PROCEEDINGS OF THE FOURTH ANNUAL CONFERENCE ON ENVIRONMENTAL TOXICOLOGY HELD AT FAIRBORN, OHIO ON 16, 17, AND 18 OCTOBER 1973

AEROSPACE MEDICAL RESEARCH LABORATORY

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This report is a compilation of the papers presented at the Proceedings of the 4th Annual Conference on Environmental Toxicology, sponsored by the University of California, Irvine and held in Fairborn, Ohio on 16, 17, and 18 October 1973. Major technical areas discussed included Toxic Substance Control Act of 1973; toxicology of halogenated solvents, aerosol propellants, and fire extinguishants; and toxicology of propellant, materials and assessment of carcinogenesis to certain materials.

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FOREWORD

The Fourth Conference on Environmental Toxicology was held in Fairborn, Ohio on 16, 17, and 18 October 1973. Sponsor was the University of California, Irvine under the terms of Contract F33615-73-C-4059 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. T. Timothy Crocker, M.D., Chairman, Department of Community and Environmental Medicine, University of California, Irvine, Irvine, California served as Conference Chairman, and Mrs. Lois Doncaster, University of California, served as Conference Coordinator.

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OPENING REMARKS

T. Timothy Crocker, M.D.

University of California, Irvine
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It is a pleasure to introduce the Fourth Annual Conference on Environmental Toxicology, and to express the gratification of the University of California in being the host of this Conference. It is also a pleasure to tell you that the members of the staff of the Toxic Hazards Research Unit are members of the Department of Community and Environmental Medicine of the College of Medicine, University of California, Irvine. This Department has undertaken to operate the Toxic Hazards Research Unit in spite of the distance separating Irvine and Dayton because we regard the Toxic Hazards Research Unit as a unique facility, ranking as a national resource in the field of inhalation toxicology. Moreover, we consider that the future balance between energy needs and environmental safety will raise many critical questions that can be served by efforts of the University of California in cooperation with industry and government. The issues will include, but not be limited to, advancement in the science and technology of assessment and control of environmental hazards and to educating the public and specialized manpower needed for these purposes. The Department of Community and Environmental Medicine in the College of Medicine has undertaken to bring together competent and interested faculty members from the University of California at large in order to begin an organized approach to this end. I am grateful to our Dean and Chancellor at UCI for support in these objectives, and for the participation of Drs. MacEwen, Hodge, and Culver as members of the Department of Community and Environmental Medicine.

The Conference has experienced its usual benefit from the excellent administrative arrangements made by the University staff in the Toxic Hazards Research Unit. I would like to acknowledge the efforts of Edmond Vernot, aided by Lois Doncaster and her associates, for having assembled the Conference so smoothly.

The field of toxicology is built upon many scientific and professional disciplines. Many of these are represented in the University, but the University has only begun to assemble its disciplines to serve the field of environmental toxicology. The participants in this Conference represent toxicology as a professional and scientific field. University of California members who have not previously been identified as toxicologists and may never acquire

that status are having an opportunity to learn about the concerns of toxicology by participating in this Conference. As faculty members of the University we need to become better acquainted with toxicology as represented by the Air Force and other programs in order to be able to bring the special skills of the University faculty members to bear on the toxicology of community and special environments.

The University, including the THRU staff, is involved in identifying the ways by which we can meet Air Force research needs and also advance the field of toxicology not only by research but also by instruction at any level, including technical, graduate academic and professional. Joint efforts in research and instruction by the University of California at Irvine and at Dayton will include the aim to prepare future manpower for the field and to increase, if needed, the depth or the range of disciplines it may be possible to bring to problems in environmental toxicology. We invite you to express opinions as individuals and as a group regarding your expectations and needs for additional academic disciplines and for new graduates in toxicology. Your readiness to advise or assist us in the educational and training process would be welcomed.

INTRODUCTORY REMARKS

Colonel William K. Douglas, USAF, MC

Vice Commander
Aerospace Medical Division
Brooks Air Force Base, Texas

I bring you greetings from the Aerospace Medical Division of the Air Force Systems Command. I specifically bring you greetings from Brigadier General George Shaffer, the Commander of that organization, who would have been here had he not been called to Washington for a variety of duties which only Generals can perfo m.

I note with interest that a significant portion of this conference is devoted to the discussion of national legislation affecting toxicological groups everywhere. The regulatory aspects of the National Environmental Policy and Occupational Safety and Health Act have already deeply affected the Air Force community. When an individual who has been elevated, or gravitated (as the case may be) to a management position is called upon to make some introductory remarks, and when he has been separated for some years from the actual operations, he can probably rely only upon the historical aspects of the subject.

