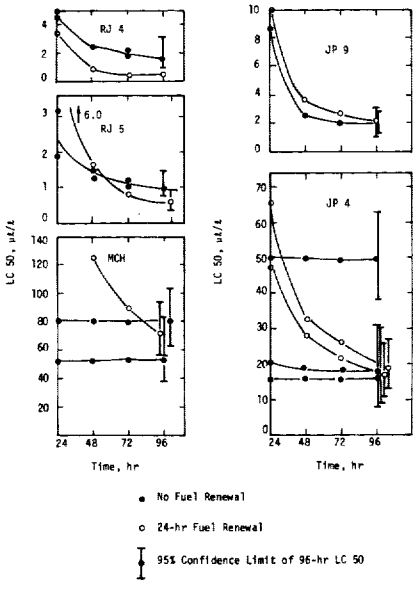

Figure 4. Fuel and fuel component emulsion toxicity in static bioassays. Test organisms: Golden Shiner.

TABLE 2. FUEL AND FUEL COMPONENT EMULSION TOXICITY IN STATIC BIOASSAYS -- TEST ORGANISM, GOLDEN SHINER

Fuel or Fuel Component	Renewal Time, hr	Water Hardness mg $CaCO_3$/liter	LC_{50}, μliter/liter 24 hr	48 hr	72 hr	96 hr	95% Confidence Limits of 96-hr LC_{50} μliter/liter
RJ-4	96	100	5.0	2.6	1.9	1.7	1.3 - 2.4
	96	25	4.4	2.5	2.0	1.7	0.94 - 3.2
	24	100	3.6	0.95	0.56	0.51	0.35 - 0.75
RJ-5	96	100	1.9	1.5	1.1	1.0	0.73 - 1.4
	96	25	3.2	1.3	1.2	1.0	0.68 - 1.5
	24	100	6.0	1.6	0.8	0.61	0.39 - 0.96
MCH	96	100	53	53	53	53	33 - 85
	96	25	81	81	81	81	63 - 104
	24	100	--	126	90	72	56 - 95
JP-9	96	100	8.8	2.5	2.1	1.9	1.1 - 3.2
	24	25	10	3.7	2.7	2.0	1.3 - 2.9
JP-4	96	100	50	49	49	49	38 - 63
	96	25	16	16	16	16	8 - 31
	96	25	20	19	18	18	9 - 31
	24	100	48	28	22	17	11 - 26
	24	25	66	33	27	19	13 - 27

The effect of renewing the contents of static bioassay vessels each 24-hr during a 96-hr test (with the objective of attempting to compensate for the loss of volatile components) was variable. With MCH and the fuel JP-4, which are (or contain) the most volatile components studied in static bioassay of emulsions, the full toxic effect observed after 96-hr exposure was virtually all exerted in the first 24 hr. This observation could support the hypothesis that an initially toxic component is being lost from the assay solution, possibly by stripping or volatilization. Further support

for this hypothesis is given by the data for the assays with 24-hr renewals of solution. For MCH and JP-4 emulsions, the toxicity continued to increase throughout the 96-hr period of exposure (Figure 4). The 24-hr renewal technique did not significantly influence the LC_{50} values of JP-9 and RJ-5; neither was the pattern of increasing LC_{50} values between 24 to 96-hr influenced by 24-hr solution renewal. The toxicity of RJ-4 as estimated by 24, 48, 72 and 96-hr LC_{50} values was significantly influenced by 24-hr solution renewal.

A comparison of the toxicity of JP-9 with the sum of the toxicities of its components was made using the Toxic Unit method of Sprague (1969). The toxic units of the JP-9 components were summed in direct proportion to the weight composition of each component in JP-9, i.e.,

$$\text{JP-9}_{TU} = 0.15\ \text{RJ-4}_{TU} + 0.45\ \text{RJ-5}_{TU} + 0.4\ \text{MCH}_{TU}.$$

The toxicity computed by this method is in good agreement with the experimentally-measured toxicity of JP-9 for all experiments of up to 48-hr duration (Figure 5). For experiments where there was no fuel renewal throughout the 96-hr bioassay period, the computed and measured JP-9 toxicities were in excellent agreement. For bioassays in which the fuel components were renewed each 24-hr the sum of the component toxicities predicted a progressively higher toxicity for JP-9 than was observed at 72-hr and 96-hr.

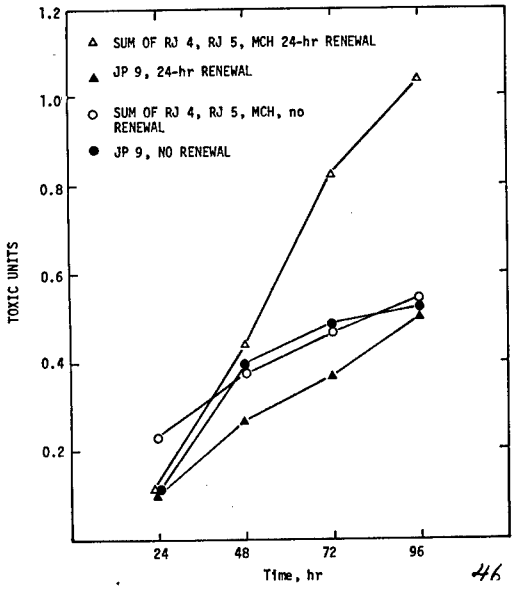

Figure 5. Computed and observed toxicity of JP-9 for 24-hr renewal and nonrenewal emulsion static bioassays.

These results are consistent with the premise that one mode of exposure is physical contact of the fish with droplets of the emulsified fuel. When fuel is not renewed during the 96-hr bioassay the emulsion is given time to separate so that physical contact is less important than intoxication from dissolved components. In this situation computed and observed toxicities are in excellent agreement.

When the fuel and fuel components are renewed each 24-hr throughout the 96-hr assay period the emulsion is not given an opportunity to separate. In this instance physical contact between the fish and fuel droplets is more important. When one sums the toxicities of the three JP-9 components, the effect of physical contact is counted three times because the component bioassays were conducted in separate vessels. The experimental measurement of JP-9 emulsion toxicity, however, only includes the effect of physical contact once. Thus, for stable emulsions the prediction of toxicity of JP-9 from its components toxicities should give larger values than measured experimentally for JP-9 and these values should become more divergent with the duration of the experiment. The results are in excellent agreement with this hypothesis and show that for the stable emulsions the toxicity of JP-9 can only be predicted from its components' toxicities up to 48 hr. Beyond that time the component toxicities overpredict JP-9 toxicity.

STATIC BIOASSAY OF NONEMULSIFIED FUEL AND FUEL COMPONENTS

Static assays, in which Golden Shiners were contacted with floating or bottom pools of fuel and fuel components, indicated lower toxicity than emulsified fuel in all instances (Table 3). Using the 96-hr LC_{50} for comparative purposes, MCH was 3 times less toxic, JP-4 was 32 times less toxic, RJ-4 was 100 times less toxic and RJ-5 was 4,700 times less toxic than the corresponding emulsified materials.

TABLE 3. FUEL AND FUEL COMPONENT TOXICITY IN STATIC BIOASSAY USING PURE FUEL OR FUEL COMPONENTS - TEST ORGANISM: GOLDEN SHINER

Toxicant	LC_{50}, μliter/liter Time in Hours 24	48	72	96	95% CL 96-hr LC_{50} μliter/liter
RJ-4	--	5000	100	100	--
RJ-5	--	8000	6100	4700	3200 - 7100
MCH	240	240	240	240	190 - 300
JP-9	560	490	490	470	340 - 640
JP-4	1600	620	570	570	400 - 800

There is considerable evidence that one important route of exposure in these tests was by direct contact of the fish with the fuel on the surface of the water. Thus for RJ-4 the percent survival of test fish was a function of time of exposure rather than the volumetric concentration of the fuel (Figure 6). Evidence exists also in the pattern of fish survival for RJ-5 that physical contact between fuel and fish is involved in the toxic response. However, since this component is more water soluble than RJ-4 the response due to physical contact is not as unequivocal as with RJ-4. For MCH, which is both more soluble than RJ-4 and RJ-5 as well as being more volatile (Table 1), the toxicity response is typical for a dissolved toxicant (Figure 6). However, since the toxic response is identical at 24, 48, 72 and 96-hr one must assume that any toxic effect is exerted immediately. This type of response would be expected of a highly volatile toxicant which was stripped out of the assay vessel rapidly. The response of fish to JP-9 is typical of a response to a soluble toxicant.

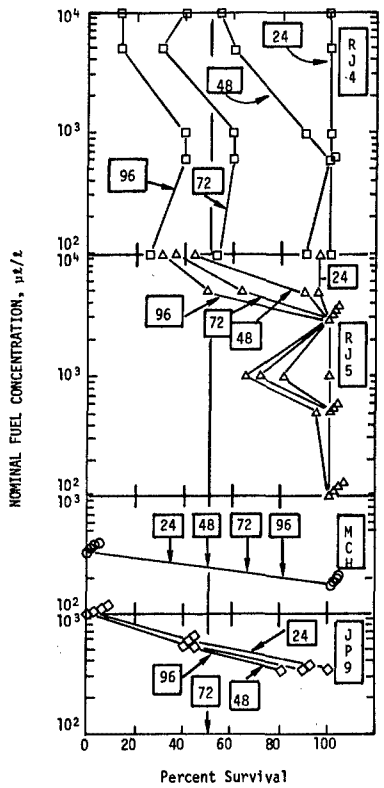

Figure 6. Relationship of fish survival to exposure time and toxicant concentration for JP-9 and its components RJ-4, RJ-5 and MCH.

Further evidence in support of the direct contact mode of exposure to pure fuels is provided by the relatively low toxicity of water soluble fractions of MCH and JP-4. These two materials were individually stirred overnight with water (fuel/water volume ratio of 13.6:86.4) and the water layer then drawn off for static bioassay determinations. The 96-hr LC_{50} values show that water-soluble MCH (Table 3) was some 979 times less toxic than pure MCH in contact with water (Table 4) while the water-soluble components of JP-4 were some 351 times less toxic than the pure fuel in contact with water.

TABLE 4. WATER-SOLUBLE FUEL AND FUEL COMPONENT TOXICITY IN STATIC BIOASSAYS -- TEST ORGANISM: GOLDEN SHINER

Fuel or Fuel Component	LC_{50}, μliter/liter				95% Confidence Limit of 96-hr LC_{50}, μliter/liter
	24 hr	48 hr	72 hr	96 hr	
MCH	309,000	243,000	239,000	235,000	188,000 - 295,000
JP-4	313,000	267,000	212,000	200,000	174,000 - 230,000

CONTINUOUS FLOW BIOASSAY

Introduction

Continuous-flow bioassays were conducted on the water-soluble fractions of fuels and fuel components to determine egg hatchability and fry development effects for the Flagfish in the presence of MCH, RJ-4, RJ-5, and JP-9. Because droplets of fuel were carried over from the head tank into the diluters and thence to the fish tanks it was necessary to terminate these experiments for RJ-4, RJ-5 and JP-9 following the egg hatchability stage. Since MCH floated on water, its droplets were continuously expelled from the head tank overflow so that MCH droplets did not reach the exposure tanks in large quantities. The data for MCH (Table 5 and Figure 7) are illustrative of two problems encountered with the use of the Mount-Grungs (1969) dilution device for poorly water-soluble, volatile compounds. Significant volatilization of MCH took place between the final head tank of the contacting device and the effluent from the diluter (the influent to be "100% Exposure Tank"). It is also evident from Table 5 that there was considerable temporal variation in the exposure tank MCH concentrations. Such variation is attributable largely to inconsistencies in the dosing and dilution apparatus because the MCH analytical technique has a coefficient of variation of only 8%. It is noteworthy that the MCH concentration in the exposure tanks did not decrease linearly with nominal dilution (Figure 7).

TABLE 5. MCH CONCENTRATIONS IN DILUTION APPARATUS AND EXPOSURE TANKS DURING CONTINUOUS FLOW BIOASSAY ON FLAGFISH

Sample Location	No. of Analyses	Mean MCH Concentration, mg/liter	Standard Deviation mg/liter	Coefficient of Variation, %
Head Tank	6	3.41	2.22	65
Diluter Effluent	7	1.85	0.82	44
100% Exposure Tank	8	0.83	0.73	88
50% Exposure Tank	6	0.67	0.65	97
12% Exposure Tank	6	0.34	0.38	112
Control Exposure Tank	6	0.01	0.01	100

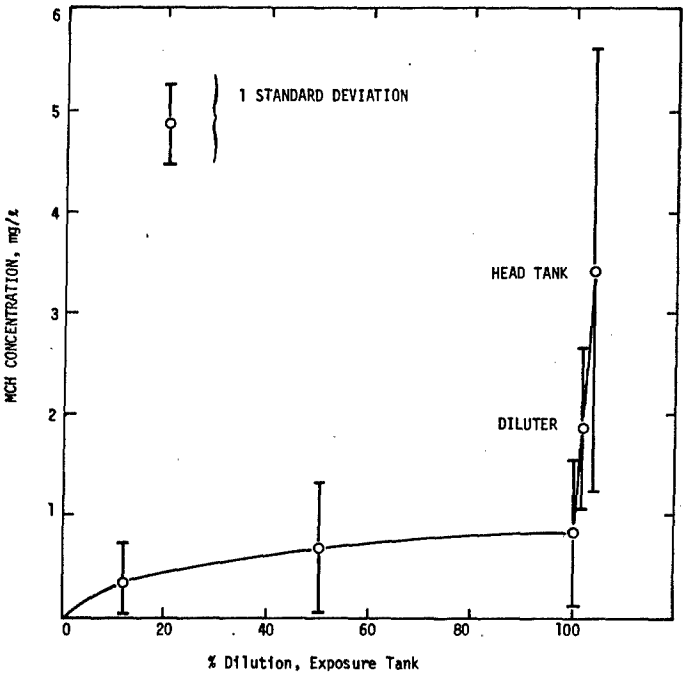

Figure 7. MCH concentration variation in dilution apparatus and exposure tanks during continuous flow bioassay on Flagfish.

Further, though less well-defined data exist for RJ-4, RJ-5 and JP-9 (Table 6), these data substantiate the problems with volatility losses in the dilution apparatus, and for JP-9 show that the volatility losses affect the relative composition of the fuel. Thus the fractions of RJ-4 and RJ-5 in JP-9 fall respectively from 14% and 56% in the head tank to 6% and 24% in the "12% Exposure Tank" while the MCH increases from 30% to 71% of the JP-9.

TABLE 6. AVERAGE CONCENTRATIONS OF SOLUBLE JP-9 COMPONENTS IN CONTINUOUS FLAGFISH BIOASSAY

Fuel or Fuel Component	Component Concentration, ±/Standard Deviation, mg/liter						
	Head Tank	Diluter Effluent	100% Exposure Tank	50% Exposure Tank	25% Exposure Tank	12% Exposure Tank	Control Exposure Tank
MCH	3.41 ± 2.22	1.85 ± 0.82	0.83 ± 0.73	0.67 ± 0.65	--	0.34 ± 0.38	0.01 ± 0.01
RJ-4	13.9 ±3.68	2.36 ±2.22	0.20 ± 0.03	0.19 ± 0.05	0.15 ± 0.05	0.089 ±0.04	0.001 ± 0.002
RJ-5	0.83 ± 0.89	0.32 ± 0.35	0.11 ± 0.09	0.12 ± 0.13	0.067 ± 0.07	0.043 ± 0.05	0.045 ± 0.06
JP-9	7.15 ± 5.53	1.39 ± 0.92	0.23 ± 0.11	0.17 ± 0.13	0.11 ± 0.07	0.15 ± 0.12	0.01 ± 0.01
MCH	2.15 ± 2.15	0.59 ± 0.37	0.13 ± 0.09	0.11 ± 0.05	0.05 ± 0.04	0.11 ± 0.09	0
RJ-4	1.00 ± 0.82	0.13 ± 0.13	0.01 ± 0.01	0.01 ± 0.007	0.02 ± 0	0.01 ± 0.007	0
RJ-5	4.00 ± 2.77	0.67 ± 0.81	0.09 ± 0.05	0.07 ± 0.06	0.05 ± 0.04	0.04 ± 0.04	0.01 ± 0.01

Flagfish Egg Hatchability

A minimum of 92, 1-4 day old eggs were placed in egg cups in each dilution. The egg cups were located adjacent to the outlet of a recirculation pump so that they were agitated by the flow from the pump. The eggs generally hatched within one week following immersion in the exposure tanks.

Water soluble fractions of MCH and RJ-4 had no significant effect on egg hatchability up to average concentrations of 0.83 mg/liter MCH and 0.2 mg/liter RJ-4 (Figure 8). It appeared that RJ-5 at concentrations of greater than 0.05 mg/liter significantly reduced Flagfish egg hatchability. The water soluble components of JP-9 at an average concentration of 0.23

mg/liter appeared to reduce Flagfish egg hatchability. The data presented in Figure 8 support the view that the effect of JP-9 on egg hatchability in this experiment was due to the toxicity of its RJ-5 component. However, because of the variability in the hatchability of the controls and in the concentrations of fuels and fuel components in the various exposure tanks, this conclusion must be regarded as tentative.

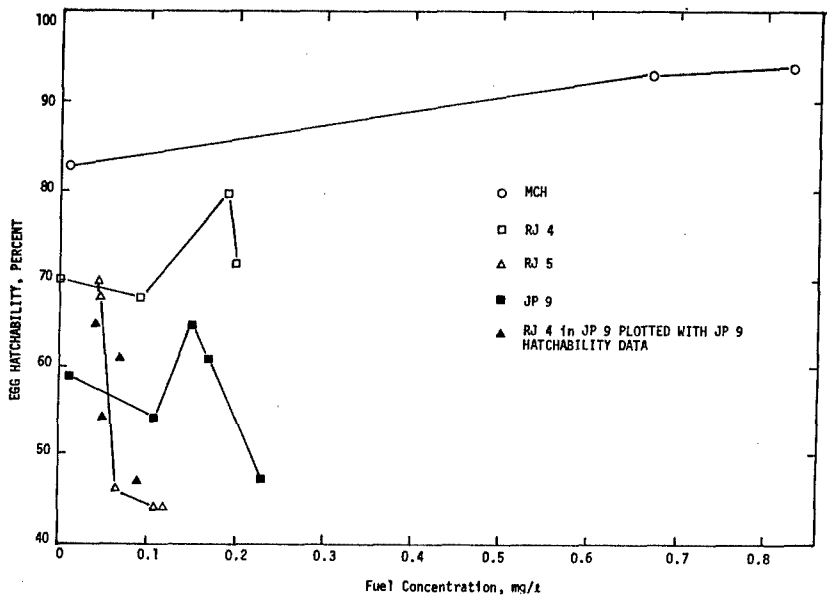

Figure 8. Flagfish egg hatchability in presence of water soluble components of fuels and fuel constituents.

Flagfish Fry Development

Because of the previously mentioned problem of fuel droplet carry-over it was only possible to conduct fry development studies on MCH. Fry were kept in fry chambers for 37 days following hatching then released to the exposure tanks for the remainder of the 87-day exposure period. No significant toxic effects as assessed by fish deaths and by measurement of fish lengths and weights following exposure were observed at any dilution of the water soluble components of MCH (Figure 9). Reference to Figure 9 will show that while it was not possible to obtain standard deviations for fish weights (because the fish were weighed in batches rather than individually) the average wet weights of fish in all MCH concentrations up to 0.83 mg/liter were within the band of values encompassed by the average wet weights for the control exposure tanks.

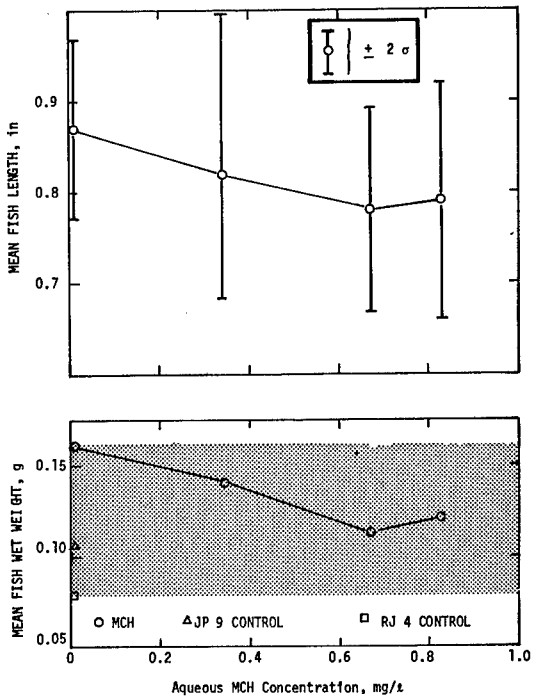

Figure 9. Relationship of Flagfish wet weight and length to aqueous MCH concentration in continuous bioassay.

These data indicate that average aqueous concentrations of 0.83 mg/liter MCH or less are not toxic to Flagfish in the egg or fry development stages. Because of the loss of MCH between the contact/dilution apparatus in the exposure tanks it was decided to conduct a static bioassay test on five Flagfish using the diluter effluent (inlet to the "100%" exposure tank). In this solution there was an average concentration of 1.85 mg/liter MCH and all of the fish died after seven days exposure. These results allow one to tentatively set the acute toxic level for MCH aqueous solutions between 0.83 mg/liter and 1.85 mg/liter.

BIOASSAY OF SOLUBLE FUEL COMPONENTS USING RAINBOW TROUT

Prior to the commencement of the continuous trout bioassay of soluble components of fuels and fuel constituents, the fuel contacting apparatus was modified to prevent the fuel droplet carryover that had caused problems in the previous Flagfish assay. Synthetic wool was placed in the separation chamber (Figure 2) to reduce turbulence and to intercept fuel globules. Water flow rates and mixing rates were reduced in the fuel chambers.