I will remind you of some of the historical aspects of toxicology in the Air Force. I can remember times when the major Air Force concern was with the occasional chemical handler or the mechanic using carbon tetrachloride in the maintenance hangar. I discussed this with Dr. Thomas yesterday, and he pointed out a perfect example of the bureaucratic unlogic of the situation in those days. It seems that this was made perfectly all right if the mechanic in the maintenance hangar officially went to the Flight Surgeon, got a prescription, took the prescription down to the fire department and got his carbon tetrachloride. Well, gentlemen, times have changed. The missile and space business required us to obtain a great deal more sophistication, especially in the areas of protection, prevention, and treatment of the effects of some of the more exotic compounds, the propellants such as the hydrazines, and chlorines. And now with new legislation, we are finding out that the old ways of doing business are no longer acceptable. I would like as an aside to point out that it is my genuine belief that this legislation is much needed and has been a long time coming. The prescription for carbon tetrachloride is no longer obtainable, I might add. In fact, if we proceeded as we did in those days, we would get into a great deal of trouble with a multitude of agencies, both government and private. We find now that we must anticipate some 3 to 5 years in advance what weapon systems will be required and what chemical exposures may be associated with them. We must set out early to define all the information needed by environmental and occupational health teams to prevent, control, and treat the effects of a host of new and exotic substances.

Another part of the problem, and no easy task, is to make sure that the information channels are kept open and data up-to-date. This job includes nearly constant interpretation of legislative regulations, the development of new toxicological data, and most important, informing the operations and systems acquisition people as to how this legislation, as well as the toxicological properties of their systems, will affect them operationally. All this must be done before the systems are deployed and before toxic exposures start showing up in medical reports from the field. This is quite a change from the days when we relied on use experience to tell us whether or not we had a problem.

One of the real benefits of a conference such as this one is that the open discussions of the issues deriving from this legislation help each participating agency to respond to the President's announced national policy directed at improving the quality of life for all of our citizens. I believe that the Air Force role in all of this must be closely tied to the policies and programs of all the various civilian agencies represented here today. This conference provides a forum for frank discussions between the scientists who develop policy and the scientists who provide the essential research on which this policy is based. In light of this stated need for coordination within the scientific community, the laboratories of our command have developed cooperative programs with other federal agencies of similar interests. Our individual scientists continue to play key roles in the formulation of recommended human exposure standards. We will seek to strengthen these relationships whenever the work is clearly of mutual interest and benefit.

Before General Shaffer left for Washington, he brought me into his office and he told me a few words that he especially wanted to send to you. He wanted me to take this opportunity to express our sincere appreciation to the University of California, our new in-house contractor for the Toxic Hazards Research Unit, for sponsoring this conference and for assembling such an outstanding scientific program. The enthusiastic participation of the University of California scientists and the scientific management team has greatly enriched our own research program and has opened up a vast resource of expertise in toxicology and related sciences which we can call upon to our great advantage. This inter-campus, if you will, coalition also offers to interested investigators from the nine campuses of the University, an avenue for access to the Toxic Hazards Research Unit, which is truly a unique research tool. We hope that such active participation at the Dayton campus will continue to develop in the future.

Ladies and gentlemen, thank you very much.

SESSION I

TOXIC SUBSTANCES CONTROL ACT OF 1973

Chairman

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INTRODUCTORY COMMENTS - SESSION I

Harold C. Hodge, Ph. D.

University of California
San Francisco, California

I'd like to say a few words in introducing the first session devoted to consideration of the Toxic Substances Control Act of 1973. I have here a copy of the Senate bill and the House bill and I thought maybe there might be enough of you in the audience who are not familiar with this legislation so that I might read to you a few selections which, to me, represent some of the key points that must be discussed and will face the panel this morning. I'm going to start with Senate Bill S. 426. This bill was introduced by Mr. Byrd for a number of other people.

First I want to read from this document some excerpts about the declaration of policies. Congress finds that "(1) man and the environment are being exposed to a large number of chemical substances each year; (2) among the many chemical substances constantly being developed and produced are some which may pose an unreasonable threat to human health or the environment; and (3) the effective regulation of interstate commerce in such chemical substances necessitates the regulation of transactions in such chemical substances in intrastate commerce as well."

"Authority over chemical substances should be exercised in such a manner as not to unduly impede technological innovation while fulfilling the primary purpose of this Act to assure that such innovation and commerce in such chemical substances does not pose an unreasonable threat to human health or the environment." This is the basis for the legislation.

There are a couple of definitions I want to read to you: " 'protect health and the environment' means protect against any unreasonable threat to human health or the environment resulting from any chemical reaction of a chemical substance taking into account the benefits of the chemical substance as compared to the risks to human health or the environment."

As to test standards, "the Administrator shall issue proposed regulations (i) for such test protocols for various chemical substances or classes of chemical substances and (ii) for the results that must be achieved therefrom, as are necessary to protect health and environment." A little further: "Such test protocols may include tests for carcinogenesis, teratogenesis, mutagenesis, persistence, the cumulative properties of the

substance, the synergistic properties of the substance and other types of hazards, and epidemiological studies of the effects of the chemical substance."

There are three points made under the heading of "Research." The Environmental Protection Agency Administrator is authorized to conduct research and monitoring and here are the things suggested: to determine proper test protocols and results; determination of what existing chemical substances might present unreasonable hazards, not new ones, existing; and such monitoring of chemical substances in man and in the environment as is otherwise necessary.

In the area of environmental prediction and assessment, the document reads: "The Environmental Protection Agency shall, in cooperation with the Council on Environmental Quality and other Federal agencies, develop the necessary personnel and information resources to assess the environmental consequences of the introduction of new chemical substances into the environment."

I would like to compare two statements. One from the Senate bill under "Premarket Screening of New Chemical Substances." "Any manufacturer of a new chemical substance to which such regulations are applicable shall submit to the Administrator, at least ninety days in advance of the commercial production of such substance, the test data developed in accordance with the regulations." That was from the Senate bill. In the House bill, which is identified as H. R. 5356, there is a similar section. Remember that this section of the Senate bill was entitled "Premarket Screening." In the House bill, it is entitled "Limited Premarket Screening of Substantially Dangerous Chemical Substances." It reads, "The Administrator shall, by rule, identify and publish in the Federal Register a list of chemical substances (or classes or chemical substances) which the Administrator finds are likely to pose substantial danger to health or environment. For the purposes of this section, 'substantial danger to health or environment' means an unreasonable risk of death, severe personal injury or illness, or severe harm to the environment."

One final and very brief statement from the Senate bill again, and again under the "Research" heading. The Administrator is authorized to do various things. For example, he may make contracts and grants and construct research laboratories, and here's the idea I want to stress, after fully utilizing the personnel, facilities and other technical support available in other Federal agencies. It seems to me, and I acknowledge the suggestion of Ralph Wands on this point, that it's reasonable to compliment the Air Force in its foresight in having available a facility, often described as unique, the Toxic Hazards Research Laboratory, which can conduct work in connection with this program.

I'm not going to talk further but I'm going to pose some questions for the panelists. What tests should be performed? What results must be achieved? How can we possibly do so much testing? Where will we find the toxicologists? Who will interpret the data? How will we justify the costs if the tests exceed screening? Will the costs reduce, perhaps drastically reduce, the production of new chemicals? You may find other questions to ask our panelists and speakers.

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PAPER NO. 1

TOXIC SUBSTANCES: LEGISLATION, GOALS, AND CASE STUDIES

Glenn E. Schweitzer

Environmental Protection Agency
Washington, D. C.

I had hoped to be able to report to you today on the passage of the Toxic Substances Control Act of 1973 and the initial steps being undertaken in the implementation of this new law. However, there are a number of complicated aspects of the legislation, and it will still be some time until the conferees for the Senate and the House are able to reconcile the differences that exist in the two versions of the bill passed in July by these bodies. We are optimistic that agreement soon will be achieved on all provisions and that this much needed regulatory authority to control toxic substances will become law.

In view of the uncertainty as to the details of the proposed legislation, I have decided to refer to its specific provisions only briefly today and then concentrate on some of the broader aspects of our overall program. Specifically, I plan to set forth some of the general goals of our approach to the control of toxic substances and then discuss several case histories of very recent concern to us, the problems toxic substances are causing in these instances, and those aspects of the problems of particular interest to toxicologists.

TOXIC SUBSTANCES CONTROL ACT

The proposed Toxic Substances Control Act would give EPA new authority for (a) information acquisition, and (b) restrictive actions. EPA could require testing of chemical substances (both existing and new) which are suspected to pose unreasonable risks and also require other information from manufacturers including the name of the substance, chemical formula, amounts produced, actual or intended uses, and known by-products. EPA could then restrict the use and distribution of chemical substances found to pose unreasonable risks. The Agency could prescribe the amounts of a chemical which may be sold to processors, limit the type of processor to whom it may be sold, restrict the amount a given type of processor may use, or limit the sale or manner in which a substance may be used, handled, labelled, or disposed by any person.

This new authority is important from two standpoints. First, the Federal Government is given direct authority to restrict substances presently in commercial use that are known to cause health or environmental hazards, and when effects information is lacking, to require testing of the substance by the manufacturer to assess human or environmental impact. Second, for substances not yet in commercial production, the Agency could require premarket testing and review of chemicals suspected to be hazardous. The Agency would have the opportunity to assess the risks before the new substances are commercially produced and to take appropriate regulatory action to prevent toxic incidents.