Restraining screens were placed over the exposure tanks in an attempt to prevent fish from jumping out of the tanks. Even with these screens in place some fish succeeded in jumping out of the tanks, especially at the higher concentrations. Thirty fish were used in each exposure tank.

In a 97-day exposure to various dilutions of an aqueous solution of RJ-4, almost all observed deaths occurred during the first 23 days exposure (Table 7). At this time there were no deaths in the control (RJ-4, 0.001 ± 0.002 mg/liter), two deaths at an RJ-4 concentration of 0.031 ± 0.023 mg/liter, 18 deaths at 0.048 ± 0.019 mg/liter RJ-4 and no survivors (29 deaths) in the exposure tank containing an average RJ-4 concentration of 0.068 ± 0.025 mg/liter. There were no survivors (29 deaths) at an RJ-4 level of 0.122 ± 0.057 mg/liter. In the remaining 74 days of the experiment only 2 further deaths occurred (one in the control and one in the tank containing 0.031 ± 0.023 mg/liter RJ-4). Nonlethal aqueous concentrations of RJ-4 could not be established but they are certainly below 0.03 mg/liter (Table 7). The LC_{50} value for this exposure time was estimated to be 0.045 mg/liter (Figure 10).

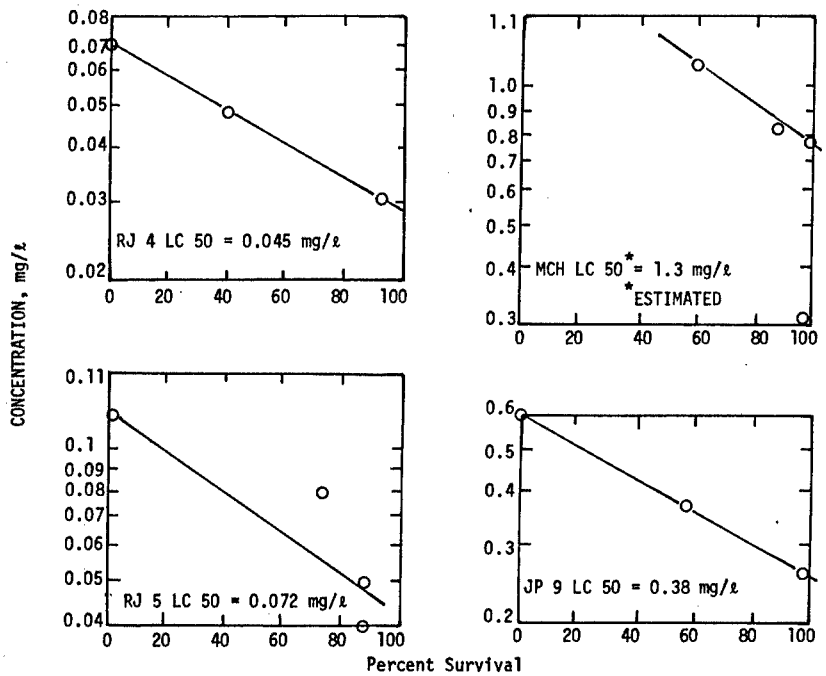

Figure 10. 23-day LC_{50} values for Rainbow Trout in continuous flow bioassays.

TABLE 7. CUMULATIVE RAINBOW TROUT DEATH TALLY FOR CONTINUOUS FLOW CHRONIC BIOASSAY ON WATER SOLUBLE COMPONENTS OF RJ-4

Exposure Time Days	Cumulative Death Tally, Numbers of Fish				
1	0	0	0	0	0
3	0	0	0	8	23
4	0	1	5	16	28
5	0	1	6	22	28
6	0	1	8	22	29
10	0	2	10	28	29
14	0	2	13	28	29
23	0	2	18	29	29
34	1	2	18	29	29
35	1	3	18	29	29
57	1	3	18	29	29
77	1	3	18	29	29
97	1	3	18	29	29
Water Soluble RJ-4 Concentration, mg/liter	0.001	0.031	0.048	0.068	0.122
Standard Deviation, mg/liter	0.002	0.023	0.019	0.025	0.057

The RJ-5 study lasted for 97 days. There were no deaths in the control exposure tank, 4 deaths each in the exposure tanks containing average RJ-5 concentrations of 0.04 and 0.05 mg/liter; 8 deaths in the presence of 0.08 mg/liter RJ-5 and no survivors in the presence of 0.12 mg/liter RJ-5 (Table 8). All fish deaths occurred during the first 14 days of the experiment. Nonlethal RJ-5 aqueous concentrations could not be established but they are certainly below 0.04 mg/liter. The LC_{50} value for this exposure time was estimated to be 0.072 mg/liter (Figure 10).

TABLE 8. SURVIVAL OF RAINBOW TROUT IN CONTINUOUS FLOW BIOASSAY OF WATER SOLUBLE COMPONENTS OF RJ-4, RJ-5, MCH AND JP-9

RJ-4			RJ-5			MCH		JP-9	
Average Concentration mg/liter	Survival % 97 Days Exposure	Survival % 23 Days Exposure	Average Concentration mg/liter	Survival % 97 Days Exposure	Survival % 23 Days Exposure	Average Concentration mg/liter	Survival % 23 Days Exposure	Average Concentration mg/liter	Survival % 23 Days Exposure
0.001	97	100	0	100	100	0.01	97	0.08	100
0.031	90	93	0.04	87	87	0.31	97	0.12	97
0.048	40	40	0.05	87	87	0.80	97	0.26	97
0.068	0	0	0.08	73	73	0.84	77	0.37	57
0.122	0	0	0.12	0	0	1.19	60	0.60	0

The experiments on both MCH and JP-9 had to be terminated after 23 days because of a failure in the refrigeration equipment that allowed the temperature of the exposure tanks to rise to 20 C and caused significant mortalities. However, on the basis of the RJ-4 and RJ-5 experiments it is likely that the deaths occurring during this period are representative of those that would have taken place over the longer (97 day) exposure time used in the RJ-4 and RJ-5 experiments. On this basis it would appear that the chronic nonlethal level for MCH is some value below 0.8 mg/liter. This value should be interpreted with caution because at an average concentration of 0.84 mg/liter MCH, significant mortality occurred. It was only possible to approximate an LC_{50} value for MCH because the highest concentration used only caused 40% mortality. However, from the data obtained a 23-day LC_{50} value of 1.3 mg/liter is estimated.

The JP-9 experiment terminated after 23 days because of temperature control problems. The experimental data indicate a nonlethal concentration of some value less than 0.37 mg/liter and a 23-day LC_{50} value of 0.38 mg/liter. In reporting these values one must be aware that the aqueous solutions of JP-9 in the fish exposure tanks are grossly different in composition from the JP-9 fuel used to make up the aqueous JP-9 solution. This is because of both differential solubility and volatility of JP-9 components.

Thus the neat JP-9 fuel contained some 27% MCH, 16% RJ-4, and 57% RJ-5 while the aqueous JP-9 solutions in the exposure tanks had an average composition of 85.4% MCH, 1.8% RJ-4, and 13.3% RJ-5. Using the toxic unit summation of Sprague (1969) to predict the toxicity of JP-9 from its components RJ-4, RJ-5 and MCH gave excellent results when based on the JP-9 composition found in the exposure tanks (Figure 11); however, if these computations had been based on the original fuel composition the prediction of toxicity would have been grossly in error (Table 9).

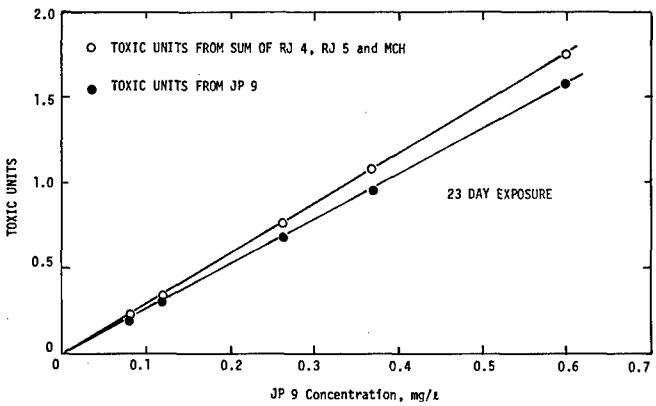

Figure 11. Comparison of toxicity of JP-9 and computer toxicity of its components. Rainbow Trout in water soluble fuel components, continuous flow assay.

TABLE 9. PREDICTED AND OBSERVED TOXICITY OF WATER SOLUBLE JP-9 COMPONENTS TO RAINBOW TROUT IN 23-DAY CONTINUOUS BIOASSAY

JP-9 "Concentration" mg/liter	Toxic Units Observed	Toxic Units Predicted: Based on Exposure Tank JP-9 Composition	Toxic Units Predicted: Based on JP-9 Fuel Feed Composition
0.08	0.21	0.23	0.95
0.12	0.32	0.34	1.38
0.26	0.68	0.77	3.04
0.37	0.97	1.08	4.32
0.60	1.57	1.75	9.25

Fuel Accumulation and Voiding by Flagfish

At the conclusion of the 87-day MCH bioassay live Flagfish were removed from the exposure tanks and analyzed for MCH. Fish from a short-term (7 day) static bioassay conducted on MCH dilution tank effluent were also analyzed for MCH. The latter fish had been exposed to an average aqueous MCH concentration of 1.85 mg/liter and were all dead. The results (Figure 12) show that there is an almost linear relationship between total MCH body burden and aqueous MCH concentration and that Flagfish can tolerate a total body burden of about 0.5 mg MCH/g wet weight without lethality. It can be deduced that lethal total body burdens of MCH lie somewhere between 0.5 and 1.19 mg/g wet weight for Flagfish. Figure 12 also includes one data point relating the total body burden of MCH to aqueous MCH concentration after 42 days exposure. It can be seen that there is excellent agreement between this point and the Flagfish data.

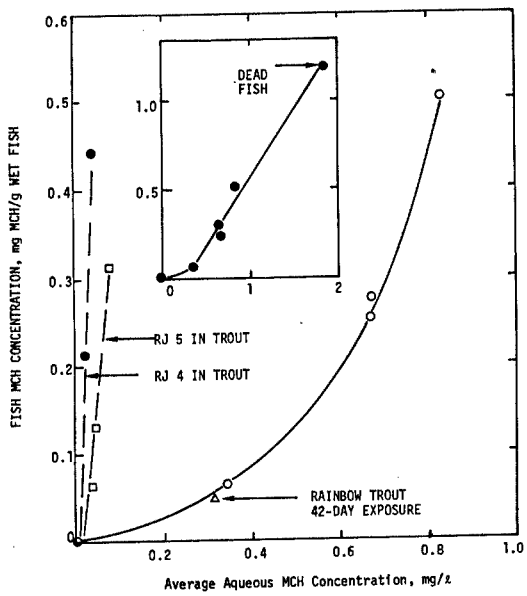

Figure 12. Accumulation of MCH in Flagfish as a function of aqueous MCH concentration (87-day exposure).

Surviving Rainbow Trout from the 97-day continuous bioassay of the soluble components of RJ-4 and RJ-5 were analyzed for their fuel content. The inclusion of a 2-5 sec pentane rinse prior to storage and extraction of the fish showed that only on the order of 0.1-0.2% of the total fuel (RJ-4 or RJ-5) found in the fish flesh could be accounted for on the surface of the fish.

The Rainbow Trout were of a sufficient size to permit separation of muscle tissue and thus allow a comparison between whole body and muscle tissue fuel burdens. The data in Figure 13 show that the total body burdens of RJ-4 and RJ-5 are related linearly to the aqueous RJ-4 and RJ-5 concentrations and that these fuels are concentrated in Rainbow Trout flesh to a much higher degree than is MCH in Flagfish flesh. If one assumes that the single data point for MCH accumulation in Rainbow Trout is valid then the degree of concentration of RJ-4, RJ-5 and MCH in Rainbow Trout (assuming a wet fish density of 1.0) is respectively 9,800 times for RJ-4, 3,900 times for RJ-5, and 150 times for MCH. The RJ-4 and RJ-5 results indicate that the muscle tissue of Rainbow Trout contains approximately half the fuel concentration that the total body contains (Table 10). Since the aqueous RJ-4 and RJ-5 concentrations from which the fish for fuel analysis were taken covered a range of concentrations up to the LC_{50} values, it is possible to make tentative statements concerning the fuel body burden that will cause 50% mortality. For RJ-4 this concentration is on the order of 0.43 mg RJ-4/g wet fish; for RJ-5 this value is approximately 0.27 mg/g (Figure 13).

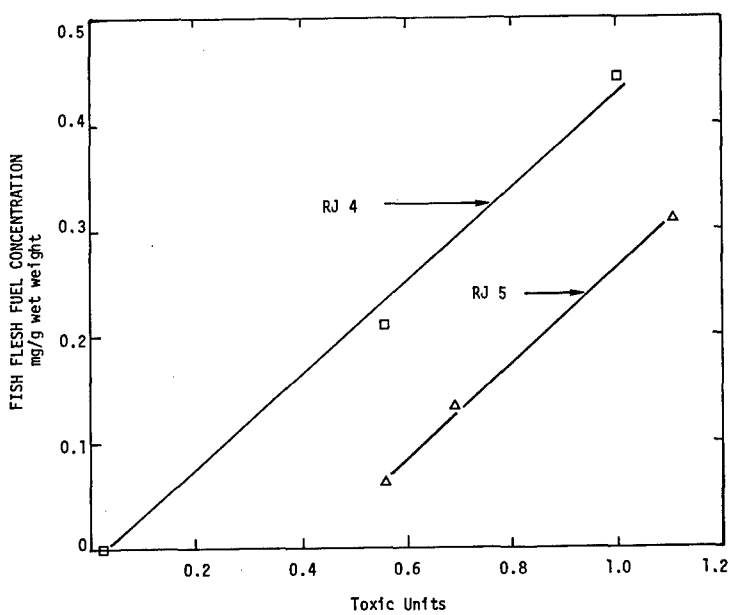

Figure 13. Relationship of fuel body burden to toxicity of water soluble RJ-4 and RJ-5 to Rainbow Trout.

TABLE 10. ACCUMULATION OF RJ-4 AND RJ-5 IN MUSCLE AND WHOLE BODY OF RAINBOW TROUT

Fuel	Mean Water Soluble Concentration mg/liter	Toxic Units	Fuel Concentration	
			Muscle mg Fuel/ g Wet Fish	Whole Body mg Fuel/g Wet Fish
RJ-4	0.045	1	0.278	0.443
	0.025	0.56	0.133	0.217
	0.001	0.022	0.001	0.001
	0.08	1.1	0.186	0.311
	0.05	0.69	0.080	0.132
RJ-5	0.04	0.56	0.035	0.062
	0.003	0.04	0.003	0.003

The voiding of fuel components from Rainbow Trout was studied for MCH, RJ-4 and RJ-5. Surviving fish from the 97-day continuous bioassay of water soluble components of these fuels were placed in fuel free water (the control exposure tanks) and after known time intervals were sacrificed, then rinsed and extracted with pentane. The total body burdens of fish from various fuel concentrations were normalized by using concentration factors (mg fuel/kg wet fish divided by mg fuel/liter exposure solution from which the fish was derived). These values, plotted in Figure 14 show that there was no evidence of any significant voiding of either RJ-4 after 8 days or RJ-5 after 6 days. However, the MCH level in the Rainbow Trout had dropped by a factor of almost 8 in a 12-hr period.

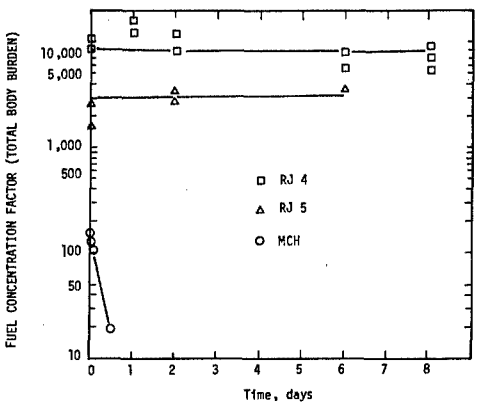

Figure 14. Voiding of fuel components from Rainbow Trout.

CONCLUSIONS

1. Water hardness in the range 25-100 $CaCO_3$/liter did not influence the toxicity of JP-9 or its components RJ-4, RJ-5 and MCH to Golden Shiners in static bioassays.

2. In static Golden Shiner bioassays of fuel and fuel components it was shown that the toxicity of JP-9 could only be predicted from the toxicity of its components when the bioassays were conducted without daily renewal of the emulsions. When emulsions were renewed each day the summation of toxic units of JP-9 components to predict JP-9 toxicity overestimated the toxicity of the fuel beyond exposures of 48 hr. Evidence existed to suggest that the cause of this overestimate was the contribution of direct physical contact with emulsion droplets to the toxic response.

3. In static Golden Shiner bioassays the toxicity of static pools of unemulsified fuel and fuel components was orders of magnitude less than the emulsified materials as assessed by the 96-hr LC_{50} values. Evidence existed to indicate that the physical contact of fish and fuel was an important mode of intoxication for RJ-4 and RJ-5.

4. The use of a Mount Brungs dilution device for dosing continuous-flow bioassay tanks with aqueous extracts of fuel results in considerable volatility losses and in carry-over of fuel droplets into bioassay exposure tanks. These factors combined with variable water solubility can strongly influence the composition of a multi-component fuel (such as JP-9) so that the composition of the material to which fish are exposed is greatly different from the composition of the neat fuel.

5. In continuous-flow bioassay aqueous solution concentrations of 0.83 mg/liter MCH and 0.2 mg/liter RJ-4 had no effect on Flagfish egg hatchability. RJ-5 concentrations of greater than 0.05 mg/liter and JP-9 levels in excess of 0.23 mg/liter reduced Flagfish egg hatchability. It was tentatively concluded that the effect of JP-9 solutions on Flagfish egg hatchability could be largely ascribed to its RJ-5 content.

6. MCH aqueous concentrations of 0.83 mg/liter did not affect Flagfish fry development as assessed by fish length or weight. A short-term experiment (7 days) allowed the level of MCH causing a lethal response to Flagfish to be set between 0.83 mg/liter and 1.85 mg/liter.

7. Nonlethal aqueous concentrations of RJ-4 to Rainbow Trout are below 0.03 mg/liter; the 97-day LC_{50} value is 0.045 mg/liter.

8. Nonlethal aqueous concentrations of RJ-5 to Rainbow Trout are below 0.04 mg/liter; the 97-day LC_{50} value is 0.072 mg/liter.

9. The nonlethal aqueous concentration of MCH appears to be below 0.8 mg/liter; an approximate 23-day LC_{50} value is 1.3 mg/liter.

10. The nonlethal level of JP-9 (of a composition similar to that obtained in the bioassay exposure tanks, i.e., 85.4% MCH, 1.8% RJ-4, 13.3% RJ-5) is some value less than 0.37 mg/liter. The 23-day LC_{50} value is 0.38 mg/liter.

11. It was possible to accurately predict the toxicity of aqueous JP-9 solutions from the toxicity of aqueous solutions of its components RJ-4, RJ-5 and MCH by the toxic unit method of Sprague. In making this prediction it was important to use the composition of JP-9 in the aqueous solution in the exposure tank, rather than the neat JP-9 composition used to prepare the aqueous fuel solution.

12. Flagfish can tolerate a MCH total body burden of some 0.5 mg MCH/g wet weight without lethality. A lethal response to MCH exists somewhere in the range of total MCH body burdens of 0.5 and 1.19 mg MCH/g wet weight.

13. Rainbow Trout and Flagfish appear to concentrate MCH from their aqueous surroundings to about the same extent (150 fold). Rainbow Trout concentrate RJ-4, 9800 times; and RJ-5, 3900 times from aqueous solution. Body burdens of 0.43 mg RJ-4/g and of 0.27 mg RJ-5/g wet fish will produce 50% mortality of Rainbow Trout.

14. Surviving Rainbow Trout exposed to the JP-9 components MCH, RJ-4 and RJ-5 will rapidly void MCH from their tissues but RJ-4 and RJ-5 are retained.

ACKNOWLEDGEMENTS

The work reported herein was supported in part by contract number F33615-73-C-4059 from Aerospace Medical Research Laboratory of the U.S. Air Force Systems Command. The technical assistance of Ms. P. C. Ulrichs, Mr. J. C. Palm, and Ms. N. Quan is gratefully acknowledged.

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AMRL-TR-75-125

PAPER NO. 25

USE OF UNICELLULAR ALGAE FOR EVALUATION OF POTENTIAL AQUATIC CONTAMINANTS

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INTRODUCTION

Increasing emphasis on environmental protection has made it necessary to determine the possible adverse effects of new chemical compounds before they are put into use. The determination of the possible adverse environmental effects includes testing against a broad range of plants and animals.

One of the key ecosystems that should be used for testing is fresh water algae because their role as primary producers is important in the food chain. Several algal species recommended for use in algal bioassays have been shown to be very sensitive to small quantities of aquatic contaminants and the methodology for using these algae as a tool in determining growth effects has been well established.

OBJECTIVES

The overall objective of this investigation was to determine possible effects of selected chemicals which are currently being proposed for use and which may be released into the aquatic environment. The specific objectives were:

1. To conduct a literature survey on the effects of petrochemicals on algae in natural ecosystems.

2. To determine the possible algal growth effects of three groups of compounds:

   Lube Additives

   - p, p' dioctyldiphenylamine (Vanlube 81)
   - phenyl-α-naphthylamine

   Jet Fuels

   - RJ-5 (petroleum base)
   - RJ-4 (petroleum base)

   Hydrazines

   - hydrazine
   - unsymmetrical dimethylhydrazine (UDMH)
   - symmetrical dimethylhydrazine (SDMH)
   - monomethylhydrazine (MMH)

The effects have been determined under standard laboratory conditions using batch cultures of the test alga Selenastrum capricornutum in the presence of bacteria.