Given the extensive scope of the legislation, the number of areas of initial emphasis will depend to a significant degree on the staff and resources available for implementation activities. In selecting areas for priority attention consideration should be given to Congressional mandates, severity and urgency of existing problems which can be alleviated by the new authority, opportunities to reduce future problems of major dimensions, and necessity for establishing long-term viability of implementation procedures.

While the final version of the legislation may influence the choice of initial activities, it seems clear that the following activities should be high on the agenda for early attention:

-- Elaboration and articulation of the criteria or sets of criteria to be used in weighing risks versus benefits, and in determining when regulatory action is needed. Clear understanding by both industry and Government of the ground rules for restrictions is essential to the viability of industrial R & D activities.

-- Determination of the character and scope of initial testing requirements, including the possibility of umbrella testing requirements for a broad range of chemical classes, and identification of specific chemicals or classes of chemicals of particular immediate concern. While the selection of substances covered by the standards for test protocols that are initially promulgated will in large measure reflect intuitive judgements concerning likely hazards and inadequacy of current data, concurrent work is needed to provide a basis over the longer term for selecting areas of concern.

-- Development of regulations setting forth timing, coverage, content, and format of the reporting requirements for chemical manufacturers and processors, including both annual reporting and premarket notification, as appropriate.

-- Establishment of a data system for handling the industrial reports and test results that are submitted. Experience in the pesticide area underscores the importance of early attention to establishing efficient and decision oriented procedures to be effective when the first reports arrive.

PROGRAM GOALS

Considering our program in its broadest sense, our goals are quite straightforward and are directed to:

-- clarification of the risks to health and the environment associated with the manufacturer, distribution, use, and disposal of new and existing chemical substances, with particular regard to chemical properties, production levels and trends, and exposure of the chemicals to man and the environment;

-- more effective utilization of regulatory authorities and related tools available to the Agency to mitigate such risks, taking into account the economic and social impact of restrictions on toxic substances; and

-- increasing the concern of and appropriate actions by the chemical and related industries to reduce risks to health and the environment associated with their activities.

Of particular interest is the emphasis placed on the responsibility of industry, for it is the course taken by industry -- with and without direct Governmental encouragement -- which will largely determine the environmental risks posed by chemical substances in the years ahead. The importance of this industrial stewardship is apparent when it is realized that the annual value of chemical substances manufactured in the United States which are not subject to existing regulatory authorities exceeds $150 billion.

Now let us turn to several specific activities of interest, starting with the concept of early warning. In this area our general goal is to identify and prioritize previously unsuspected chemicals entering the environment which are most likely to pose a significant hazard to man or the environment in the near future. Subgoals include:

-- Criteria: To develop criteria and techniques for determining on the basis of minimal information which chemical substances should be of greatest concern.

-- Expert Opinion: To mobilize and use expert opinion to assist in rapidly screening large numbers of chemicals and predicting potential problem substances.

-- Data Analysis: To collect, collate, and synthesize data from sources such as monitoring, trend assessment, and industrial reporting on those chemicals which appear to deserve the highest priority in a manner that will facilitate judgements as to whether the chemicals should be candidates for further investigations, testing, and/or control.

A second activity which is of particular interest to this group is testing. In general, we are attempting to improve the approaches by Government, industry, and the scientific community to testing of chemical substances entering commerce. Again, I would like to underscore the role of our industrial firms as we delineate our subgoals:

-- Industrial Stewardship: To encourage increased industrial concern and appropriate action in testing both new and existing chemicals.

-- Regulatory: To require or encourage, as appropriate, industrial testing of specific chemicals for which inadequate data concerning the risks associated with the chemicals is available but which are suspected to pose a hazard to man or the environment.

-- Experimental: To provide experimental data needed to determine appropriate standards or restrictions for specific chemicals of near-term concern.

-- Public Awareness: To bring test data concerning the safety of chemicals into public view, in a way that will not compromise trade secrets, thus facilitating a broader base of understanding and inputs for evaluating the necessity for restrictions for such chemicals.

A third area of interest is research, recognizing the overlaps between activities that are classified as research and programmatic activities. While the research interests of EPA encompass many areas, the three types of activities of priority interest to my Office are:

-- Test Methods: To stimulate development of faster, cheaper, and/or more reliable test methods, with particular emphasis on approaches that provide data needed for specific types of regulatory actions.

-- Trend Assessment: To improve assessments and forecasts of technological developments, economic and market trends, and material production and use patterns which can assist in anticipating environmental problems resulting from chemical substances entering the environment.

-- Estimation of Exposure: To develop and apply methods and background data needed for assessing the extent of environmental and human exposure to selected chemical substances, including consideration of environmental transport, persistence, routes of entry into the environment, bioconcentration and bioaccumulation, environmental degradation, and retrospective monitoring through sample banking.