METHODS AND PROCEDURES

Experimental Program

The overall experimental program included both batch and continuous culture algal assays. In addition, preliminary investigations were conducted to test the methodology of light/dark Warburg kinetic studies with both bacterized and axenic algal cultures.

Rationale

The laboratory experimental program was designed to estimate the environmental effects of the compounds under the following conditions:

Natural Water Quality: Oligotrophic or Eutrophic
Type of Water Body: Lake or Stream
Quantity of Compound Released: Large or Small

Natural Water Quality

The natural water quality was modeled in the experimental program by using two levels of nutrients in the algal growth medium. The low nutrient level used corresponded to one percent of the Standard Algal Assay Medium (SAAM) nutrient levels and the high level corresponded to thirty-three percent of the SAAM nutrient level.

Previous investigation has shown that the algal growth in the assay medium at the low (one percent) nutrient level is about the same as in Lake Heather or Tamarack, both high Sierra lakes and that growth in the high nutrient level is similar to Lake Arrowhead or Big Bear Lake (James, 1975).

Types of Water Body

Batch cultures are characteristic of bodies of water such as lakes with long hydraulic residence time relative to the algal life cycle. Continuous culture resembles lakes with short residence times as well as flowing streams. In addition, the continuous culture assay will also provide kinetic constants which quantitatively describe the changes in algal growth rates. It is hoped that such growth rate changes can be correlated with the future Warburg studies.

Quantity of Compound Released

The quantity of the compound is important because there may be a substantial difference in the effect caused by the release of small and large concentrations of a given compound. This difference can be related to the natural removal by processes such as adsorption on suspended matter, evaporation (especially for the volatile fuels tested), and bacterial decomposition. The concentration levels selected for testing were 1 mg/l and 100

mg/l (or 1 μl/l and 100 μl/l for liquid compounds) except for the hydrazine compounds which were tested at 1 mg/l and 10 mg/l (or 1 and 10 μl/l for liquid compounds) levels. These levels correspond to the concentrations which would result from a spill of 100 kg (220 lbs), 1000 kg (2200 lbs) and 10,000 kg (22,000 lbs) respectively, in a body of water with a volume of 10^6 m^3 (26,400,000 gal or $\cong$ 100 acre feet). This is the approximate volume of many small lakes or impoundments.

Correlation Between Material and Test Conditions

Specific attention was given to developing test conditions which resembled natural conditions as closely as possible. This was accomplished by using bacterized batch cultures which were open to the atmosphere to allow for both bacterial decomposition and evaporation of the test compound as would occur under natural conditions. In addition, no attempts were made to use emulsifiers to bring the relatively insoluble compounds into solution. Instead the appropriate quantity of the compound was placed in each culture flask and allowed to equilibrate.

Experimental Design of Batch Assays

All batch assays were conducted with replicates and controls as shown in Table 1 so that a common method of statistical analysis could be used.

Batch Assay Procedure

Batch algal assays were conducted in accordance with the "Algal Assay Procedure: Bottle Test (1971)."

Response Parameters

Two response parameters, algal cell number and total algal volume were used to determine the effect of the compounds. These parameters were measured every 3 to 5 days until growth stopped. The maximum values for these growth parameters were then used in the data analyses, irrespective of the day on which they occurred.

TABLE 1. EXPERIMENTAL DESIGN OF BATCH ASSAYS

(Values in Table Indicate Number of Replicates)

	Nutrient Concentration		
	Low (Oligotrophic Lakes)	High (Eutrophic Lakes)	Reference Medium
No Test Compound Added (Control)	5	5	2
1 or 10 mg/l Test Compound Added	5	5	
10 or 100 mg/l Test Compound Added	5	5	

Chemical Analyses

Routine chemical analyses were performed on the assay media using the procedures described in Standard Methods for the Examination of Water and Wastewater (1971).

Specific analyses of the actual concentrations of the test compounds were performed for the following compounds using the methods discussed below:

> p, p'Dioctyldiphenylamine was analyzed using a modification of the method described by Kinder (1968) where the compound is extracted into isooctane and the concentration determined by reading the absorbance at 287 mμ using a Beckman DU-2 spectrophotometer. The detection limit was about 0.010 mg/l after a ten-fold concentration from water to isooctane. The method was linear from 0.1 to 0.5 mg/l and had a standard deviation of ± 0.025 mg/l.

The analysis of RJ-5 was performed using a modification of the method described by Butler (1973) whereby RJ-5 is extracted into isooctane and the organic layer analyzed by gas chromatography using a 5' x 1/8" column packed with 5% SF-96 or 60/80 mesh Chromosorb. This method was linear from 0.1 to 100 mg/l with a standard deviation of ±0.8 mg/l.

The following compounds were not analyzed specifically in this year's program because they were added quantitatively to the growth media and were completely soluble in the concentration ranges tested in both batch and continuous culture assays:

- hydrazine
- unsymmetrical dimethylhydrazine
- symmetrical dimethylhydrazine
- monomethylhydrazine.

The actual concentrations of the compounds RJ-4 and phenyl-α-naphthylamine were not analyzed. The bioassays were completed before columns of appropriate dimensions for gas chromatography were received and the methodology perfected.

Test Alga

The standard test alga, Selenastrum capricornutum Printz, as recommended in Algal Assay Procedures, was used throughout the investigations.

RESULTS

Batch Assays

p, p'Dioctyldiphenylamine

The statistical significance of the effects of 1 and 100 mg/l concentrations of p, p'dioctyldiphenylamine was determined for cell number and total cell volume data from the day of maximum standing crop. Both chemical

concentrations were tested at nutrient levels of 1% and 33% Standard Algal Assay Medium (SAAM). These nutrient levels are similar to those found in oligotrophic and eutrophic lakes as discussed above. Algal growth curves are shown in Figures 1 and 2. Results of the analysis of variance are summarized in Tables 2 and 3. These results show that there was no statistical difference in the maximum amount of algal growth produced in the presence of 1 or 100 mg/l concentrations of the chemical as compared with the controls. Those flasks with a concentration of 100 mg of p, p'dioctyldiphenylamine per liter of 1% SAAM produced an average of 23% to 28% more algae than the controls, as measured by volume and number respectively. This difference was not significant under the test conditions.

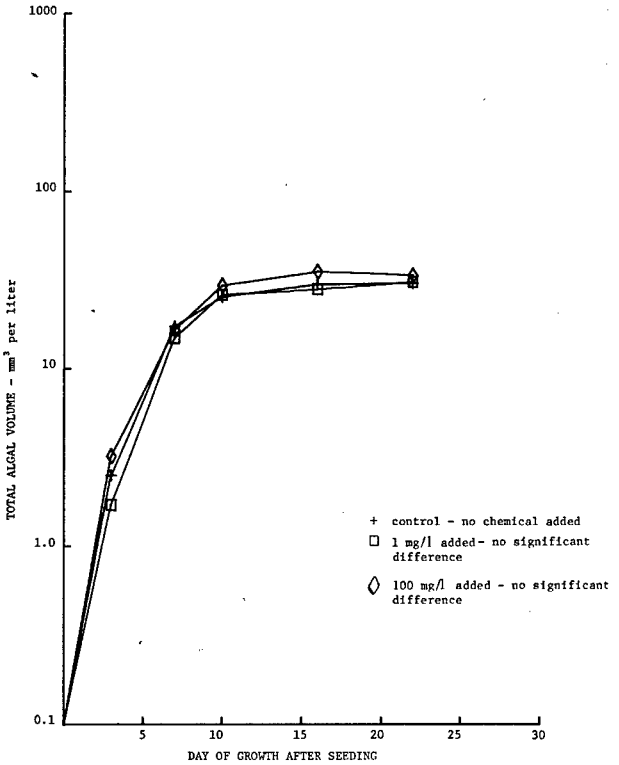

Figure 1. Growth of Selenastrum in low nutrient medium in the presence of p, p'dioctyldiphenylamine.

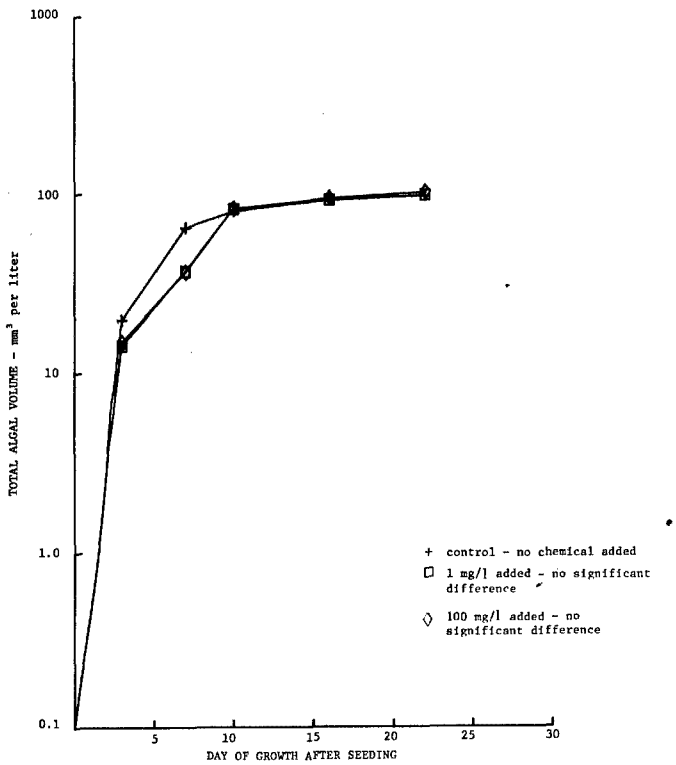

Figure 2. Growth of Selenastrum in high nutrient medium in the presence of p, p'dioctyldiphenylamine.

TABLE 2. EFFECT OF p, p'DIOCTYLDIPHENYLAMINE ON SELENASTRUM CAPRICORNUTUM IN LOW NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume				Algal Cell Number			
	Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (o mg/l)	30.2	2	100	----	594	2	100	----
(1 mg/l)	30.6	6	101	N.S.	701	6	118	N.S.
(100 mg/l)	37.2	6	123	N.S.	758	6	128	N.S.

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. =Not Significant.

TABLE 3. EFFECT OF p, p'DIOCTYLDIPHENYLAMINE ON SELENASTRUM CAPRICORNUTUM IN HIGH NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. *
Control (0 mg/l)	96.4	2	100	----	2252	2	100	
(1 mg/l)	97.0	6	101	N.S.	2251	6	100	N.S.
(100 mg/l)	101.1	6	105	N.S.	2227	6	99	N.S.

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

Phenyl-α-naphthylamine

Algal growth curves are presented in Figures 3 and 4. Summarized results of the statistical analyses are shown in Tables 4 and 5. This chemical showed a different pattern at the low (1% SAAM) nutrient concentration. Algal growth in 1% SAAM in the presence of 1 mg/l of the chemical was 3% greater for total algal volume and 14% greater for algal cell number as compared to the controls. The increase in cell number was statistically significant at the 95% level. The presence of 100 mg of phenyl-α-naphthylamine per liter of 1% SAAM caused a 93% reduction of cell number and 91% reduction in algal volume. Both of these results are significant at 99.9% level.

Algal cell numbers in the 33% SAAM in the presence of the 1 mg/l concentration of the chemical were significantly less (at the 95% level) than the controls. There was no difference in the maximum volume produced. The 100 mg/l concentration of this chemical did not cause a statistically different amount of growth in the 33% SAAM as compared to the controls. However, there was an average 50% reduction in the mean volume and a 61% reduction in cell number. The lack of statistical significance is due to the extreme variation in the effects of the chemical which resulted in large standard deviations for each group of flasks.

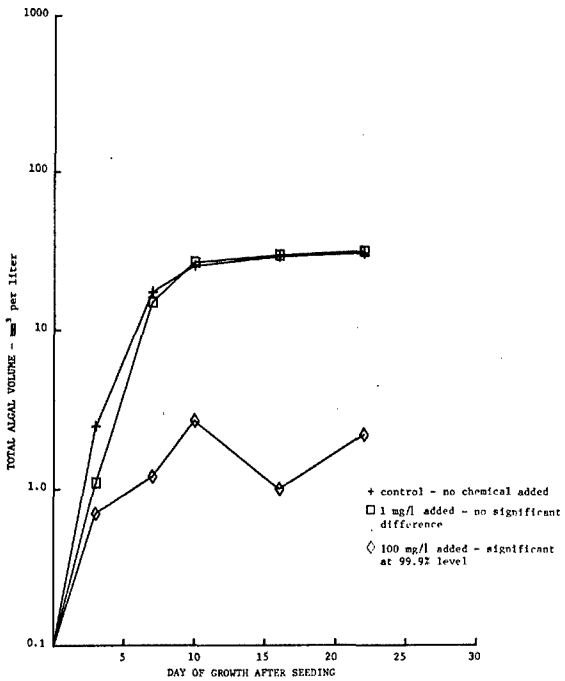

Figure 3. Growth of Selenastrum in low nutrient medium in the presence of phenyl-α-naphthylamine.

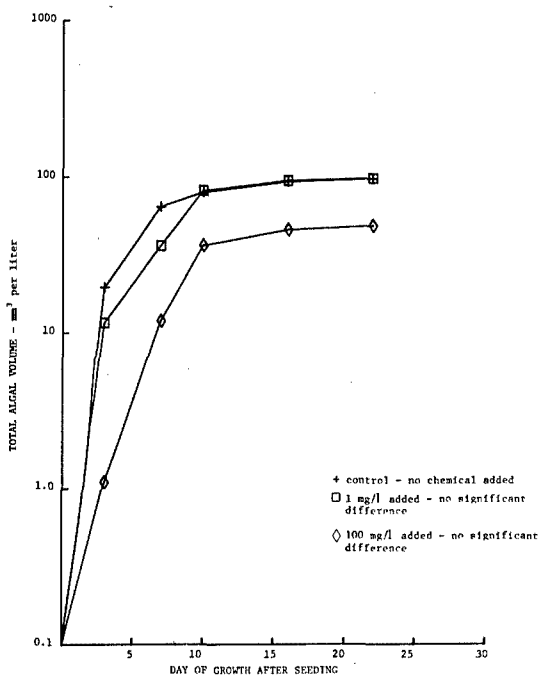

Figure 4. Growth of Selenastrum in high nutrient medium in the presence of phenyl-α-naphthylamine.

TABLE 4. EFFECT OF PHENYL-α-NAPHTHYLAMINE ON SELENASTRUM CAPRICORNUTUM IN LOW NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 mg/l)	30.2	2	100	----	594	2	100	----
(1 mg/l)	31.0	6	103	N.S.	673	6	114	*
(100 mg/l)	2.9	6	9	***	44	6	7	***

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

TABLE 5. EFFECT OF PHENYL-α-NAPHTHYLAMINE ON SELENASTRUM CAPRICORNUTUM IN HIGH NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 mg/l)	96.4	2	100	----	2251	2	100	---
(1 mg/l)	96.9	6	101	N.S.	2044	6	91	*
(100 mg/l)	48.4	6	50	N.S.	868	6	39	N.S.

† Indicate that growth in the presence of test chemical in concentrations shown in statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

RJ-5

The statistical significance of the effects of 1 and 100 µl/l concentrations of RJ-5 was determined for cell number and total cell volume data from the day of maximum standing crop. Algal growth curves showing growth as total cell volume versus time are presented in Figures 5 and 6, and the summarized results of the statistical analyses are shown in Tables 6 and 7. The only statistically significant effect of RJ-5 occurred in the 1% SAAM in the presence of the 100 µl/l concentration which caused a 22% reduction in algal volume. This reduction is significant at the 95% level.

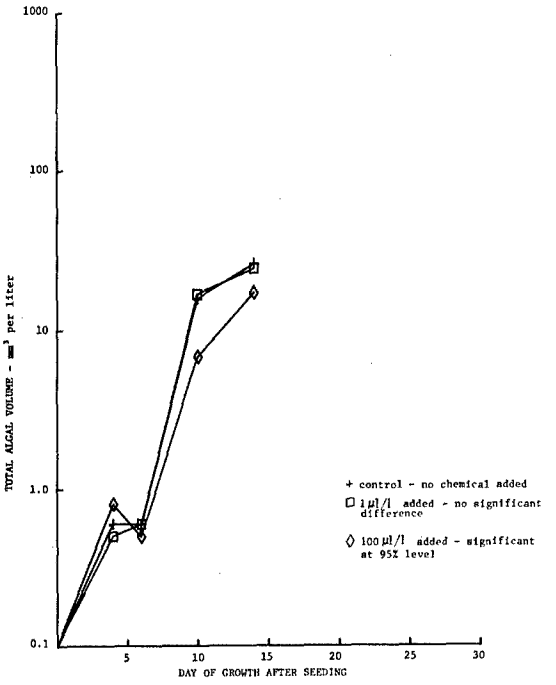

Figure 5. Growth of Selenastrum in low nutrient medium in the presence of RJ-5.

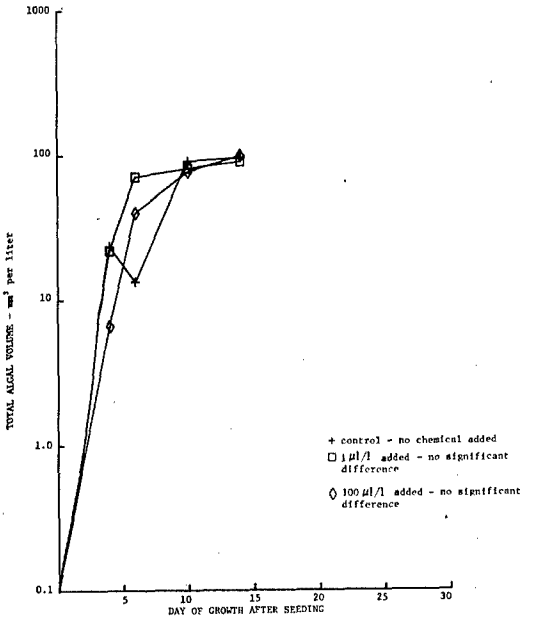

Figure 6. Growth of Selenastrum in high nutrient medium in the presence of RJ-5.

TABLE 6. EFFECT OF RJ-5 ON SELENASTRUM CAPRICORNUTUM IN LOW NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	29.2	5	100	---	600	5	100	---
(1 µl/l)	27.8	5	95	N.S.	540	5	90	N.S.
(100 µl/l)	22.9	5	78	*	443	5	74	N.S.

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

TABLE 7. EFFECT OF RJ-5 ON SELENASTRUM CAPRICORNUTUM IN HIGH NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	95.9	5	100	----	1957	5	100	----
(1 µl/l)	90.4	5	94	N.S.	1761	5	90	N.S.
(100 µl/l)	99.0	5	103	N.S.	1887	5	96	N.S.

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

RJ-4

Summarized results of the data analysis are presented in Figures 7 and 8 showing total algal volume as a function of time. The results are summarized in Tables 8 and 9. Results of the analyses show that there was some inhibition of growth in the 100 µl/l concentration of RJ-4. This effect was statistically significant at the 95% level for both number and volume data in the 1% SAAM and for cell number data only in the 33% SAAM, when compared to the controls. The 1 µl/l concentration of RJ-4 did not have any significant effect on the algal growth.

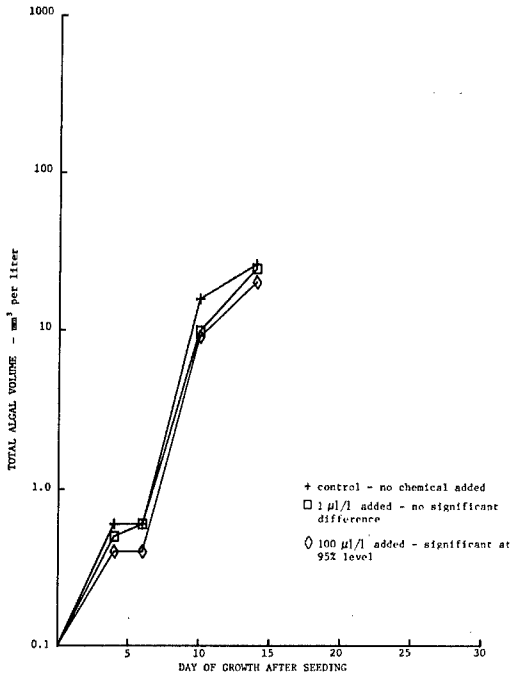

Figure 7. Growth of Selenastrum in low nutrient medium in the presence of RJ-4.

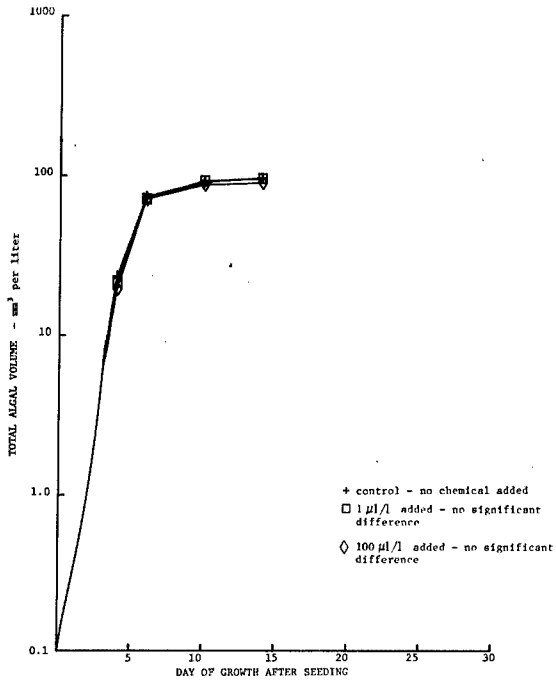

Figure 8. Growth of Selenastrum in high nutrient medium in the presence of RJ-4.