CASE HISTORIES

Recent discovery of hexachlorobenzene (HCB) in the fat of animals brought to slaughter has focused attention on the hazards of this material. The most serious case involved cattle in central Louisiana. Other incidents of HCB contamination in the past year have involved sheep in western Texas and in eastern California. Sources of HCB in Louisiana appear to be airborne emissions of manufacturing plants which produce chlorinated hydrocarbons and waste disposal practices of these plants. The Texas and California situations have been associated with pesticides which were contaminated with HCB.

An immediate problem confronting EPA earlier this year was the determination of a permissible level of residues of HCB in food. In response to a request from the Department of Agriculture, EPA reviewed available data on the toxicology of HCB, and concluded that it was not adequate to permit establishment of a tolerance level for HCB in food. This was confirmed in consultations with scientists in the Departments of Agriculture and Health, Education, and Welfare, and with other members of the scientific community.

Nevertheless, HCB occurs in food frequently enough that some guidance is necessary to protect public health. An experience in Turkey in the mid-fifties vividly demonstrated the health effects of HCB when ingested over a long period. There is, however, no experience which indicates the effects of low doses on humans. Attempts in the United States and abroad to determine the safe level of HCB in experimental animals have been extremely limited and sporadic, and at present there is a lack of authoritative data. Experiments on a variety of animals have been limited in design, scope, and duration. Meaningful inferences are difficult to draw although there are indications that repeated dosages of HCB at low levels may be harmful.

Related to these uncertainties is the unknown extent which meat products with low levels of HCB residues are likely to reach individual consumers on a repetitive basis. The sources of HCB of immediate concern are confined to very small geographic pockets which traditionally disperse a significant portion of their products to markets around the country. Similarly, little information is available for assessing the economic impact of alternative tolerance levels. In the original Louisiana quarantine area, for example, there were up to 20,000 food animals with HCB in their fat at levels ranging from 0.1 to 6.0 ppm. However, the extent that these levels could be reduced through fattening with clean feed and through natural processes prior to slaughter is uncertain. Similarly, the cost and success of biochemical methods to reduce the levels are difficult to assess. There are many other economic costs related to establishing a tolerance including the impact on the value of contaminated land, the costs of alternative feed supplies and the long-term impact on animal herds. Data are not available to assess these types of impact. Furthermore, the extent and levels of HCB residues which will appear in the months and years ahead in many areas of the country cannot be predicted.

On June 1, EPA recommended an interim action guideline of 0.5 ppm HCB in the fat of cattle, swine, sheep, horses, and goats at the time of slaughter. This was based on analysis of the best data available, including toxicological studies and economic considerations. At the same time several toxicological, epidemiological, ecological, and economic studies are being launched to provide an improved basis for determing the permissible level as the guideline is periodically reviewed.

A second set of interesting case histories relates to the emission standards for hazardous pollutants established in April of this year under the Clean Air Act. Limitations were set on mercury, asbestos, and beryllium. While toxicologists have been concerned with these standards, toxicology data which is usable in quantifying an acceptable level of risk for these substances has been conspicuous by its absence.

In the case of asbestos, the association between occupational exposure to asbestos and higher-than-expected incidence of bronchial cancer was recognized. Also, asbestos has been identified as a causal factor in development of cancers of the membrane lining the chest and abdomen. However, the lack of adequate quantitative data correlating asbestos exposure to these and other malfunctions, together with the difficulty in measuring asbestos emissions, made establishment of allowable numerical concentrations impractical. Therefore, the standard relies largely on limitations on visible emissions and on the specification of related air cleaning technologies.

With regard to beryllium, the AEC limit of 0.01 micrograms/cubic meter, which was set in 1949, has been adopted as the basis for the emission standard. In the period since the implementation of the AEC guideline, no reported cases of chronic beryllium disease have occurred as a result of community exposure, and the Committee on Toxicology of the National Academy of Sciences has concluded that the AEC guideline represents a safe level of exposure.

Now turning to mercury, it seems clear that the airborne burden must be considered together with the water- and food-borne burdens. An expert group concluded, based on its analysis of several episodes of mercury poisoning in Japan, that 4 micrograms of methylmercury per kilogram of bodyweight per day would result in the intoxication of a sensitive adult; application of a safety factor of 10 yielded an acceptable exposure of about 30 micrograms per day for a 70-kilogram man, and this level is also believed to provide satisfactory protection against genetic lesions and poisoning of the fetus and of children. It has been estimated that from average diets, over a considerable period, mercury intakes of 10 micrograms per day may be expected, so that, in order to restrict total intake to 30 micrograms per day, the average mercury intake from air would have to be limited to 20 micrograms per day. Assuming inhalation of 20 cubic meters of air per day, the air could contain an average daily concentration of no more than 1 microgram of mercury per cubic meter. Thus, this level serves as the basis for the standard.