TABLE 8. EFFECT OF RJ-4 ON SELENASTRUM CAPRICORNUTUM IN LOW NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	29.2	5	100	----	600	5	100	----
(1 µl/l)	27.8	5	95	N.S.	584	5	97	N.S.
(100 µl/l)	23.8	5	82	*	463	5	77	*

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

TABLE 9. EFFECT OF RJ-4 ON SELENASTRUM CAPRICORNUTUM IN HIGH NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	95.9	5	100	----	1957	5	100	----
(1 µl/l)	95.1	5	99	N.S.	1913	5	98	N.S.
(100 µl/l)	89.6	5	93	N.S.	1713	5	88	*

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

Hydrazine

The statistical significance of the effects of 1 and 10 µl/l concentrations of hydrazine was determined for cell number and total cell volume data from the day of maximum standing crop. The 10 µl/l concentration was used instead of the 100 µl/l concentration because of the expected high toxicity of hydrazine. Plots of algal growth as a function of time are shown in Figures 9 and 10 and the detailed results are summarized in Tables 10 and 11.

Analysis of algal growth in the 1% SAAM shows that there is a highly significant (99.9% level) statistical difference in the maximum algal cell numbers and volumes produced in the presence of 1 and 10 µl/l concentrations of hydrazine when compared with the controls without the chemical.

The presence of hydrazine in concentrations as low as 1 µl/l resulted in a 200 times decrease in the total amount of algae growing at the end of the test period, indicating a high persistent toxic effect of this compound in a low nutrient water.

The results obtained in more nutrient rich 33% SAAM, which more closely represents eutrophic lake conditions, are more complex. The presence of 1 µl/l and 10 µl/l showed an initial growth reduction compared to the controls. Toward the end of the growth experiment this toxic effect began to disappear in the flasks to which 1 µl/l had been added but persisted in the 10 µl/l flasks. The maximum volume of algal cells produced in the presence of 1 µl hydrazine per liter of 33% SAAM was statistically different from the controls at the 99% level of significance. Since algal cells usually enlarge somewhat with age, this difference might have become insignificant if the experiment had been continued for a few more days. There was no difference in the number of cells produced in the presence of the 1 µl concentration of hydrazine. The 10 µl concentration was extremely toxic resulting in a 98% to 99% lower algal growth than in the controls. The difference is significant at the 99.9% level.

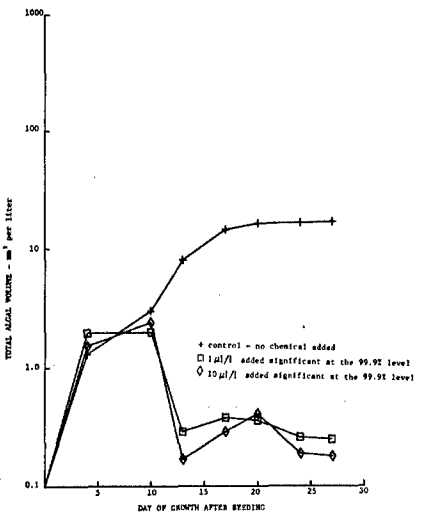

Figure 9. Growth of Selenastrum in low nutrient medium in the presence of hydrazine.

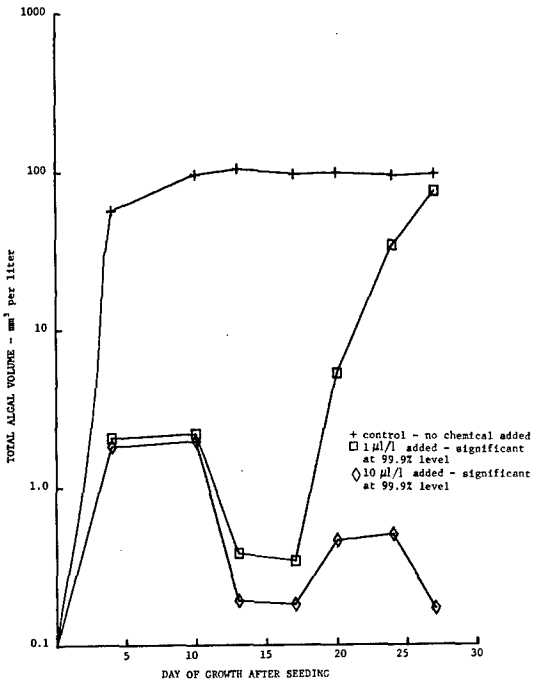

Figure 10. Growth of Selenastrum in high nutrient medium in the presence of hydrazine.

TABLE 10. EFFECT OF HYDRAZINE ON SELENASTRUM CAPRICORNUTUM IN LOW NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	16.6	3	100	----	219	3	100	----
(1 µl/l)	2.0	5	12	***	9	5	4	***
(10 µl/l)	2.4	5	14	***	10	5	5	***

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

TABLE 11. EFFECT OF HYDRAZINE ON SELENASTRUM CAPRICORNUTUM IN HIGH NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Agal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	103.9	5	100	----	1706	5	100	----
(1 µl/l)	74.4	5	72	**	1925	5	113	N.S.
(10 µl/l)	2.0	5	2	***	1	5	1	***

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; ***= 99.9%; N.S. = Not Significant.

Unsymmetrical Dimethylhydrazine

Results of the statistical analyses to determine the effects of 1 and 10 µl/l concentrations of UDMH are summarized in Tables 12 and 13. Algal growth curves showing cell volume as a function of time are presented in Figures 11 and 12. The addition of the 1 µl/l UDMH to the 1% SAAM resulted in a 15% increase in algal volume, which is not statistically significant, and a 55% increase in cell number as compared with the controls. The increase in cell number is significant at the 99% level. An addition of 10 µl UDMH/l of 1% SAAM resulted in a decrease in algal growth which is significant at the 99% and 99.9% levels for volume and cell data respectively.

Growth results in the 33% SAAM were somewhat different. The 1 µl/l concentration of UDMH did not have a significant effect on either cell number or volume as compared with the controls. The addition of the 10 µl/l concentration caused a 57% (significant at the 99% level) to 67% decrease (significant at the 99.5% level) in algal growth.

TABLE 12. EFFECT OF UNSYMMETRICAL DIMETHYLHYDRAZINE ON SELENASTRUM CAPRICORNUTUM IN LOW NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	17.3	4	100	----	166	4	100	----
(1 µl/l)	19.9	5	115	N.S.	258	5	155	**
(10 µl/l)	10.1	5	58	**	84	5	51	***

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

TABLE 13. EFFECT OF UNSYMMETRICAL DIMETHYLHYDRAZINE ON SELENASTRUM CAPRICORNUTUM IN HIGH NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	111.8	5	100	----	1908	5	100	----
(1 µl/l)	105.2	5	94	N.S.	1723	5	90	N.S.
(10 µl/l)	48.1	5	43	**	633	5	33	***

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

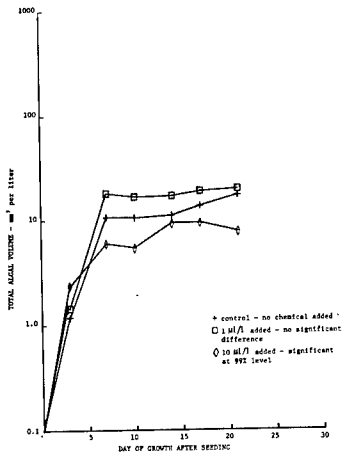

Figure 11. Growth of Selenastrum in low nutrient medium in the presence of unsymmetrical dimethylhydrazine.

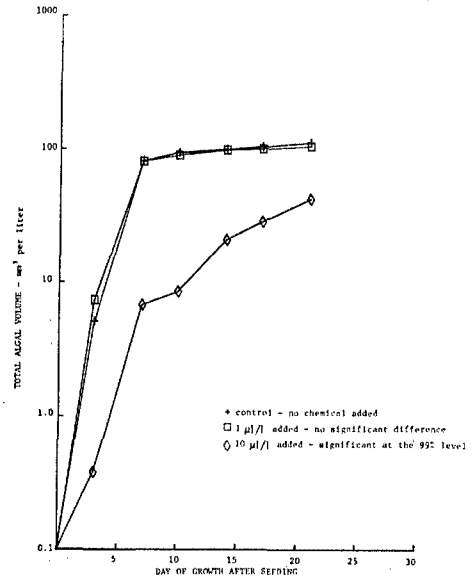

Figure 12. Growth of Selenastrum in high nutrient medium in the presence of unsymmetrical dimethylhydrazine.

Monomethylhydrazine

Algal growth curves are presented in Figures 13 and 14 and the summaries of the statistical analyses are in Tables 14 and 15. This compound showed a different pattern in the low 1% SAAM nutrient concentration as compared with 33%. There was no statistically significant difference in the amount of algae produced in the presence of 1 and 10 µl/l of monomethylhydrazine when compared to controls. The presence of 1 µl/l of monomethylhydrazine in 33% SAAM resulted in a 50% decrease in total algal volume and a 63% decrease in algal cell growth when compared with the controls. This decrease in growth is significant at the 99.9% level. An addition of 10 µl of MMH resulted in a 99% decrease in algal cell number and volume which is significant at the 99% level.

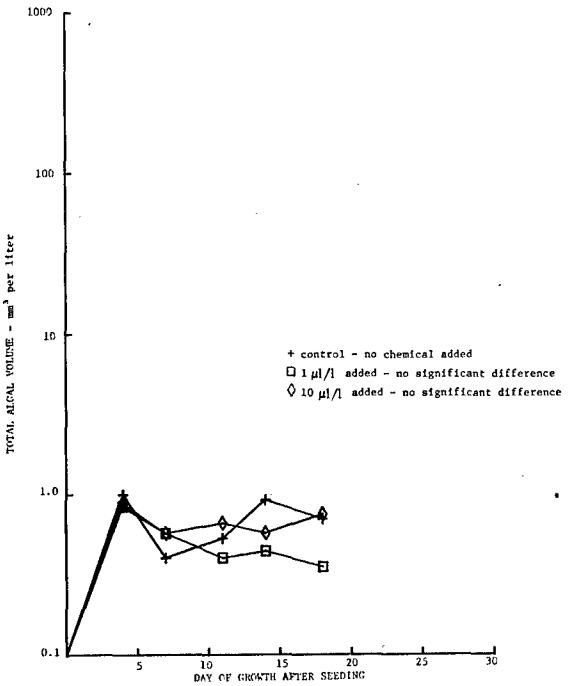

Figure 13. Growth of Selenastrum in low nutrient medium in the presence of monomethylhydrazine.

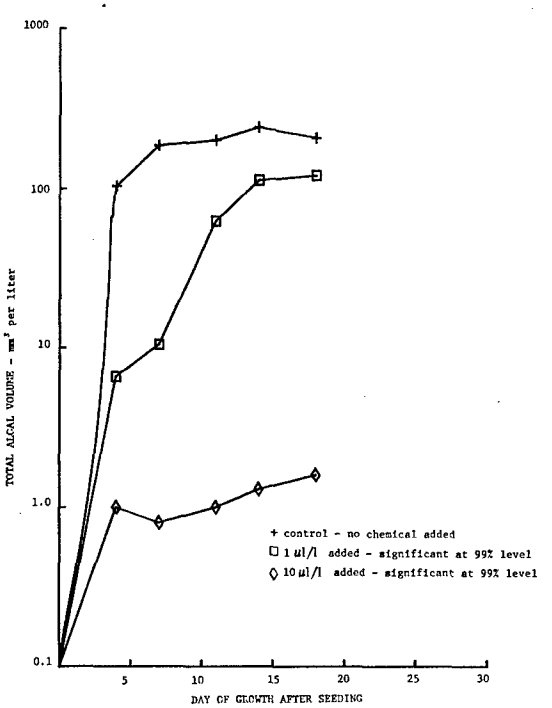

Figure 14. Growth of Selenastrum in high nutrient medium in the presence of monomethylhydrazine.

TABLE 14. EFFECT OF MONOMETHYLHYDRAZINE ON SELENASTRUM CAPRICORNUTUM IN LOW NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	1.10	5	100	----	22.7	5	100	----
(1 µl/l)	1.01	5	92	N.S.	17.6	5	77	N.S.
(10 µl/l)	1.01	5	92	N.S.	18.2	5	80	N.S.

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

TABLE 15. EFFECT OF MONOMETHYLHYDRAZINE ON SELENASTRUM CAPRICORNUTUM IN HIGH NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 µl/l)	242	5	100	----	2355	5	100	----
(1 µl/l)	121	5	50	***	861	5	37	***
(10 µl/l)	1.7	5	1	***	25	5	1	***

† Indicate that growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

Symmetrical Dimethylhydrazine

Algal growth curves showing cell volume versus time are seen in Figures 15 and 16. Summarized results of the statistical analyses of the maximum standing crop data are presented in Tables 16 and 17. These results indicate that neither the 1 nor 10 mg/l concentration of SDMH had a statistically significant difference on the amount of algal growth in 1% SAAM. The presence of SDMH at the 1 mg/l concentration did not result in a significant difference in the amount of algae produced in 33% SAAM. However, the 10 mg/l concentration resulted in a 50% decrease in cell number and 71% decrease in total algal volume as compared with the control flasks without SDMH. This decrease in algal growth is statistically significant at the 95% level for cell number and at the 99.9% level for cell volume.

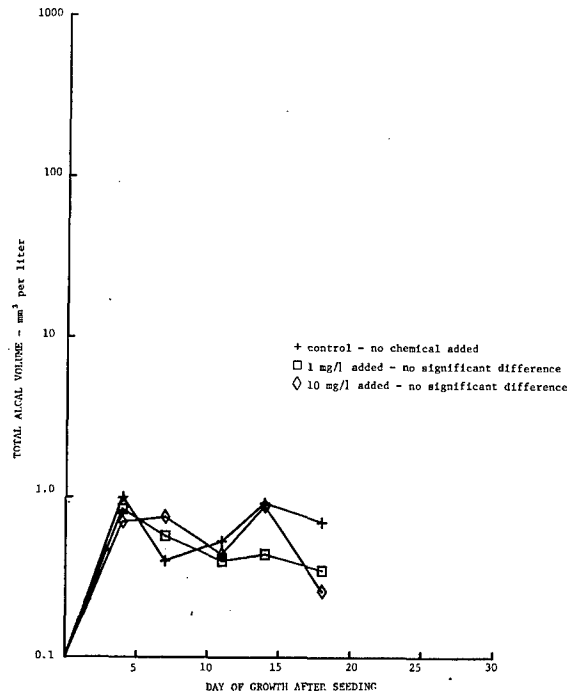

Figure 15. Growth of Selenastrum in low nutrient medium in the presence of symmetrical dimethylhydrazine.

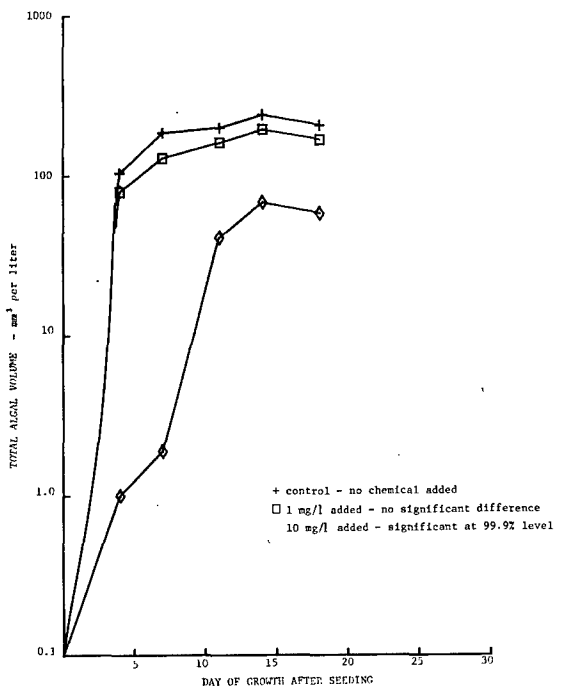

Figure 16. Growth of Selenastrum in high nutrient medium in the presence of symmetrical dimethylhydrazine.

TABLE 16. EFFECT OF SYMMETRICAL DIMETHYLHYDRAZINE ON SELENASTRUM CAPRICORNUTUM IN LOW NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 mg/l)	1.10	5	100	----	22.7	5	100	----
(1 mg/l)	0.84	5	76	N.S.	15.3	5	67	N.S.
(10 mg/l)	1.23	5	112	N.S.	18.1	5	80	N.S.

† Indicate that growth in the presence of test chemical in concentrations shown is statistically-different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

TABLE 17. EFFECT OF SYMMETRICAL DIMETHYLHYDRAZINE ON SELENASTRUM CAPRICORNUTUM IN HIGH NUTRIENT MEDIUM

Amount of Test Comp. Added	Algal Cell Volume: Average Max. Standing Crop mm^3/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †	Algal Cell Number: Average Max. Standing Crop 10^6 cells/l	No. of Repl.	Percent of Growth in Controls	Level of Signif. of Diff. †
Control (0 mg/l)	242	5	100	----	2355	5	100	----
(1 mg/l)	196	5	82	N.S.	1924	5	82	N.S.
(10 mg/l)	69	5	29	***	1186	5	50	*

† Indicate the growth in the presence of test chemical in concentrations shown is statistically different from the control at the following levels of significance: * = 95%; ** = 99%; *** = 99.9%; N.S. = Not Significant.

SUMMARY

The investigations presented in this paper were conducted as the first year of a five year program to determine the possible effect of various chemical compounds on the fresh water algal ecosystems.

The possible effects were determined for the following chemicals under the test conditions shown:

COMPOUND TESTED	TEST CONDITIONS USED
Lube Additives	
p, p'Dioctyldiphenylamine (Vanlube 81)	Batch
Phenyl-α-naphthylamine	Batch
Jet Fuels	
RJ-5 (Petroleum base)	Batch
RJ-4 (Petroleum base)	Batch
Hydrazines	
Hydrazine	Batch
Unsymmetrical Dimethylhydrazine (UDMH)	Batch
Monomethylhydrazine (MMH)	Batch
Symmetrical Dimethylhydrazine (SDMH)	Batch

The possible effects were determined under standard laboratory conditions by comparing the algal growth in the presence of the test chemical with the algal growth in the absence of the chemical.

The laboratory test conditions were selected to simulate the following conditions:

Natural Water Quality: Oligotrophic/Eutrophic
Type of Water Body: Lake/Stream
Quantity of Compound Released: Large/Small

CONCLUSIONS

The conclusions for the individual compounds are summarized below. It is important to realize that these conclusions are reached on the basis of only one algal species under laboratory conditions. Subsequent testing during the remainder of the program will include verification of these conclusions by testing some of the typical compounds with other algae and under other conditions.

Lube Additives

p, p'Dioctyldiphenylamine

The addition of this compound showed no effect on algal growth under batch or continuous culture conditions in either concentration of 1 mg/l or 100 mg/l.

Phenyl-α-naphthylamine

Phenyl-α-naphthylamine was only tested under batch conditions. The presence of this compound in a concentration of 1 mg/l had no significant effect on algal cell volume but caused a 14 percent increase (significant at the 95% level) on the number of algal cells grown.

The presence of phenyl-α-naphthylamine at 100 mg/l resulted in only 7 and 9 percent respectively of the growth in the controls for the low nutrient medium representing olitogrophic mountain lakes. In the high nutrient medium (eutrophic lakes), the presence of 100 mg/l phenyl-α-naphthylamine reduced the growth to less than 50 percent of that in the controls based on the average of the individual test flasks. However, this difference was not statistically significant because of the high degree of variability between the individual replicates.

Comparison of Lube Additives

Based on the results obtained in this investigation and the understanding that these tests are not representative of all fresh water algal systems, it can be concluded that p, p'dioctyldiphenylamine appears to be better than phenyl-α-naphthylamine with respect to having the least effect on fresh water algal growth.

Jet Fuels

RJ-5

The presence of 1 μl/l RJ-5 reduced the average growth by five to ten percent under the low and high nutrient conditions, but the difference was not statistically significant at the 95 percent level.

The presence of 100 µl/l RJ-5 reduced the average growth to 78 (cell volume) and 74 percent (cell number) of the controls in the low nutrient medium (oligotrophic mountain lakes). This reduction was statistically significant at the 95 percent level for the cell volume but not for the cell number results. There was no significant effect of 100 µl/l RJ-5 in the high nutrient medium (eutrophic lakes).

RJ-4

The results for RJ-4 were quite similar to those obtained for RJ-5. There was no statistically significant effect observed with 1 µl/l RJ-4 in either the high or low nutrient medium. The addition of 100 µl/l RJ-4 reduced the growth in the low nutrient medium to about 80 percent of the growth in the controls and to about 90 percent in the high nutrient medium. The differences at 100 µl/l RJ-4 were significant at the 95 percent level with the exception of cell volume under the high conditions.

Comparison of Jet Fuels

The results of this testing do not indicate any substantial difference between the effects of RJ-4 and RJ-5 in the aquatic algal systems tested here.

Hydrazines

Hydrazine

Hydrazine was found to be very toxic to the algal test system. With addition of 10 µl/l the algal cell volume growth was only 14 percent of the controls in the low nutrient medium and only two percent of the control in the high nutrient medium. These differences were all statistically significant at the 99.5% level. At the low concentration of 1 µl/l hydrazine, the effect was equally pronounced in the low nutrient medium. However, in the high nutrient medium, the initial growth inhibition appeared to disappear with time as seen in Figure 10. It is possible that this reduction in toxicity is a result of bacterial decomposition or adsorption of the hydrazine on the first cells grown, thus removing the hydrazine from the test system.