A third type of case history concerns current efforts to set toxic pollutant effluent standards under the Federal Water Pollution Control Act. On September 7, EPA gave notice that standards would be set on nine toxic pollutants. This standard setting activity is currently underway with a publication date of early December for the proposed standards. Included on the list of pollutants are cyanide whose acute toxicity properties are of major concern, cadmium and mercury which are associated with major chronic toxicity problems, and benzidine, a known carcinogen. The adequacy of toxicological data for supporting these standards varies from substance to substance, but I can assure you that in some instances it is sorely lacking. Nevertheless, in all nine cases we are faced with the necessity to make judgements as to an acceptable level of risk. When the proposed standards are promulgated, the

toxicology community hopefully will take the time to offer suggestions for improving the proposed approaches, for refining the interpretations of test data, and for generally pointing the way for future standard setting activities as they relate to the use of toxicological data.

In short, there are two types of problems related to toxicological data in setting standards in the near-term. First, how can the minimal test data which is available be most effectively used; and, secondly, how can future testing activity be best oriented to provide usable data for refining at a later date the standards that are being set? As an outsider with only brief exposure to the standard setting process and to the use of toxicological data, I have the general impression that there is not a good impedance match, at least in some areas, between standard setting needs and the orientation of testing activities. Furthermore, it appears that this difficulty is largely attributable to the failure of those of us responsible for programs to articulate our needs sufficiently far in advance to enable the scientific community to respond.

As we prepare to set standards for test protocols and to consider restrictive actions under the Toxic Substances Control Act, the importance of clear articulation of program needs takes on even greater significance. I am confident that in the years ahead, we will erode the communication difficulties between the program operators and the research community with society being the beneficiary.

Scientific data is interesting, but only if it is usable. Or in the words Frank Lloyd Wright, "Science can only give us the tools in a box . . . . but of what use to us are miraculous tools until we have mastered the human, cultural use of them?"

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PAPER NO. 2

REASONABLE DATA BASES FOR EVALUATION OF ACUTE TOXICITY

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INTRODUCTION

The primary goal of the Toxic Substances Control Act is to prevent unreasonable threats to human beings or the environment from the use of chemical substances and products containing chemical substances. The Environmental Protection Agency (EPA) would regulate such substances by restricting their use and distribution if pre-market testing indicates that there is an unreasonable threat to the public health or environment. To carry out this mission, the EPA must deal systematically with a broad spectrum of chemical compounds. It has been estimated, in the Magnuson hearings, that there are, in existence, about two million recognized chemical components with approximately another quarter million new compounds being added annually. However, in this latter category most will probably never be produced commercially. It is the several hundred chemical compounds which will be produced in commercial quantities that this legislation is designed to control.

The plethora of chemical products currently available, in addition to those that will enter commercial channels, will require physical, chemical, and biological research efforts to develop methods of analysis and hazard evaluation to meet the needs of both the manufacturer and the regulator. Intervention by government control is a natural corollary to our National concern over the problems of environmental pollution. Historically, government has sought to control toxic materials. In addition to statutory controls, various other aids are essential if government is to pursue an effective regulatory policy. The need for an adequate surveillance system, as well as a basic toxicity information storage and retrieval system, is of fundamental importance. There is also a need for adequately trained personnel to plan and carry out the research or regulatory programs. In order to handle this statutory control, the regulator must have adequate resources at his disposal to investigate new toxic hazards as they come to light. The type of enforcement activity depends, in large, on the quality of the research information.

REASONABLE DATA BASES FOR EVALUATING ACUTE TOXICITY

The regulating agency must make a prior determination that an unreasonable risk is, at least, suspect before it can begin proceedings to restrict a chemical's sale and distribution. In order to accomplish this, the enforcement agency needs to broaden and standardize the currently available toxicological testing protocols so that the data required to be submitted for regulatory review will be meaningful.

The regulator must weigh a compound's or product's utility against its toxicological hazards. This is most difficult, particularly because the worst conditions of abuse or misuse cannot always be foreseen. Nor has there been devised any set of toxicological, pharmacological, biochemical, or physiological tests which can accurately predict all the possible harmful or deleterious effects of a new substance or product in th an.

Based on the assumption that elaborate testing schemes would be impractical, one then must ask, what is a reasonable approach to developing acute toxicity data? What are the factors that we should be concerned with?