Unsymmetrical Dimethylhydrazine (UDMH)

The addition of 10 µl/l UDMH reduced the growth of algae to about one-half of the growth in the controls under both high and low nutrient conditions. This difference was statistically significant at least at the 99 percent level. The addition of 1 µl/l showed an average 10 percent reduction under high nutrient conditions but the reduction was not statistically significant. Under the low nutrient conditions, the addition of 1 µl/l UDMH resulted in an increase in algal growth reflecting a possible bacterial decomposition of UDMH and a release of nutrients (nitrogen?).

Monomethylhydrazine

Monomethylhydrazine had no statistically significant effect in the 1% SAAM, but was found to be extremely toxic to the algae in the 33% nutrient. The addition of 1 µl/l of MMH reduced the algal growth to about one-half, whereas the addition of 10 µl/l reduced growth to about 1%. These differences were all statistically significant at the 99% level.

Symmetrical Dimethylhydrazine

The 1 mg/l concentration of SDMH had no effect at either nutrient level, nor did the 10 mg/l concentration affect algal growth in the 1% SAAM. However, in the high nutrient medium, 10 mg/l SDMH reduced the cell numbers to 50% and the cell volume to 29% of the controls.

Comparison of Hydrazines

The effects of the four hydrazines are summarized in Table 18. The hydrazine and the monomethylhydrazine showed the greatest effects, with the unsymmetrical and symmetrical dimethylhydrazines showing significantly less effect on growth of test algae under standard conditions. In the cases where the high concentration of the test compound was added (10 ppm), the most significant result was noted in the high nutrient test medium (eutrophic lakes). In the cases where low concentration of the test compound was added, there was no clear pattern of toxic effects observed and only in three cases was any statistically significant effect observed at all in either nutrient level.

TABLE 18. SUMMARY OF EFFECTS OF TEST COMPOUNDS ON MAXIMUM ALGAL VOLUME PRODUCED

Test Compound	Low nutrient (1% SAAM) % of Control Growth	Low nutrient (1% SAAM) Statistical Significance	High nutrient (33% SAAM) % of Control Growth	High nutrient (33% SAAM) Statistical Significance
Hydrazine				
1 µl/l	12	+	72	+
10 µl/l	14	+	2	+
Unsymmetrical dimethylhydrazine				
1 µl/l	115	-	94	-
10 µl/l	58	+	43	+
Monomethylhydrazine				
1 µl/l	92	-	50	+
10 µl/l	92	-	1	+
Symmetrical dimethylhydrazine				
1 mg/l	76	-	82	-
10 mg/l	112	-	29	+

On an overall basis, the results indicate that if it is possible to choose any one of the four different hydrazine compounds for a given use which can lead to aquatic contamination, then preference should be given to one of the two dimethylhydrazines.

ACKNOWLEDGEMENTS

Dr. Barbara Gittins, Mrs. Carol Justice, and Mrs. Cecilia Kiang have been Staff Research Associates for the project, responsible for the detailed measurements and data collection. Richard Appleman and Patrick O'Brien have performed part of the investigations in connection with their doctoral research.

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AMRL-TR-75-125

PAPER NO. 26

EFFECTS OF POLLUTANTS ON EMBRYOS AND LARVAE OF FROGS: A SYSTEM FOR EVALUATING TERATOGENIC EFFECTS OF COMPOUNDS IN FRESH WATER ENVIRONMENTS

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INTRODUCTION

The objective of this study was twofold: (1) to establish a pollutant test system using embryos and larvae* of amphibian species, and (2) to use this system to screen a number of compounds designated by the Air Force. Efforts focused on determination of lethal and teratogenic levels of exposure. Two species of frog were utilized as test animals, Rana pipiens, the leopard frog, and Xenopus laevis, the South African clawed toad. Both of these species live and breed in aquatic habitats exhibiting a wide range of temperature and varying water quality, they are commercially available, and they are adaptable to laboratory conditions.

Tadpoles have been shown to be sensitive to a number of environmental pollutants of demonstrated toxicity such as pesticides (Cabejszek and Wojcik, 1968; Cook, 1972, 1973; Bancroft and Prahled, 1973), herbicides (Pravda, 1973), and heavy metals (Birge and Just, 1973); and to known teratogens such as trypan blue (Greenhouse and Hamburgh, 1968), LiCl (Hall, 1942), and tetracycline (Greenhouse, 1975).

Thus two factors, sensitivity to chemicals in the environment and adaptability to laboratory conditions, make embryonic and larval stages of frogs excellent indicators of water quality.

*See Appendix 1 for definition of embryo and larvae.

The above test system was used to screen two groups of compounds designated by the Air Force as potential environmental pollutants. The first group consisted of three substituted amines, N-phenyl-α-naphthylamine, octyl-phenyl-α-naphthylamine, and p,p'dioctyldiphenylamine. Only N-phenyl-α-naphthylamine was found to have toxic effects on developing frog embryos and larvae. The second group of compounds tested included hydrazine and its methylated derivatives (mono)methylhydrazine, symmetrical dimethylhydrazine, and unsymmetrical dimethylhydrazine. All four of these compounds were found to have toxic effects on developing frog embryos and larvae.

RESULTS

Effects of N-phenyl-α-naphthylamine, octyl-phenyl-α-naphthylamine, and p,p'dioctyldiphenylamine on Frog Embryos and Larvae

These amines are relatively insoluble in water. Regardless of whether they were added to the water as a dry powder or as an acetone solution, some insoluble residue remained. Therefore, two types of test media were prepared: suspensions and solutions. Solutions were prepared by agitating suspensions of amine on a reciprocating shaker and then filtering out the undissolved particles. The amount of amine in solution was then determined by spectroscopy.

N-phenyl-α-naphthylamine

This was the only one of the three amines which was toxic to frog larvae and embryos. Exposure to suspensions of this compound produced death or abnormal development in both R. pipiens and X. laevis. The actual response of the test animals varied with the concentration of the amine, the duration of exposure, and the developmental stage of the animals.

Exposure to concentrations of N-phenyl-α-naphthylamine $\geq$ 6.0 mg/l was lethal to larvae. When exposed to lethal concentrations of the amine the following syndrome was observed. Swimming activity decreased during the first hour of exposure and ceased by the end of the second. However, the larvae still responded to pricking. Continued exposure led to a gradual decrease in heart rate which eventually became irregular. Death occurred within 24 hours.

Larvae growing in lethal concentrations of N-phenyl-α-naphthylamine which were transferred to uncontaminated water after only brief exposures usually survived and developed normally. Survival rate was inversely proportional to duration of exposure. Data on toxicity of amines to frog larvae are summarized in Tables 1 and 2.

TABLE 1. EFFECT OF EXPOSURE TO SOLUTIONS OF NAPHTHYLAMINES ON VIABILITY OF XENOPUS LARVAE

Compound	Conc. mg/l	No. Larvae Exposed	No. Larvae Surviving 124 hours	No. Larvae Surviving 168 hours
Control	0.0	90	84	82
N-phenyl-α-naphthylamine	5.6	85	85	80
	6.0	75	75	0
	6.2	20	0	0
	7.1	36	0	0
N-phenyl-α-naphthylamine Tris buffer pH 7.6	6.0	75	75	0
Octyl-phenyl-α-naphthylamine	0.67	46	46	43
	1.00	15	15	15

TABLE 2. EFFECT OF EXPOSURE OF RANA PIPIENS LARVAE TO SUSPENSIONS OF ORGANIC AMINES

Compound	Conc. mg/l	No. Larvae Exposed	No. Larvae Surviving 24 hours	No. Larvae Surviving 48 hours
Control	0	125	125	125
N-phenyl-α-naphthylamine	5	125	125	0
Octyl-phenyl-α-naphthylamine	50	125	125	125
Dioctyldiphenylamine	100	125	125	125

Exposure to N-phenyl-α-naphthylamine was also toxic to embryonic stages. Data are summarized in Tables 3 and 4. Exposure of Xenopus embryos to concentrations of N-phenyl-α-naphthylamine ≤ 5.2 mg/l produced no observable effects. Concentrations ≥ 6.2 mg/l were lethal.

TABLE 3. EFFECT OF EXPOSURE TO SOLUTIONS OF OCTYL-PHENYL-α-NAPHTHYLAMINE AND N-PHENYL-α-NAPHTHYLAMINE ON XENOPUS EMBRYOS

Compound	Conc. mg/l	No. Emb. Exp.	No. Emb. Malf.	% Surv. Malf.	No. Emb. Died
Control	0.0	100	1	1.4	28
Octyl-phenyl-α-naphthylamine	1.0	100	8	8.9	10
N-phenyl-α-naphthylamine	5.2	100	17	23.3	27
	6.2	100	21	100	79
	7.2	100	10	100	90

TABLE 4. TERATOGENIC EFFECT OF EXPOSURE TO SUSPENSIONS OF ORGANIC AMINES ON DEVELOPMENT OF RANA PIPIENS EMBRYOS

Compound	Conc. mg/l	No. Emb. Exp.	No. Emb. Malf.
N-phenyl-α-naphthylamine	20	100	100
	200	100	100
Octyl-phenyl-α-naphthylamine	20	100	0
	200	100	0
Dioctyldiphenyl-amine	20	100	0
	200	100	0
Control		100	0

Embryos of R. pipiens or X. laevis showed no effects from exposure to N-phenyl-α-naphthylamine until they reached stages* 18 and 24 respectively. These stages are characterized by motor reactions to external stimuli. Embryos exposed to toxic levels of this compound usually failed to respond to external stimuli. Embryos exposed to toxic levels of this compound usually failed to respond to external pricking. Continued exposure resulted in death of embryos of either species.

Concentrations of N-phenyl-α-naphthylamine $\geq$ 5.2 mg/l were teratogenic to Xenopus embryos. Malformed embryos died before completing metamorphosis. The syndrome of anomalies was complex, but most resembled micromyelia.

Octyl-phenyl-α-naphthylamine

This compound was not toxic to either embryos or larvae of R. pipiens or X. laevis. Suspensions of up to 1 g/l were tested. Highest concentrations of this compound appeared to retard the developmental rate of tadpoles. However, histological examination indicated that these animals were not malformed. Development in continuous contact with octyl-phenyl-α-naphthylamine did not prevent larvae from metamorphosing into normal froglets.

p, p'Dioctyldiphenylamine

This compound was not toxic to either embryos or larvae of R. pipiens or X. laevis. Suspensions of up to 1 g/l were tested. Fertilized eggs allowed to develop in continuous contact with dioctyldiphenylamine completed embryogenesis and metamorphosis normally.

*See Appendix 2 for method of staging embryos and larvae.

Effects of Hydrazine, (Mono)methylhydrazine, and Dimethylhydrazine on Xenopus Larvae

Hydrazine

Xenopus larvae exposed to hydrazine at concentrations of 0.2 mg/l or greater exhibited irreversible toxic effects and died within 24-48 hours (Table 5). At concentrations of 0.1 mg/l hydrazine was not toxic to Xenopus larvae. Animals exposed to this concentration of hydrazine throughout their development metamorphosed normally (Table 6).

Methylhydrazine

Larvae exposed to 1 mg/l of this compound exhibited irreversible toxic effects and died within 48 hours (Table 5). Larvae in continuous contact with 0.1 mg/l hydrazine throughout their development metamorphosed normally (Table 6).

Symmetrical Dimethylhydrazine (SDMH)

Exposure of larvae to 1 mg/l of SDMH was lethal to 100% of the animals within 7 days (Table 5). Larvae in continuous contact with 0.1 mg/l SDMH throughout their development survived and metamorphosed normally (Table 6).

Unsymmetrical Dimethylhydrazine (UDMH)

UDMH was not toxic to larvae at concentrations of 1 mg/l. Animals exposed to this concentration of UDMH throughout development survived and metamorphosed normally (Table 6). Higher concentrations were lethal (Table 6).

TABLE 5. EFFECT OF ACUTE EXPOSURE TO HYDRAZINES ON VIABILITY OF XENOPUS LAEVIS LARVAE

Compound	Conc. mg/l	No. Larvae Exposed	No. Larvae Surv. (120 hr.)	% Surv.
Control		125	123	98
Hydrazine	0.01	50	50	100
Hydrazine	0.10	55	48	87
Hydrazine	0.20	50	0	0
Control		265	263	99
Methylhydrazine	0.10	50	50	100
Methylhydrazine	1.0	50	0	0
Control		65	65	100
SDMH	0.1	5	5	100
SDMH	1.0	62	0	0
Control		69	67	97
UDMH	1.0	69	68	98
UDMH	2.0	50	0	0

TABLE 6. EFFECT OF CHRONIC EXPOSURE TO HYDRAZINES ON DEVELOPMENT OF XENOPUS LAEVIS LARVAE

Compound	Conc. mg/l	No. Larvae Exposed	No. Larvae Metamor.	% Metamor.
Control		150	110*	73
Hydrazine	0.1	50	36	72
Methylhydrazine	0.1	50	34	68
SDMH	0.1	50	39*	78
UDMH	0.1	50	42*	84
UDMH	1.0	50	37	74

*One animal from each of these groups had abnormal hindlimbs.

Effects of Hydrazine, Methylhydrazine, and Dimethylhydrazine on Xenopus Embryos

Determination of teratogenic concentrations of Hydrazines

All four of these compounds were teratogenic. Table 7 summarizes representative data used to determine safe and toxic levels of exposure. Cleavage stage embryos were placed in solutions containing various concentrations of the desired compound. Embryos were allowed to develop in continuous contact with the hydrazine (or its methylated derivative) until hatching.

TABLE 7. TERATOGENIC EFFECT OF HYDRAZINES ON DEVELOPMENT OF XENOPUS LAEVIS EMBRYOS

Compound	Conc. mg/l	No. Emb. Exp.	No. Emb. Malf.	% Malf.
Control	0	700	43	6
Hydrazine	1	100	3	3
Hydrazine	10	150	52	35
Hydrazine	25	100	100	100
Methylhydrazine	3	100	1	1
Methylhydrazine	5	200	104	52
Methylhydrazine	10	200	189	94
Methylhydrazine	15	50	50	100
SDMH	40	100	5	5
SDMH	50	50	40	80
SDMH	55	100	100	100
UDMH	2	100	10	10
UDMH	5	200	109	54
UDMH	10	150	73	49
UDMH	20	100	100	100

Hydrazine

Exposure to less than 10 mg/l of hydrazine had no observable effect on the development of embryos. Concentrations of hydrazine in excess of 10 mg/l were always teratogenic. The threshold of tolerance to this compound appeared to be at 10 mg/l, as exposure of embryos to this concentration of hydrazine was teratogenic in some instances but not in others. Embryos exposed to concentrations of hydrazine as high as 100 mg/l completed gastrulation and continued development.

Methylhydrazine

Exposure to less than 5 mg/l of methylhydrazine had no effect on the development of embryos. Concentrations in excess of 5 mg/l of this compound were invariably teratogenic. The threshold of tolerance to this compound appeared to be about 5 mg/l. This compound appeared to be the most toxic of the four. Embryos exposed to 28 mg/l of methylhydrazine died without completing gastrulation.

Symmetrical Dimethylhydrazine (SDMH)

This compound was the least teratogenic of the four. Exposure of embryos to 40 mg/l of SDMH had no effect on development. At 50 mg/l or greater SDMH was teratogenic.

Unsymmetrical Dimethylhydrazine (UDMH)

Exposure of embryos to less than 5 mg/l of this compound was never toxic. Concentrations of UDMH in excess of 5 mg/l were usually teratogenic.

Embryos exposed to nontoxic concentrations of these compounds developed normally to hatching. If they were then transferred to uncontaminated water, the embryos developed into normal larvae. This transfer to uncontaminated water was necessary because larval stages are more sensitive to the toxic effects of hydrazines than are the embryonic stages (compare Tables 6 and 7).

The Syndrome of Malformations Caused by Exposure to Hydrazines

In discussing the malformations induced in frog embryos by exposure to hydrazine or methylated derivatives, these compounds may be grouped as a unit. Although the teratogenic concentration of the individual compounds varied, the syndrome of abnormalities induced by these compounds was identical. Regardless of when exposure to the teratogen was initiated, abnormalities did not become apparent until after tail bud stage when the embryos began to elongate. The types of malformations observed can be grouped into two classes: those that affect the axial skeleton, tail, and trunk, and those that affect the head.

Malformations of the Axial Skeleton, Tail, and Trunk

The most prevalent malformation induced by exposure to hydrazines was kinked tail. The kinks were observed in both the dorso-ventral plane and the horizontal plane. The occurrence of more than one kink per animal was not unusual (Figure 1). Although these kinks were not intrinsically lethal in laboratory raised animals, they did interfere with mobility and would probably affect viability in a competitive environment. Kinks occurring very close to the base of the tail sometimes displaced a hind limb bud and secondarily resulted in an abnormal pelvic girdle. But most larvae with kinked tails and no other abnormalities metamorphosed into normal frogs.

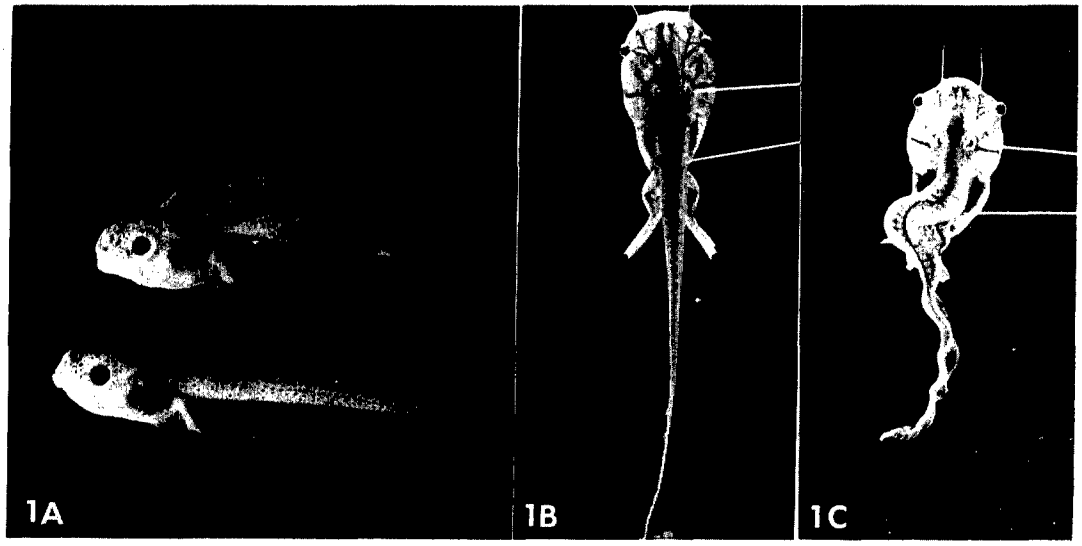

Figure 1. Tail kinks caused by exposure of embryos to hydrazine(s) during neurulation. A. Lower animal is a control, upper animal was exposed to methylhydrazine. B. Control at stage 57. C. Larva exposed to hydrazine.

Macroscopic observation indicated that the appearance of kinks was preceded by the formation of what seemed to be fluid filled cysts in the region between the axial skeleton and the epidermis. However, microscopic analysis of serially sectioned embryos revealed that what had looked like cysts under the dissecting scope were actually outgrowths of the notochord. These notochordal outgrowths increased in size with time displacing both segmental muscle and neural tube (Figure 2).

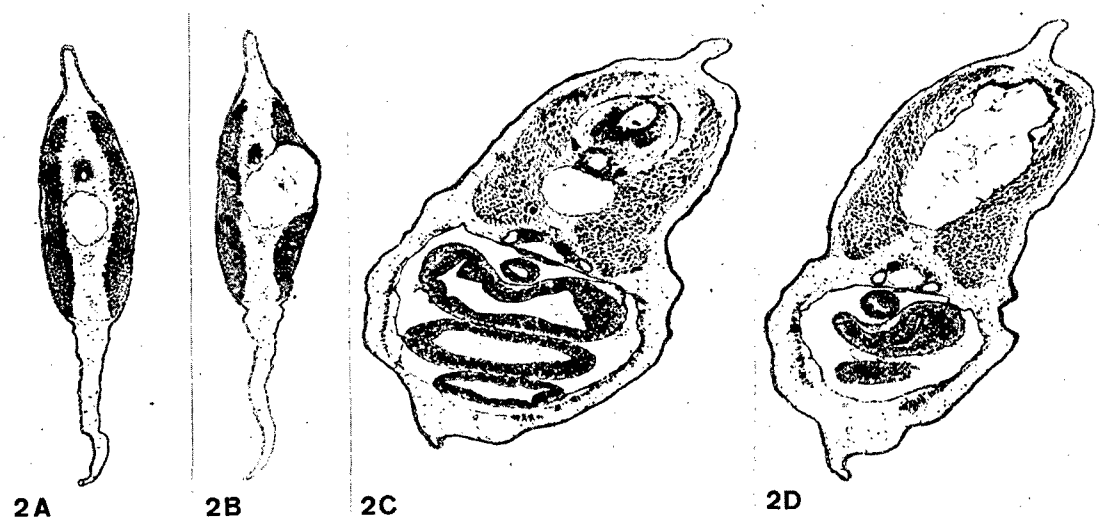

Figure 2. Sections through the caudal region of Xenopus larvae. A. Section through the tail of control. B. Section through the tail of larva exposed to methylhydrazine. Note enlarged notochord which has perforated the lateral muscle layer. C. Section through the level of the hindgut of a control larva. D. Section through the level of the hindgut of a larva exposed to methylhydrazine. Note the enlarged notochord which has replaced the neural tube.

A less common, but nevertheless interesting, aberration affecting the caudal region of embryos exposed to hydrazines was duplication of the tail. The anomaly was always accompanied by shortening of the trunk. The two tails were always gnarled and twisted.

Microscopic analysis of serially sectioned embryos revealed the presence of a bifurcated notochord in the caudal region of all two-tailed animals. If the bifurcation occurred anterior to the tail bud, two neural tubes were present as well as two notochords and two tails.

The second most common malformation observed was micromyelia, abnormal shortness of the spinal chord (Figure 3).

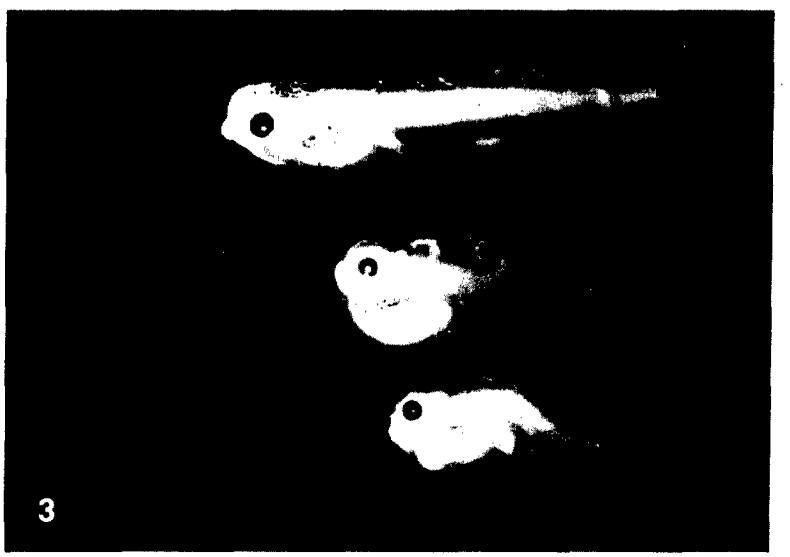

Figure 3. Micromyelia caused by exposure of embryos to symmetrical dimethylhydrazine. Upper larva is a control. Lower larvae have shortened torsos and tails.

Abnormalities of the trunk appeared to be due to failure of the embryo to elongate and to edema. The torsos of such animals became distended with fluid which displaced the visceral organs. In most cases the intestinal tract was also reduced in size.

Abnormalities of the Head

Abnormalities of the head included all grades of microcephaly including microphthalmia, cyclopia, and a few cases of anencephaly. Most embryos had a disorganized pharynx, and the external nares and eyes were located medially rather than laterally (Figures 4 and 5).

4

Figure 4. Control larva (below) and larva with reduced forebrain and medially placed eyes caused by exposure of embryo to hydrazine.

5A

5B

Figure 5. Sections through the head of larvae. A. Control larva. B. Larva with reduced forebrain and medially located eyes.

Cyclopic embryos exhibited the full syndrome of "cyclopia perfecta" as described by Adelmann (1936): the eye single in all its parts or incomplete, the forebrain greatly reduced, external nares absent, and the mouth and pharynx absent or aberrant. The midbrain and hindbrain tended to be normal (Figure 6).

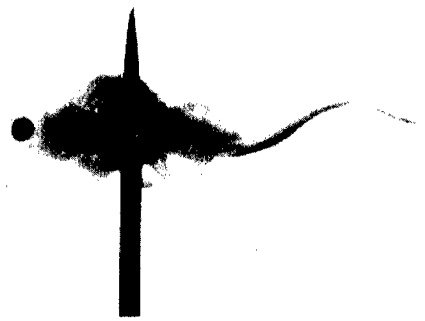

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Figure 6. Cyclops larva caused by exposure of embryo to unsymmetrical dimethylhydrazine.

Edema

Abnormal embryos and larvae often developed edema. Edema occurred in the head, torso, and/or fin of affected embryos. Most such embryos exhibited structural abnormalities as described prior to the appearance of the edema, indicating that swelling, in itself, was probably not the primary cause of the other defects. Thus, larvae with reduced forebrains and medially placed eyes tended to develop edema of the head and pharyngeal region 24-48 hours after the structural abnormalities could first be diagnosed. The only exception to this may be in the case of secondary displacement of visceral organs by edema.

Notochordal abnormalities

One ubiquitous deformity common to all embryos and larvae exhibiting the above syndrome was excessive growth of notochord. The extent of notochordal hyperplasia varied from small outgrowths (associated with tail kinks) to massive enlargement which displaced muscle and nervous

tissues (Figure 2). However, all the malformed embryos had abnormal notochords, suggesting that dysfunction of this organ may have been the primary factor leading to the pathogenesis of the other tissues.

That the presumptive notochord and later the notochord itself profoundly affect the pattern of differentiation and growth of the vertebrate embryo is an unquestioned tenet of developmental biology. The role of the presumptive notochord in the induction of the neural tube has been recognized since the classical experiments of Spemann and Mangold (1924), whereas the forebrain is induced by the prechordal endomesoderm. The prechordal endomesoderm differentiates into the pharynx and gill pouches (See Nieuwkoop, 1973, for review). The elongation of the embryo at tailbud is also dependent upon concomitant elongation of the notochord and fails to occur in notochordectomized embryos (Kitchin, 1949; Mookerjie, 1953). Elongation of the tailbud itself also seems to be dependent upon the presence of the notochord (Kitchin, 1949).

There can be little doubt that some of the malformations in embryos exposed to hydrazines were caused by notochordal dysfunction, i.e., kinks in the axial skeleton as a result of notochordal hyperplasia, and the development of secondary neural tubes and two tails in embryos with bifurcated notochords. All embryos with shortened bodies had abnormally thickened notochords and often contained additional areas of notochordal tissue along what should have been the long axis of the embryo. It appeared as if the notochord increased its mass but failed to elongate.

The anomalies affecting the forebrain and eyes, such as cyclopia and microcephaly, cannot be directly attributed to the notochord, as these organs are not dependent on the notochord but on the prechordal notochord for their induction. However, there is no doubt that the prechordal endomesoderm, which is an anterior extension of the tissue destined to differentiate into notochord, is also abnormal in embryos with reduced forebrains or eyes. All such embryos had abnormalities of the pharynx and/or gill structures.

DISCUSSION

These data compared favorably with results obtained by an Environmental Protection Agency approved system utilizing algae to test the effects of pollutants on water quality. Using that system, Scherfig and Dixon (1975) found all four of the hydrazines to be inhibitory to growth at roughly the same concentrations that affect viability of tadpoles. The same system also indicated N-phenyl-α-naphthylamine to be toxic and dioctyldiphenylamine to be nontoxic (Lerman et al., 1974; Scherfig and Dixon, 1975), again in agreement with the data obtained with frog larvae.

In comparing the data on the relative sensitivity of embryos and larvae to chemicals in their environment, it can be seen that embryos withstood higher concentrations of a given compound than did larval stages. This difference in relative sensitivity is probably explained by the fact that the embryonic stages are practically impermeable to compounds in their environment whereas larval stages are very permeable.

In addition to data on viability, the embryo-tadpole system provided data on teratogenicity of compounds. This information is not available when unicellular or adult multicellular organisms are used as test animals. Given the high incidence of congenital malformations in human populations (Kennedy, 1967) and the paucity of information concerning their etiology, a system able to predict teratogenicity is of more than academic interest.

The data on toxicity of N-phenyl-α-naphthylamine was calculated using solutions containing known quantities of amine. This compound is not very soluble in water. The relative solubility of the compound increases with time and with temperature. Thus, a given amount of N-phenyl-α-naphthylamine will be more toxic in warm water than in cold water and in standing water than in running water. Clean-up operations which make use of detergents or other solubilizers may also exacerbate toxic effects due to this compound.

Initially, two species of frog were utilized for our experiments. Although preliminary results indicated that both of these species were suitable test organisms, we subsequently restricted our studies to Xenopus. This animal offered several advantages as a laboratory species when compared to Rana. These may be summarized as follows: (1) They are easily

maintained in a disease-free condition whereas Ranid species are very often overcome by bacterial infections when kept in the laboratory, (2) Xenopus will thrive on commercially available trout food whereas Rana must be kept in cold storage or fed live food, (3) each female Xenopus will lay eggs several times per year for a period of several years (some have lived 15 years) in response to commercially available human chorionic gonadotropin, whereas each Rana female can be used only once and requires Rana hormone which must be prepared in the lab, and (4) Xenopus embryos metamorphose to froglets in 8-12 weeks whereas Rana pipiens embryos require 6-12 months.

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APPENDIX 1 - GLOSSARY

Anencephaly	- Absence of the cerebral and cerebrellar hemispheres, with only a rudimentary brain stem.
Embryo	- The developing frog is an embryo from fertilization until feeding begins. This period is characterized by organogenesis with little or no change in mass.
Larva	- Once the embryo has emerged from its external membranes (hatched) and commences feeding, it is a larva. By the time an embryo becomes a larva the main organ rudiments are present. This stage is characterized by rapid growth.
Microcephaly	- Abnormal smallness of the head and brain.
Micromyelia	- Abnormal shortness of the spinal cord.
Microphthalmia	- Abnormal smallness of the eyes.

APPENDIX 2 - STAGING OF FROG EMBRYOS AND LARVAE

Rana pipiens were staged according to Shumway (1940).

Xenopus laevis were staged according to Nieuwkopp and Faber (1956).

AMRL-TR-75-125

PAPER NO. 27

MEASUREMENT OF SOLID ROCKET MOTOR EFFLUENTS IN THE ATMOSPHERE

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INTRODUCTION

In response to the concern for understanding the fate of exhaust products emitted in the troposphere by launch vehicles and the National Environmental Policy Act of 1969, the National Aeronautics and Space Administration has initiated an extensive program of supporting research within the agency to investigate means of predicting the interactions of the solid rocket motor exhaust cloud constituents with the atmosphere and the environmental effects of these constituents. To predict the downwind dispersion and ground-level deposition of exhaust effluents from launch vehicles, NASA has developed a tropospheric diffusion model to determine those conditions under which the deposition of exhaust effluents may present a potential environmental impact.

Typically during the launch of a rocket vehicle the initial exhaust from the vehicle generates a ground cloud in the immediate vicinity of the launch pad. As a result of the heat content of this cloud, it rises to a stabilization altitude and drifts and diffuses with the prevailing winds. This stabilization altitude is typically 500 to 3000 meters depending upon the buoyancy of the cloud and the height of the surface mixing layer. When the cloud reaches the stabilization altitude, it is termed "a stabilized ground cloud" and is trapped between the Earth's surface and the top of the surface mixing layer. Initially, the cloud is composed of those species generated by the rocket-motor exhaust; however, as the cloud rises, stabilizes, and drifts with the wind, it entrains large quantities of atmospheric air.

The objective of the launch vehicle effluent program is to refine the existing diffusion model that describes the exhaust cloud behavior, check it against experimental measurements, develop models where none exist,

and therefore minimize the uncertainty in the knowledge of exhaust behavior and effects in order to insure that only necessary operational constraints will be considered for NASA launches. Experimental work to establish confidence limits for the model has been underway for three years. Measurements have been made of the concentrations of constituents in exhaust clouds for launches of existing vehicles and the results are being compared to predictions. Measurements have been made for Saturn V, Scout, Delta, and Titan III launches. The early experiments served to checkout the experimental methods and instrumentation. More recently the launch vehicle effluent measurement program has employed a sampling aircraft and an extensive network of ground level samplers in several Titan III launches to obtain the measured data for comparison with the diffusion model. A preliminary analysis of this data indicates that a qualitative agreement has been obtained between the model and experiments in the direction of travel, in the time of arrival of the exhaust cloud, and for the presence of the constituents in question. The detailed comparisons between the measured data and the diffusion models remain to be completed.

Source Description

All current and near future launch vehicles are powered by chemical rocket engines that operate by the combustion of a self-contained fuel and oxidizer. The effluents from the launch vehicles using solid propellant rockets may be derived from the nominal propellant composition. These propellants generally consist of ammonium perchlorate oxidizer and aluminum powder fuel, dispersed in a rubber binder-fuel. The major chemical species emitted at the nozzle exit plane are: water, carbon dioxide, carbon monoxide, hydrogen chloride, nitrogen, and aluminum oxide (NASA, 1972; NASA, 1973).

Although the exit plane exhaust composition is usually shown for the launch vehicles, it is necessary to carry out calculations of the afterburning of the fuel-rich exhaust in the expanding plume to accurately account for the exhaust products deposited in the troposphere. These calculations were carried out using the turbulent plume program (Mikatarian et al., 1972) and the calculations for the typical solid propellant (Gomberg and Stewart, 1976) show that the major exhaust products are:

Product	Percent by Weight	
	Exit Plane	1 km Downstream
Al_2O_3	30.2	0.2
CO	24.2	
HCl	20.9	0.1
H_2O	9.4	0.2
N_2	8.7	78.4
CO_2	3.4	0.2
H_2	2.1	
O_2		20.8
Other	0.6	0.1

Of the major constituents, carbon monoxide and hydrogen chloride are generally recognized as air pollutants. Aluminum oxide is emitted as a particulate and may also be of concern. Laboratory experiments have shown that a small fraction of the total hydrogen chloride ($\leq 6\%$) is also predicted to react with, and partially chloride the surfaces of numerous metastable aluminum oxide particles ($0.01 \lesssim \cdot\ d \lesssim 2\ \mu m$) in the plume at temperatures below $\approx 1000^\circ K$ (Pellett, 1974).

In the typical launch the exhaust products are distributed along the trajectory with the greatest quantities at ground level where the launch vehicle acceleration is low. A large "ground cloud" is usually formed from the exhaust products emitted during the first 20 seconds of the trajectory as shown in Figure 1. Progressive expansion of the resulting elevated ground cloud should continue rapidly, during the first 3 to 5 minutes; at this point the mass dilution ratio should exceed 10^4. For example, recent analysis of photographic coverage from three positions of a ground cloud from a Titan III launch led to mass dilution ratios of 1, 2, 3 and 4×10^4 at 2.4, 3.4, 5, and 6.5 minutes, respectively (Pellett, 1974). The anticipated source strength of hydrogen chloride which would be contained by a stabilized ground cloud at 2 km altitude would be:

Scout	2×10^6 gm
Delta	2×10^6 gm
Titan III	1.5×10^7 gm
Shuttle	3×10^7 gm

Formation and growth of an aqueous acid aerosol population in the stabilized ground cloud is viewed as a major complication in predicting short-term hydrogen chloride partitioning when the relative humidity of cloud-dilution air exceeds 85 to 90 percent. Furthermore, the acid aerosol problem as described by Pellett (1974) appears to be complicated by the influence of

chlorided aluminum oxide in at least two ways: (a) enhancement of the potential for condensation-nucleation, and (b) alteration of subsequent acid aerosol growth, as a result of solvation processes which alter water and hydrogen chloride vapor pressures just above the droplet surface.

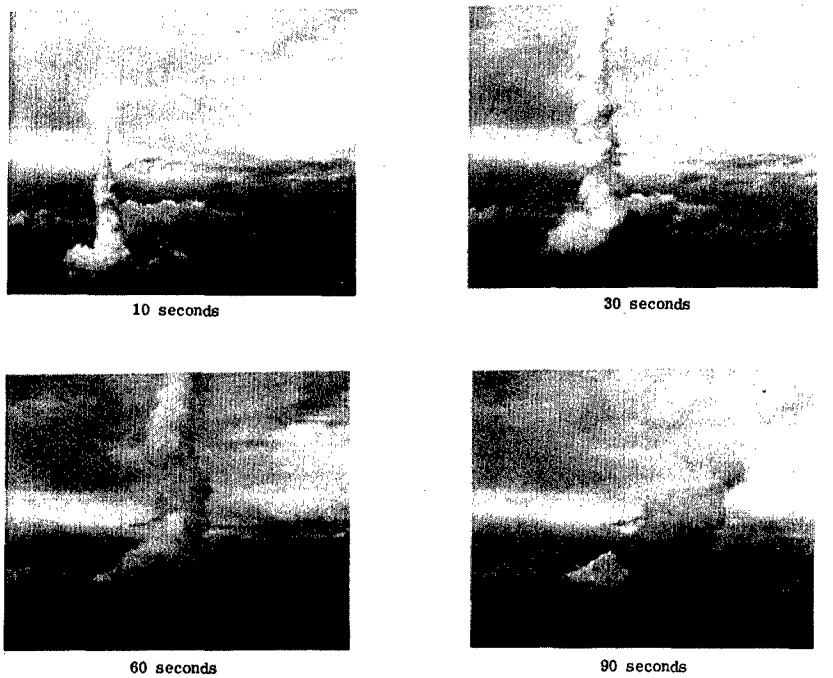


Figure 1. Ground cloud from Delta launch, February 1975.

An example prediction of the transient partitioning of hydrogen chloride in a launch vehicle exhaust cloud is shown in Figure 2. This highly oversimplified prediction, which shows significant "potential" for acid aerosol in the first few minutes, was based on the approach taken by Rhein (1974) for exhaust products with carbon monoxide and hydrogen afterburning and on the ground cloud volume measurements and resulting mass dilution calculations derived from the previously mentioned Titan III launch. Ground observers and airborne sampling personnel (Pellett, 1974) flying through the stabilized ground cloud provided both indirect and direct evidence that substantial aqueous acid aerosol existed in the cloud for 10 to 15 minutes after launch.

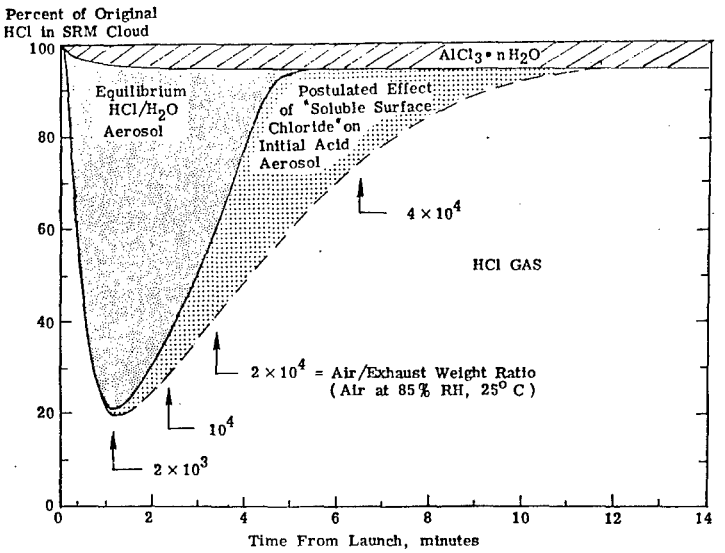


Figure 2. Example of an idealized prediction of HCl partitioning in a SRM exhaust cloud.

Thus, although we still lack critical understanding of many important details of the $Al_2O_3/HCl/H_2O$ interaction in a tropospheric ground cloud, it seems clear that the solution of aluminum in aqueous hydrogen chloride may increase the rate of acid aerosol growth initially, but then retard further acid aerosol growth at long times, relative to a pure HCl/H_2O system, hastening subsequent evaporation of the initial aerosol at large exhaust cloud dilutions.

Effluent Monitoring Program

The launch vehicle effluent monitoring program included in situ ground-level measurements of the dispersion of launch vehicle exhaust effluent from 100 meters to approximately 14 kilometers from the launch point. Instrumentation consisted of measurement systems for hydrogen chloride (HCl), carbon monoxide (CO), carbon dioxide (CO_2), and particulates (Al_2O_3). In addition, ground-based tracking systems were employed to monitor exhaust-cloud rise, growth, and movement. Measurement systems located in a sampling aircraft are used to define the material budget in the stabilized ground cloud which is the source term for the diffusion model. Instruments along the ground track of the exhaust cloud will be compared with the ground level concentrations, which are the output of the model.

The measurements are used for comparisons with the model in several areas: (1) assumption of cloud geometry at stabilization; (2) prediction of cloud stabilization altitude; (3) prediction of the path of cloud travel; (4) incloud concentrations; and (5) surface concentrations and dosages.

Measurement Systems

Nine sets of instruments were assembled to measure the major effluents from solid propellant rocket launch vehicles. The instruments used in each set are listed in Table 1 along with the sampling capabilities of each type of unit. For those instruments that collect samples for subsequent analysis, the type of analysis is shown. All instruments are commercially available and the operating characteristics are documented (Hulton et al., 1974). Each set of instruments was assembled so that they could be deployed in a truck or onboard a seacraft along the predicted path of the stabilized ground cloud immediately prior to launch. Typical installations are shown in Figures 3 and 4. In addition to the nine instrument sets which were manned in normal operations, 30 dosimeters for hydrogen chloride and particulates were used. These sets were remotely activated to turn the time-integrated samplers on and off as the stabilized exhaust cloud was carried over each site. For each launch that was monitored, the dispersion model was used with meteorological forecasts to design the effluent sampling experiment. Starting at T-12 hours and continuing until T-1 hour, meteorological forecasts and diffusion predictions were used to select the azimuth and distance from the launch site for each of the 39 ground level instrument sets used in each effluent sampling experiment. The T-1 hour data were used to direct the sampling aircraft to the initial penetration of the stabilized ground cloud. Following this penetration, the aircraft continued to probe the visible cloud in both the alongwind and crosswind directions until the cloud could no longer be discerned. The general arrangement of the airborne sampling system is shown in Figure 5. The inlet probes, sampling lines, and sampling instrument position were established to provide isokinetic flow to each instrument over the shortest and straightest flow path possible. The gas effluent sampling instruments included separate chemiluminescent detectors for nitric oxide/nitrogen dioxide and hydrogen chloride. These instruments were selected mainly for their fast response and ability to operate within the aircraft vibrational environment. The particle sampling instruments included three quartz crystal microbalance mass monitors, a nephelometer, and an aerosol concentrator. The combination of these particle measuring instruments provided real-time mass loading, size distribution, and collection for laboratory analysis.