Some of the following questions may serve as guidelines in planning such studies:

1. What is the product's intended use?
2. Under what conditions will the product be used?
3. What is the age and sex of those most likely to be exposed?
4. What is the maximum expected exposure?
5. What is the method of dispensing the product? (physical form i.e., liquid, paste, aerosol, powder, etc.)
6. What are the effects of climatic conditions? (humidity, sunlight, etc.)
7. What are the potential physiological effects and metabolic intermediates?
8. What are the product's chemical and physical properties?
9. Is the product biodegradable? Under what conditions?

Biological safety testing will represent a good fraction of the cost of developing new chemicals and expanding their potential market. There is little value in conducting costly toxicological studies on materials that have not been chemically and physically characterized. Toxicological studies are, in most cases, extremely complicated biological research programs. It is, generally speaking, impractical to spell out specific sets of tests and rules that one should follow. However, once a decision has been made with respect to what type of information is required, the protocols should be designed and the laboratory studies initiated. The number of animals, as well as the animal of choice, for a particular test is influenced by the type of information desired. The effects produced by any substance in a biological system must be interpreted in light of factors that produce biological variation. The regulatory definitions used to interpret the test results, as well as the procedures used, are of the utmost fundamental importance in developing acceptable screening data. Therefore, an appropriate emphasis should be allocated to developing these procedures and definitions. Statutory control, in order to be effective, must meet a specific need and be capable of effective enforcement.

During preliminary experimental product development research, the simpler, cheaper, "all-or-none" type acute or very short term tests, are used to predict the quantity of a new material to which exposure on a one time basis might be hazardous.

As research on potential utility of the experimental product is developed, toxicity testing is expanded to include consideration of metabolism studies and cumulative injury.

Once the development proceeds to large, field scale studies related to practical utility and for determining environmental distribution and/or persistence of the product and its metabolites, toxicity screening should be tailored to include more sophisticated testing i.e., teratogenicity, mutagenicity, carcinogenicity, etc. These tests should reflect the nature and degree of anticipated human exposure during manufacture, formulation, distribution and use of proposed products, as well as animal or human exposure to any residues in the environment.

As I pointed out earlier, the physiochemical characteristics of the compound play an important part in the toxicologist's decision as to what testing programs and what priorities will be assigned. Moreover, it should be stressed that the product, as it will be used and marketed, is tested; not the individual components.

The following types of test procedures should be considered in evaluating the chemical's acute toxicity:

1. Oral Ingestion - LD_{50}
2. Cutaneous Toxicity
   a. percutaneous absorption
   b. eye irritation
   c. skin irritation
   d. sensitization
3. Inhalation

ORAL TOXICITY

Because ingestion is, by far, the most likely route of exposure that might be encountered, it would appear sensible to determine the oral ingestion hazard for a given compound. The median lethal dose or LD_{50}, is an estimation of the single oral dose (gm/kg) that will kill 50 percent of a significant number of exposed test animals observed for a specified time. This dose is not absolute value and can vary considerably with experimental conditions. Such factors as concentration, weight, sex, preparation of test material, solubility of the substance, number of animals, species, dose interval, and period of observation are of critical importance in evaluating the data. The LD_{50} value, while not an absolute number, is extremely useful in ranking the potential hazard of a new chemical.

In these acute oral toxicity studies, additional information on a chemical's pharmacologic mechanism of action can be obtained on a gross basis by careful observation of the signs produced after administration of the chemical. It is equally important to also note their onset and duration.

Necropsy of the test animal, with or without histopathology, can also provide valuable data on injury to vital organs. For example, gross inspection of the intestinal tract may reveal irritant or corrosive effects.

Finally, the mechanism of action of the compound may be assessed in a very general way by noting the size of the dose that produced the median lethal effect. Compounds with LD_{50} values less than 50 mg/kg are thought to react through interference with vital enzymatic mechanisms while large doses, i.e., 4-5 gm/kg, may produce their lethal effect via nonspecific means.

CUTANEOUS TOXICITY

When considering systemic injury via percutaneous absorption, eye or skin irritation, or skin sensitization, as a result of contact with a chemical, it is well to consider simulating use conditions.

Prior to any consumer exposure, however, there should be some valuable data on industrial exposure available. Whether or not the chemical ever gets beyond the developmental stages depends on a number of factors which are primarily concerned with industrial hygiene, the initial concerns about toxicity, irritation, sensitization, etc., being at the chemist's bench. The amounts of material available, however, are frequently small and would probably be used for crucial chemical and physical tests. If the material proves worthy, a larger quantity will be made. The next step in development would be the pilot plant production. Here again, the industrial toxicologist must consider the manner of exposure and possible effects on the worker's environment. Therefore, before considering what animal models should be used to simulate use conditions, the industrial exposure data should be thoroughly evaluated.