TABLE 1. INSTRUMENT CAPABILITIES

Instrument/species	Range	Detection Limit	Response to 90 Percent Reading	Required Analysis
Chemiluminescent detector/HCl	0.05-50 ppmv	0.01 ppmv	1 to 5 sec	None
Microcoulometer HCl	0.1-20 ppmv	0.1 ppmv	1 to 5 sec	None
Bubbler/HCl	Greater than 50 ppmv-sec	50 ppmv-sec	Not applicable	Coulometric
pH paper/HCl	Qualitative	1 ppmv	Not applicable	None
Infrared gas* analyzer CO_2	1 to 50 ppmv above ambient	1 ppmv above ambient	2.5 sec	None
Mass monitor particles	0.1 < diameter < 10 μm	10 $\mu g/m^3$	Less than 5 sec	None
Andersen/ particles	0.43 < diameter < 11 μm	50 μg	Not applicable	Gravimetric, neutron activation
Nuclepore filter particles	Diameter greater than 0.01 μm	10 μg	Not applicable	Gravimetric, neutron activation
High volume sampler/ particles	Diameter greater than 0.01 μm	200 μg	Not applicable	Gravimetric

*Instrument specifications based on manufacturer's data.

Figure 3. Typical instrument van for land development.

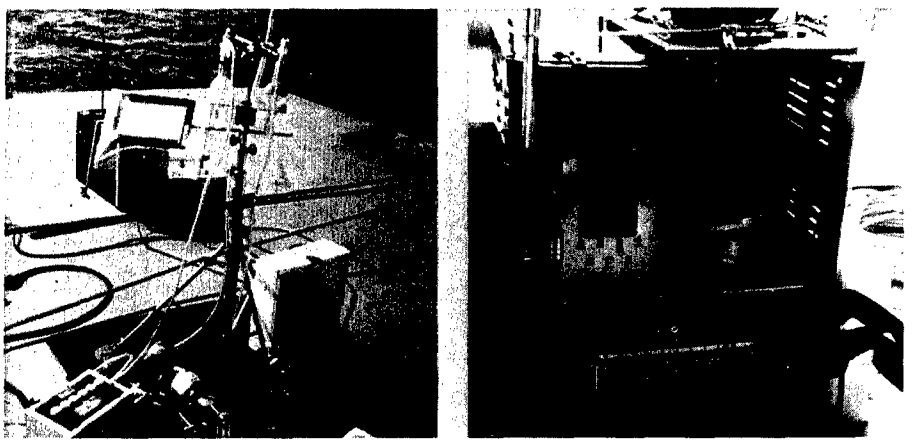
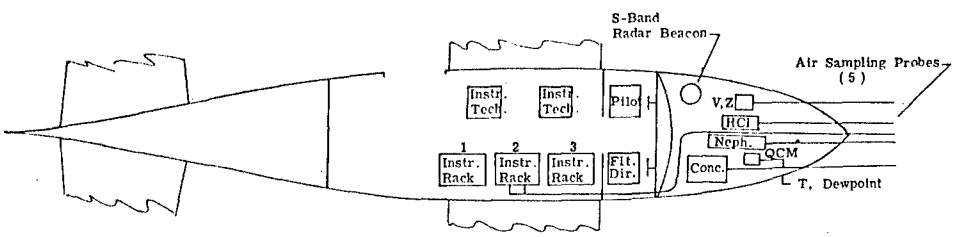


Figure 4. Typical instrument deployment on seacraft.



CESSNA 402 B TWIN ENGINE AIRCRAFT

Speed	100 to 230 knots
Altitude	Up to 26,000 feet
Range	600 miles
Duration	3 hours

1. Flight Data
   T, Dewpoint
   V, Z, θ, t
   Data Acquisition

2. Gas Sampling Instrumentation
   NO/NO_2 Analyzer (Monitor Lab)
   HCl Controls/Readout (Geomet)

3. Particle Sampling Instrumentation
   QCM (Celesco)
   Nephelometer (MRI)
   Concentrator (ERC)
   } Controls/Readout

Figure 5. SATD atmospheric sampling aircraft, launch effluent configuration.

Two tracking systems were used to document the formation, rise, growth, and downwind track of the ground cloud. The first, an optical system, consisted of three ground-based tracking cameras located at least 4 kilometers from the launch site. These were used to track the centroid of the cloud from launch until dissipation. Tracking was done virtually by the camera operators and cloud photographs were taken at 10-second intervals. The tracking-camera readouts were used to calculate the location of the cloud centroid in three-dimensional space as a function of time. The three cameras were synchronized, giving the cloud location at 10-second intervals. The tracking-camera locations were selected so that the cloud, regardless of the direction of travel, was always in the field of view of at least two cameras. A second tracking system consisting of an infrared imaging system has proven to be a useful technique for monitoring cloud growth, thermal history and to accomplish position fixing during night launches. Although it is not known what species in the cloud contribute to the detected radiation, clear images have been obtained for nearly an hour following a Titan III rocket launch. The wavelength region of the detector was from 2 to 5.6 microns which includes HCl, CO_2, and H_2O radiation bands. This region is relatively free from the usual interference from methane and water. Questions regarding atmospheric scattering and absorption remain; however, these pertubations have not proven prohibitive during a daytime monitoring of a Titan III cloud.

Because hydrogen chloride and aluminum oxide are the principal effluents, the surface sampling instrument selection concentrated on these species. A chemiluminescent hydrogen chloride detector as described by Gregory (1974) was selected to continuously measure the hydrogen chloride concentration in ambient air. The detection technique is based on a chemiluminescent reaction in which visible light is generated in an alkaline solution of 5-amino-2, 3 dihydro-1, 4-pthalazinedione (luminol) during oxidation. The unique feature of the chemiluminescent hydrogen chloride detector is the coated alumina tube which makes use of hydrogen chloride adsorption of the inlet at the low ppm concentrations. A fritted bubbler was used to determine the total dosage of hydrogen chloride at all sampling sites. The distilled water from the bubbler was analyzed for chloride using a microcoulometer. Squares of pH paper were also exposed at each site to obtain a qualitative indication of the presence of an acidic aerosol.

The pertubation of the ambient particulate concentration was monitored with quartz crystal microbalances. These were supplemented at several of the manned sites by light scattering photometers. The integrated particulate burden was obtained from 47 mm diameter collection filters that were used at all sampling sites. Samples collected by the mass monitor

and the filters are analyzed for elemental composition by using techniques such as X-ray fluorescence, X-ray, proton scattering, neutron activation analysis, atomic absorption and electron spectroscopy for chemical analysis (ESCA). The instruments were operated before, during, and after the launch to differentiate between atmospheric background and launch effluents.

RESULTS AND DISCUSSION

Typical measurements taken from several launches at Kennedy Space Center are used to illustrate the concentration levels experienced in the stabilized ground cloud as well as at ground level downwind of the launch sites. Penetrations of the rocket ground cloud have been made with the sampling aircraft as early as four minutes after lift-off and for periods exceeding one hour. Depending on the visibility, it has been possible to track the ground clouds with the optical and infrared systems for periods up to one hour. Ground level samples of the rocket effluent have been obtained at distances from 100 meters to 14 kilometers downwind of the launch site.

The initial rise of the ground cloud to a stabilized altitude has been documented for six Titan III launches. The initial rise rates in the first four minutes, which are shown in Figure 6, were between 4.2 and 5.3 meters per second with the final stabilization altitude, between 1 and 2 kilometers, being determined by the meteorological conditions at the time of launch. The measured ground cloud growth for four of the daylight launches is shown in Figure 7. As can be seen there is considerable variation in the volume versus time histories of the several ground clouds.

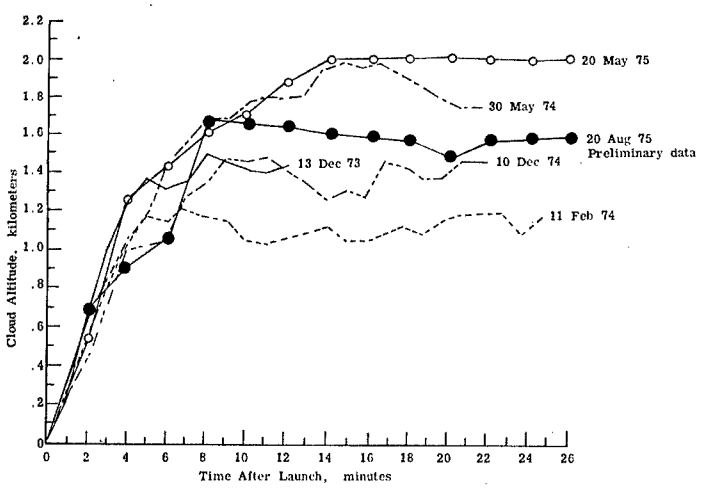


Figure 6. Measured Titan III cloud rise to stabilization in the atmosphere - KSC launches.

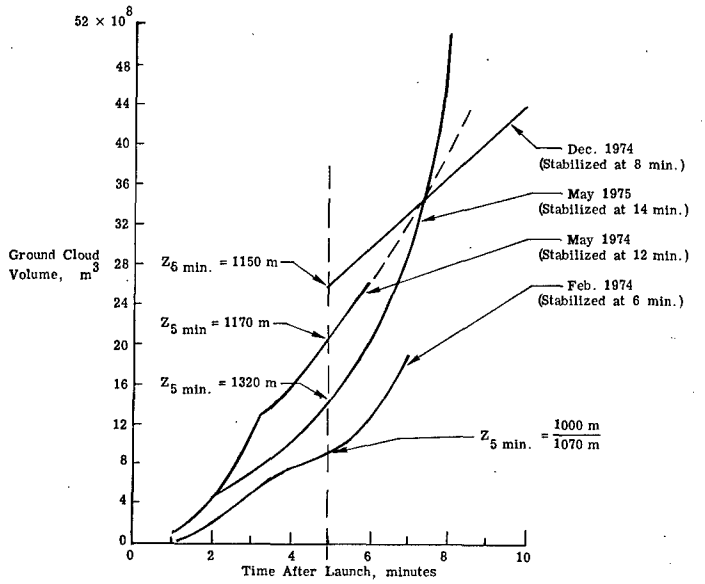


Figure 7. Measured Titan III ground cloud growth.

The concentration of hydrogen chloride and particulates in a ground cloud produced by a Titan III launch vehicle was measured over a 55 minute period as the effluent cloud was transported about 39 kilometers from the Titan III launch pad. The sampling aircraft made repeated penetrations of the effluent cloud in both the alongwind and crosswind directions. The residence time in the cloud varied from 8 to 22 seconds and the average time between penetrations was 3 minutes. Figure 8 shows four typical data sets for penetrations early and then midway through the sampling period.

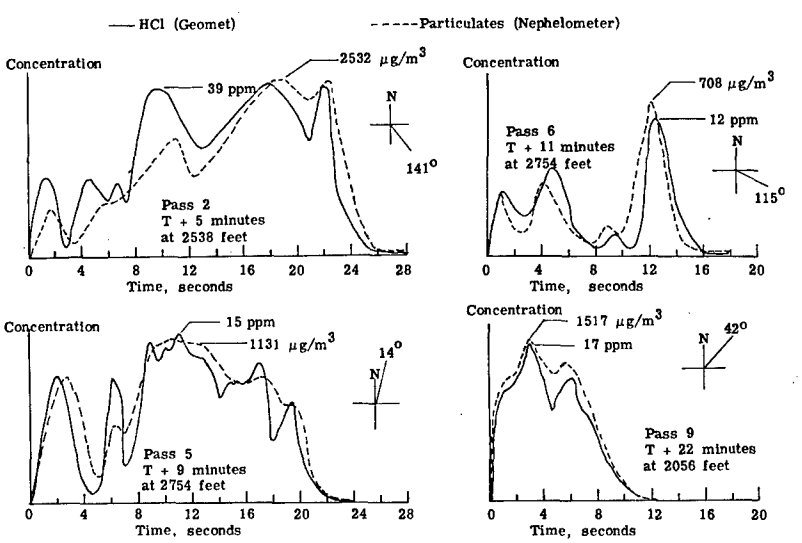


Figure 8. Airborne measurements of HCl and particulates.

The launch pertubation of the ambient particulate concentration at ground level can be seen in the mass monitor results by Hulten (1974), Figure 9, from a site located 3.18 km from the launch point of a Delta vehicle. The time change of the mass loading ($\mu g/m^3$) indicates the cloud arrival at this site of approximately T + 14 minutes from launch as indicated by a greater than factor of 3 increase in mass. The model predicted the cloud would arrive 3 km downwind at T + 13 minutes after launch. The natural atmospheric background before launch was in the range of 20 to 60 $\mu g/m^3$. The crystal from the mass monitor was examined with the scanning electron microscope (SEM). A group of photographs showing the particles analyzed with the SEM are shown in Figure 10. The upper photograph shows a group of particles collected on the mass monitor with magnification x 200. The three particles circled were selected for elemental analysis. Particle 1 is irregular in shape, 2 is spherical, and 3 has a cubical shape. The bottom photograph of Figure 10 is a x 2000 magnification of particle 1. Since this particle was irregular in shape, scans were made at two separate locations on the particle (1-1 and 1-2). At 1-2 there appears to be a small particle which adhered to the large particle. The results of the analysis, as shown in Table 2, indicate that the elemental composition was different at the two positions. The upper photograph in Figure 10 is a x 1000 magnification of Particle 2. It is spherical in shape and from the analysis it consisted of 70 percent aluminum. It was concluded that this particle was aluminum oxide from the rocket exhaust. Particle 3 is shown in the bottom photograph of Figure 10 with x 1000 magnification. The analysis as shown in Table 2 indicates that the particle is mostly sodium chloride as was suspected from the cubical shape.

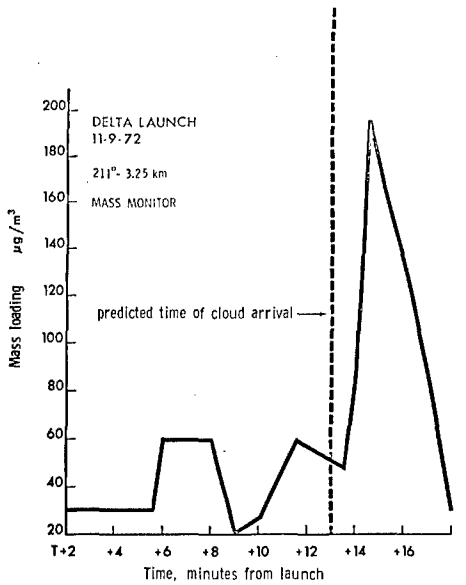
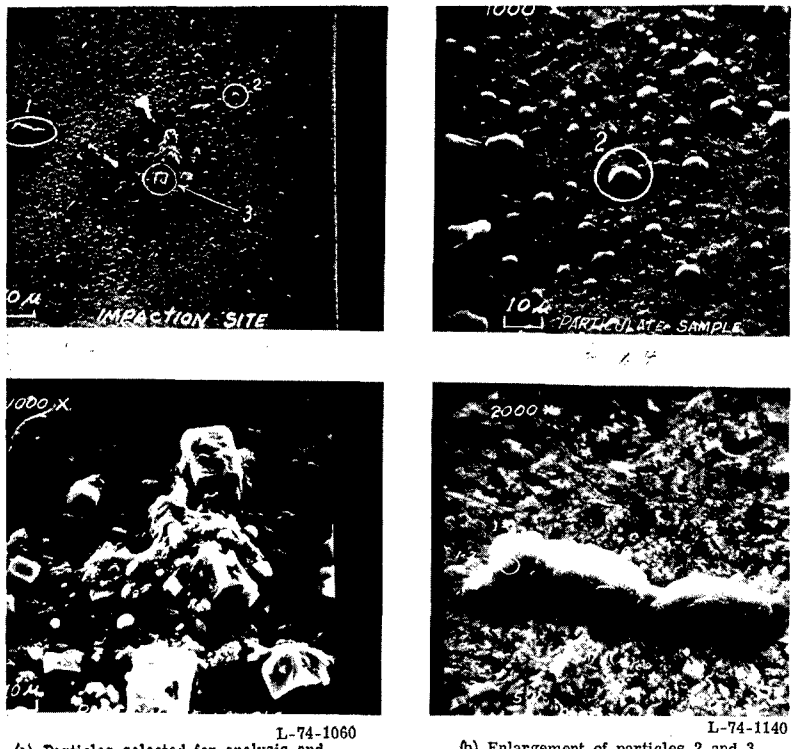


Figure 9. Mass loading as a function of time measured by the mass monitor at site S, 318 km, 209 downwind from launch point.



L-74-1060
(a) Particles selected for analysis and an enlargement of particle 1

L-74-1140
(b) Enlargement of particles 2 and 3

Figure 10. SEM photomicrographs of particles collected.

Figure 11 shows the light-scattering photometer measurements conducted at a site 5 km from the Delta launch point. The 100 channels of data are grouped into four major particle size ranges and the mass loading calculated and plotted as a function of time. The arrival of the cloud at this site approximately 23 minutes after launch is clearly evident by the change in particles which was much more marked in the smaller sizes. Measurements were made for an hour after launch, although by that time the air at the site (as seen from the plots) had not yet returned to normal prelaunch background. Prelaunch model prediction data indicated that the cloud would be 5 km downwind at T + 23 minutes. Figure 12 shows the mass loading data as a function of time as determined by the total number of particles measured in the size range of the instrument (0.6 to 6 μm).

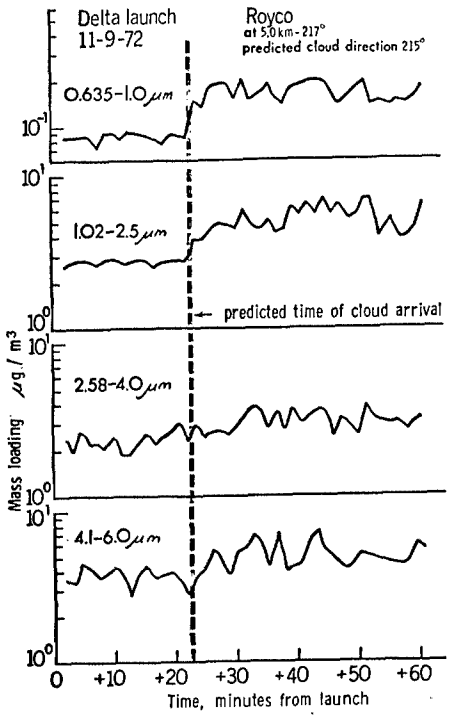


Figure 11. Mass loading as a function of time obtained from Royco particle measurements at site 1808, 5.25 km, 217° downwind from launch point.

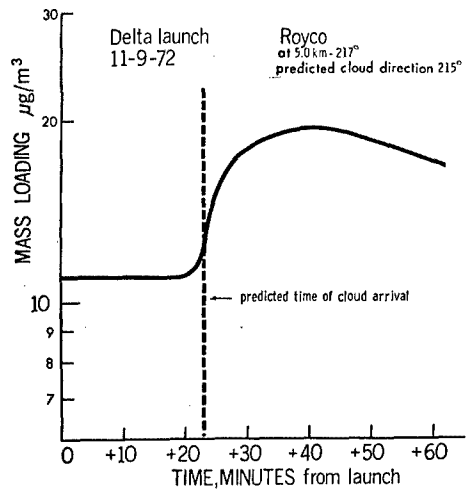


Figure 12. Mass loading determined from total number of particles in the size range 0.6 to 6 μm, measured at site 1080, 5.25 km, 217° downwind from launch point.

The surface concentrations of hydrogen chloride can be seen in the results from sites located 2.6 km, 4.2 km and 7.7 km from a Titan IIIC vehicle. As shown in Figure 13, the time of arrival of the cloud at each site can be readily determined from the increase in the hydrogen chloride concentration. The maximum concentration observed was less than 0.5 ppm with the peak dosage less than 20 ppm-seconds.

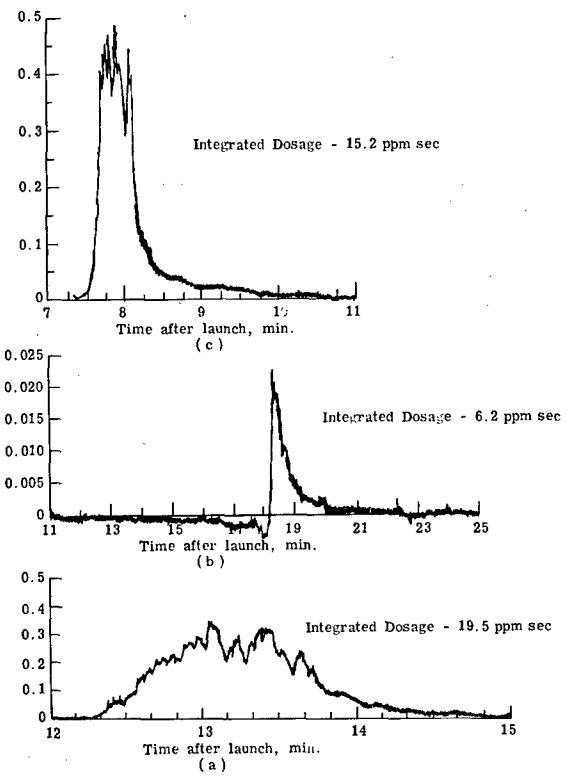


Figure 13. Hydrogen chloride results, Helios A launch, 10 December 1974.

The second application of the hydrogen chloride detector was similar to that of the launch vehicle effluent program. The purpose of the program was to monitor ambient hydrogen chloride concentrations 25 m to 1 km downwind from a static firing of a small solid rocket motor. In this study, five chemiluminescent hydrogen chloride detectors were placed in the field. Figure 14 shows a typical field installation of the detectors. Figure 15 shows typical data obtained during the measurement program from a location 0.3 km downwind from the motor firing. The maximum observed hydrogen chloride was 1.1 ppm and the total dosage was 11.7 ppm-sec.

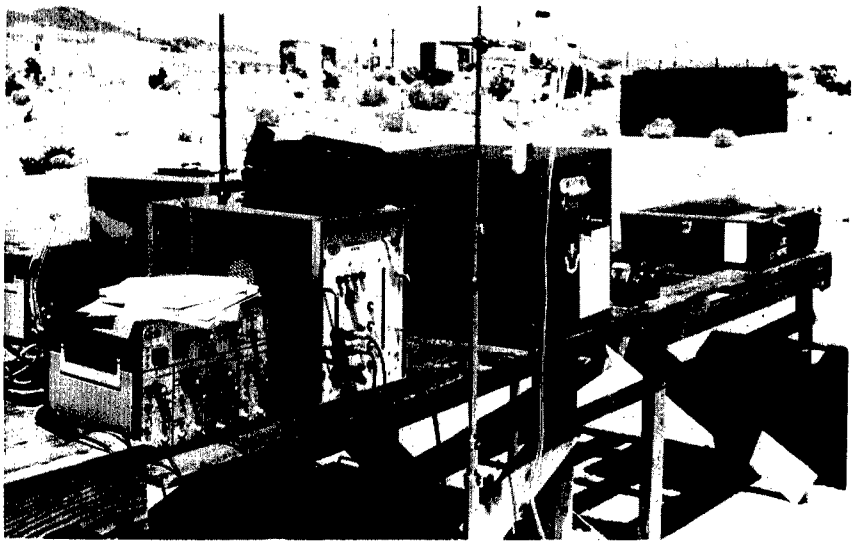


Figure 14. Field installation of hydrogen chloride detector.

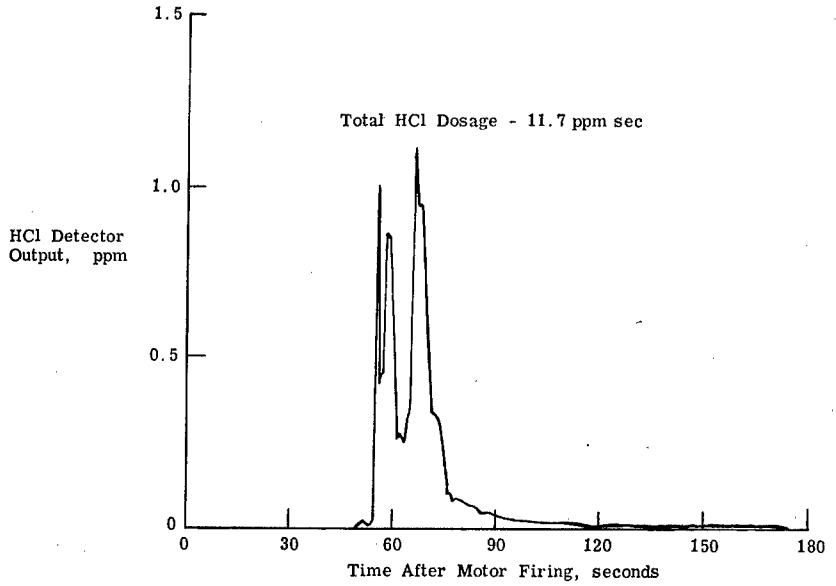


Figure 15. Ambient HCl concentrations. Source: static firing of small rocket motor.

Hydrogen chloride measurements have also been obtained from monitoring the incineration of chlorinated hydrocarbon waste materials. All measurements were made from the deck of the NOAA research ship in the Gulf of Mexico under guidelines established by participating EPA officials. In each case, sampling was of a plume that was not visible. Background ambient hydrogen chloride measurements were made at various times during the cruise, including measurements downwind from the incinerator ship prior to the burning of the chlorinated hydrocarbon waste material. The results from the ambient background measurements showed the normal hydrogen chloride concentration in the Gulf of Mexico to be less than 10 ppb, the lower detection limit of the instrument. The duration of the contacts was typically five minutes with the maximum observed hydrogen chloride concentration being about 7 ppm as shown in Figure 16.

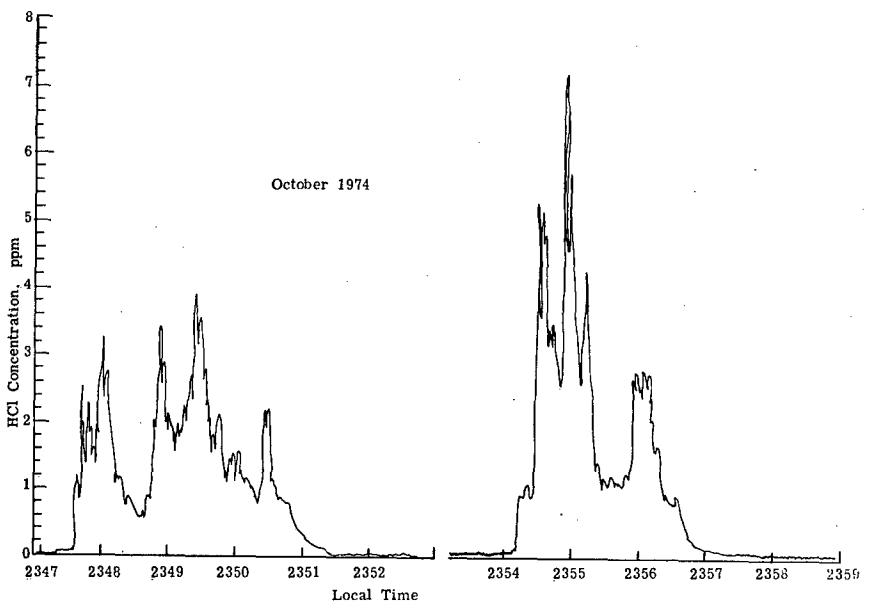


Figure 16. Hydrogen chloride concentrations from waste incineration.

CONCLUSIONS

The applicability of instrumentation to monitoring the effluents from launch vehicle was demonstrated in large scale experiments during normal launches from the Kennedy Space Center. The launch vehicle tropospheric ground cloud has been followed with tracking systems and with a sampling aircraft for periods up to one hour while the effluents cloud was transported by the prevailing winds for distances up to 40 kilometers from the launch site. The initial rise and growth characteristics of ground clouds produced by Titan III launch vehicles have been documented.

Both hydrogen chloride and particulates concentrations well above the minimum detectable limits of the instruments have been obtained at ground level beneath the path of the stabilized ground cloud. The dispersion data are being used by NASA to analyze the models for predicting the transport and dispersion of launch vehicle effluents in the vicinity of the Kennedy Space Center.

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OPEN FORUM

DR. WINSTEAD (National Academy of Sciences): I'd like to make a couple of comments and then direct a question to Dr. Greenhouse. I think most toxicologists are familiar with rapid screening tests for mutagenicity and carcinogenicity. A number of these methods were referred to in the Open Forum yesterday. There is thought to be a very high correlation between mutagenicity of chemicals and their carcinogenicity. It might be helpful if we could extend this correlation to teratogenicity, but I think Dr. Greenhouse's data clearly indicate this is not possible. He found that unsymmetrical dimethylhydrazine was less teratogenic than the other hydrazines and yet it's certainly the stronger carcinogen. I'd like to ask Dr. Greenhouse if there is a possibility of his technique being developed into a practical and rapid screening technique for teratogenicity. Can the method be developed so that a technician can perform the test procedure?

DR. GREENHOUSE (University of California, Irvine): Yes, it can. Right now just one technician and I are doing this work but it could be developed into a screening method which would require a technician trained to recognize normal and abnormal development in frogs. I think we've shown that the system is very amenable for use as a teratogenic screening procedure.

MR. WANDS (National Academy of Sciences): As we think about developing standardized screening tests such as this, one has to define the reproducibility in order to have a procedure that is meaningful and useful. What kind of variability does one get from one technician to the other, one laboratory to the other? Could you comment on that?

DR. GREENHOUSE: As far as I know, we are the only group using frogs for teratogenic testing, so there is no data available for defining reproducibility between individuals or between laboratories. There are only one technician and myself working on this program. There really isn't enough data to compare between individuals on how they score the results of the test. My impression after having worked with frog embryos and teratogenesis over a number of years is that we have good reproducibility within the lab itself. I've shown you representative experiments where the reproducibility is quite good. Certainly there's some variability between repeat

experiments in the exact concentration that causes teratogenesis but in frogs it tends to be an all or none affair, more so than we found in working with mice. When I worked with mammalian systems, a teratogenic response in 25% of the offspring was a good number. Frequently we would get a change of only a few percent. We also get total resorption occasionally which makes a screening test more difficult. Since frogs have no intervening maternal metabolism, the response to a teratogenic chemical ranges from practically no effect to essentially a 100% effect and appears to have a response threshold.

MR. WANDS: How well do frogs correlate with the mammalian systems? Have you had any opportunity to run the same teratogenic chemicals in both frog and mouse systems in your own personal experience?

DR. GREENHOUSE: No, I haven't. The only comparative data available on these chemicals is in the report of Dr. Scherfig. As far as I can tell, where Dr. Scherfig's results can be compared with mine, they gave surprisingly similar results.

DR. CROCKER (University of California, Irvine): We have been discussing teratogenesis screening with two animal systems, amphibian and mammalian. Is there also a possibility of doing mutagenesis analyses in the amphibian? Is it possible, for example, to collect white cells and culture them in an appropriate manner for a chromosome mutagenesis type of analysis? Now this is not mutagenesis testing in the same sense as analysis for dominant lethals or other types of mutations but at least it is a level of chromosomal hit that might be possible to measure in mammalian white blood cells. I'm sufficiently ignorant of what the amphibian white cell is like that I don't really know whether there's any evidence of a response that's similar. Can they be tested in this manner?

DR. GREENHOUSE: Yes. In fact, we have suggested this approach to the Air Force. Chromosomal screening can be done in frogs. The white blood cells can be cultured and karyotyped in the same manner as mammalian systems. The amphibian embryonic system also offers some interesting advantages in that tadpole tails are very much like onion root tips. You may know that onion root tips are used to demonstrate all of the mitotic stages in teaching a general biology laboratory course. Aside from only looking at the

effect of a chemical on the adult organisms, you can cut the tail tips of tadpoles off, squash them on a slide and do karyotyping. This is how, in fact, the initial karyotyping of the frog was done.

DR. CULVER (University of California, Irvine): I want to pursue the line of questioning that Mr. Wands initiated just a step further. I know that you have tested some known teratogens in your frog system. Have you found any known teratogens, as identified in mammalian systems, which do not produce teratogenesis in your amphibians?

DR. GREENHOUSE: We have exposed frog embryos to three compounds which have been identified as teratogenic by mammalian test systems. These three, tetracycline, trypan blue and lithium chloride produced similar teratogenic effects in frog embryos as were found in mammalian or other systems. With lithium chloride, which is a teratogen that causes cyclopia, we get the same types of eye and forebrain abnormalities in frogs as were found in mammals.

DR. HAYS (U. S. Department of Agriculture): Dr. Greenhouse, at what stage of embryonic development do you test chemicals for teratogenicity?

DR. GREENHOUSE: It depends on the individual chemical. We normally look for the critical period and different agents tend to produce their effects at different periods. They all occur during organogenesis which is the period between cleavage and the end of neurulation. Most teratogens produce their effect between the beginning of the gastrula stage and on through neurulation.

DR. HAYS: Have you tried adding it after the gastrula stage?

DR. GREENHOUSE: Yes. The hydrazines exert their effect between the beginning of the formation of the neuroplate and the end of the closure of the neural tube. There seems to be no effect during gastrulation. If you expose the embryo to high concentrations of hydrazines during gastrulation, then remove and wash them and put them back into fresh water, these tend to develop quite normally. The critical stage for the effect of hydrazines on frog embryos seems to be around the neuroplate stage.

MR. WANDS: I have a question for Dr. Scherfig. When do you add the hydrazines to the nutrient medium? Is it continuously added during the entire 20 days or so of the growth cycle or is it added at the beginning when the media is made up and inoculated?

DR. SCHERFIG (University of California, Irvine): The hydrazines are added only at the beginning in batch cultures, and in continuous cultures, they are added continuously with the medium.

MR. WANDS: The reason I'm asking about this is that hydrazine is commercially used as an oxygen scavenger and your systems are very well oxygenated. How do you account then for the persistence of the effect of hydrazine in your batch cultures that run for 20 days with all of that chemical decomposition of hydrazine? I'm throwing a new question at you, I know, but it's a very critical one as we examine the problem of the stability of hydrazine in water.

DR. SCHERFIG: I think part of the answer to that question is that when we tested a low concentration of some of these hydrazines, we observed what appeared to be a complete decomposition, and the effects of those compounds disappeared. At higher concentrations, even though there may be chemical changes, we still got positive effects. Some of the decomposition products may be just as toxic. We have not looked into that in detail.

MR. WANDS: I think that's a point that does need to be pursued either by you or some of the Air Force biologists at Wright-Patterson. That delay in response that you reported with SDMH could also have been due to adaptation or the development of resistant subspecies or strains.

DR. SCHERFIG: That is definitely possible.

MR. WANDS: Did that delay phase correlate with the observed shift in morphology? Perhaps the spherical cells were suddenly beginning to proliferate.

DR. SCHERFIG: That's one of the other questions that we want to look into. This finding occurred only a couple of months ago. We are now setting up a detailed testing program to repeat this experiment and see if we can reproduce this effect. If we can again get this abnormal type of cell, we

want to follow very carefully the population growth because this was something that happened very suddenly during the experiment. When we checked our cultures we saw, all of a sudden, that there was a mismatch between cell volume and cell number. When we investigated we found this abnormal cell and we started culturing it to see if it was just a short-term effect that would revert back to the normal shape. It did not. This has, of course, made us very interested in it and that's why we want to go back now and study the actual change in detail.

DR. PIER (University of Texas): A comment for Mr. Wagner. I know you're acutely aware of the problems in establishing diffusion or dispersion models for air pollutants. The Texas Air Control Board is in the process of critically evaluating several diffusion models to handle some special situations in Texas. They've found with some very sophisticated calculation and checking, that a 5% error in the measurement of the wind direction will produce a 100% error in the downwind concentration. You might find it useful to check their results because it's the result of very difficult work.

MR. WAGNER (National Aeronautics and Space Administration): I appreciate that information.

DR. SLONIM (Aerospace Medical Research Laboratory): Relative to Mr. Wands question to Dr. Scherfig, did I understand that no analysis was made in the water to determine whether hydrazine was there or not? Did any chemist analyze your water sample?

DR. SCHERFIG: In the first group of experiments, that is correct. We did not monitor throughout the experiment to determine how much of the hydrazine was left.

DR. SLONIM: I'd like to make a comment to Dr. Klein. I'd like to suggest that before you rule out any effects due to water hardness in your studies with jet fuel, you test at hardness values greater than 100 mg/liter. Most of the water pollution people would not consider 100 even moderately hard. As a matter of fact, in the classical work on acute toxicity by Deuteroff, Katz and Carzwell, they used water with a hardness different by a factor of 20. In other words, they were using 20 mg/liter versus 400. It's not uncommon in this area of the United States to have a water hardness of

between 400 and 500 mg/liter. In our own work, we found a significantly high correlation between hardness and the toxicity of beryllium and hydrazine. In one case, we were able to report a linear correlation between the LC_{50} and water hardness. I suggest, therefore, that you might repeat this experiment and use water hardness of at least 300. It's pretty well known that there is a difference in toxicity of lead, copper and all the other heavy metals in hard and soft water.

MR. KLEIN (University of California, Berkeley): We ran our studies in the water that was available in our area. It has a hardness of approximately 100 mg/liter.

DR. SLONIM: How do you quantitate your aufwuchs sample? What constitutes the unit of your aufwuchs colony? I realize it's quite a heterogenous colony but how do you know your replicates are all phytoplankton and not all zooplankton? How homogeneous are your units?

MR. KLEIN: We suspend a rack of units in the river. We have 30 aufwuchs units on each rack suspended 50 centimeters below the river surface. We take whatever growth we get.

DR. SLONIM: How do you divide them up to get a statistically valid population when you're subjecting it to a chemical? Do you do this by weight?

MR. KLEIN: We subject replicates to different concentrations of the fuels in BOD bottles, measure the photosynthetic response and the respiration response. We take some of the other replicates and determine the average weight. Then we determine the photosynthetic response and apply either to the weight or to the chlorophyll content.

DR. PASI (University of Zurich): Dr. Scherfig, the finding of morphologic transformations after exposure of algae to hydrazine and the ballooning effect that was still present at the 100th generation could be indicative of a mutational effect.

DR. SCHERFIG: That is certainly possible although mutations in unicellular algae are quite rare.

DR. PASI: Were those altered cultures morphologically uniform in the sense that there was only a round form present or were there also intermediate forms?

DR. SCHERFIG: There was a change in the population after the addition of the symmetrical dimethylhydrazine and then only round forms were present. That change in the morphology and in color which would probably indicate some physiological change also remained constant after we removed the test algae from the medium that had hydrazine in it and transferred it back to our standard growth medium and maintained it in the standard medium for a hundred generations.

DR. PASI: Do these round forms also occur without hydrazine treatment in the control population?

DR. SCHERFIG: We have not observed them yet and this is one of the reasons why we want to go back and redo the experiment and very carefully follow the change in population, get good size distribution, and look for these specific organisms.

DR. PHALEN (University of California, Irvine): My question also concerns the change in morphology of your cells, Dr. Scherfig. You mentioned the mismatch between the cell volume and number toward the end of your experiments. The volume in one experiment, for example, was 14% while the number of cells remaining was only 5. This indicates a shift in cell volume such that your median cell had 3 times the volume of the initial starting cell. Also, as I recall, you mentioned that these cells were actually mutated or the population changes to a smaller cell. This seems inconsistent to me. If the cells are getting larger, is there a possibility that cell division is being inhibited?

DR. SCHERFIG: First with respect to the question about cell volume and cell number, even in normal cultures there are some shifts in the average cell size, and growth occurs and we do see normal variations that we can't account for. This is the first time that we have ever seen a drastic morphological change rather than just a change of cell size. It certainly is true that abnormally large cell size indicates that something has inhibited cell division.

DR. LERMAN (University of California, Riverside): Mr. Wagner, you indicated that you used the crystal microbalance to measure the particles in the air. This system is known to be very strongly affected by moisture and is especially troublesome when used in the vicinity of the ocean. Your scanning electron micrograph indicated quite a few particles of sodium chloride which make the system even worse. Did you do anything to correct for moisture effects? On the same scanning electron micrograph you showed spheres. Did you positively identify them as aluminum oxide and if so, what method did you use?

MR. WAGNER: Yes, we did positively identify the spherical particles as being elemental aluminum using the beta probe. We did not take any special precautions for moisture effects other than keeping the instrument temperature elevated. The instrument we used had a compensating crystal to take care of temperature effects.

DR. LERMAN: You said elemental aluminum. It's quite possible that it's aluminum chloride and not aluminum oxide.

MR. WAGNER: It could be.

DR. LERMAN: This is very important because it's quite possible to change a particle of aluminum oxide into sphere form by passing it through a very high temperature environment. If somebody positively can identify this as aluminum oxide, it would be very helpful for plant and animal study because it's much simpler to work with spheres since most of the instruments which measure particle size are based on Stoke's Law which treats all particles as spheres.

CAPTAIN LIND (Aerospace Medical Research Laboratory): I have another instrument question for Mr. Wagner. In earlier presentations at this meeting, Mr. Wohlslagel reported that he measured atmospheric HCl concentration with a specific ion electrode and Dr. Lerman used a chloride titrator. Was your instrument one of these two types or was it different from that?

MR. WAGNER: The instrument we used for the continuous monitoring of hydrogen chloride was a Chem-illuminescence instrument. We used a coulometric technique as well. These instruments were operated in parallel.

DR. PHALEN: Mr. Wagner, in the scanning electron micrograph, you showed the sample was from an impactor stage which would mean there would be some sort of cutoff diameter and particles smaller than that diameter would not have been collected. When dealing especially with solid aerosols and trying to describe their characteristics, I think most people would agree that electron microscopy is really indispensable and if possible, one should obtain an unbiased sample. Is there any plan for including collection of samples of these aerosols for electron microscopy?

MR. WAGNER: Yes. In a separate program, samples have been collected in the lower stratosphere and these samples will be examined by electron microscopy.

DR. PHALEN: You probably back up the impactor with a filter. You may actually be able to process all of your samples by electron microscopy and thereby gain a little more confidence in the shape and size distribution.

MR. WAGNER: Our experience has shown that the samples collected in the troposphere are generally contaminated extensively by debris and hence the desire to sample at higher altitudes where one wouldn't expect to find the large amount of debris.

DR. BACK (Aerospace Medical Research Laboratory): Dr. Lerman, I'm not quite sure what you used as your basis for determining minimum damage to leaves. I've seen some very nice techniques using infrared photography to show internal damage to leaves before you see anything externally at all. I do know that you used electron microscopy but how did you determine minimal damage?

DR. LERMAN: The criteria we use for minimal damage are those signs of injury that are visible to the human eye. We do not use infrared, just visible signs of injury. Occasionally right after the exposure you can see temporary wilting. You can also see some tissue collapse as I showed on the scanning electron micrograph, but in many cases the effects were only temporary and the plant recovered. Twenty-four hours after exposure, if there is any permanent injury, it will show in the form of discoloration, loss of chlorophyll or a necrotic spot.

DR. HODGE (University of California, San Francisco): Dr. Klein, you reported some data on the accumulation of methylcyclohexane in trout and also reported that after you put these fish back in the control tank, there was a rapid loss of the compound. Did you expect this?

DR. KLEIN: No, this finding surprised us. We wanted to see whether the effect was reversible and we were amazed that overnight almost 90% of the methylcyclohexane was leached out of the trout.

DR. JENKINS (University of California, Berkeley): We proved conclusively that the methylcyclohexane was not on the exterior of the body of the fish. Washing the fish off didn't remove the material but keeping them in clear water overnight did.

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