Penetration in vivo may lead to systemic poisoning or allergic sensitization. Many chemicals penetrate the skin barrier with difficulty, while others have essentially no difficulty at all. It has been suggested by Tregear (1966) that animal skin is more permeable to chemicals than human skin. However, one should bear in mind that the permeability of skin to any specific compound will vary with its chemical structure. Percutaneous absorption should be quantitated. The evaluation of dermal toxicity can be simplified when it can be shown that a compound is not significantly absorbed.

In making a safety evaluation on a particular substance, any adverse effects noted in industrial exposure, or the animal toxicity studies, help to define specific toxic effects and may indicate the possible mechanism of action. Data from human experience, when available in documented form, is given preference over animal data.

INHALATION

The primary areas of concern are gases, volatile liquids and aerosols. Inhalation studies are time consuming, expensive, require highly trained personnel and equipment, and the results are difficult to interpret. It is especially difficult to define inhalation exposure for animal and man in appropriate dosage terms. Moreover, much consideration must be given to the differences in the anatomical and physiological characteristics of the respiratory systems between animal species. In general, the choice of experimental animals has to be tailored to meet the objectives of the experiment and the mode of action of the test compound.

Before embarking on any inhalation study, one should consider the following:

1. Are the exposure conditions realistic?
2. Are there any other routes of administration that could be used to predict systemic inhalation effects?
3. Is the animal model appropriate for extrapolating the results to man?
4. What physiological and biochemical parameters will be measured?
5. What are the physiochemical characteristics of the inhalant?
6. Exposure techniques - static or dynamic conditions
   - head or whole body of the animal
7. Exposure concentration and duration.

It is strongly recommended that the guidelines for inhalation studies be extremely flexible so that appropriate protocols can be developed for individual compounds or classes of materials.

SUMMARY

The insidious nature of poisons and the inability of the public to detect the toxicity of products merely by inspection call for statutory controls. The potential hazard of any chemical substance is directly related to the degree of exposure. One of the prime issues in evaluating a chemical's hazard potential is the "margin of safety." This can be best expressed as a ratio by comparing the predicted human exposure level to the highest dosage level in animals that produced no adverse effects. The comparison, of course, should be for similar conditions of exposure. The larger the quotient, the greater the potential risk.

A variety of test methods have been developed to evaluate the acute toxicity of chemical substances. There are inherent problems associated with such methods which are, for the most part, well known and certainly a full discussion is well beyond the scope of this presentation.

Despite their shortcomings, these tests will, by necessity or lack of interest, continue to be used. Let s not encourage changing test procedures for the sake of change, but change for the sake of improvement. In addressing the need for standard test protocols for various classes and uses of chemicals, as well as the results to be achieved, we find ourselves faced with a dilemma. There seems to be a divergence of opinion among the respected members of the scientific community as to what constitutes the best test method. There appear to be gaps in our ability to develop reproducible tests. Since the government is charged with the regulatory responsibility to protect the public and the environment, we need to identify the means by which these hazards can be consistently identified. Furthermore, the government has an obligation to the regulated industries to recommend test procedures that will be technically feasible, practical and appropriate for ascertaining the potential hazard of a chemical. If we communicate our needs and offer our respective talents to solve some of these problems, we can essentially guarantee our chances of success. But the real beneficiaries are the future generations because we will, hopefully, ensure for them a safer environment.

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PAPER NO. 3

REASONABLE BASIS FOR EVALUATION OF CHRONIC TOXICITY

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Toxicology has been defined as the science dealing with the adverse effects of chemical and physical agents on living systems. Chronic, long-term (or lifetime) exposures of living systems, including man, are necessary to define the adverse effects which may occur. Because of the potential for induction of genetic changes by exposure to toxic agents, certain studies (beyond the life span of the exposed systems) may be necessary. These can include multi-generation reproduction studies.

The classical approach to the study of chronic toxicity of substances has been described in documents of the National Academy of Sciences, the United States Food and Drug Administration, the World Health Organization, and others. This approach involves studies in several species of animals for periods of time, ranging from several months to several years, during and after exposure to the agent in question at appropriate routes of administration. A variety of specific tests, conducted at prescribed intervals during each study, allows assessment of anatomic and functional changes that may occur. These changes are measured by the use of biochemical, physiological, and histopathological methods which permit quantitation, to a degree, of the adverse effects as compared to changes which may occur spontaneously in concurrent control animals.

In the classical sense the results of such highly structured animal studies provide a basis for safety evaluation which includes definition of no-effect levels, safety factors, acceptable daily intakes, threshold limit values, etc. as related to the pattern of use.

Within the past few years, the study of chronic toxicity of substances has become more sophisticated. This has been the result of the refinement of knowledge derived from research stimulated in part by such things as the thalidomide tragedy and by the appearance of toxicologic and environmental problems associated with materials formerly judged to be safe for use when evaluated according to older classical procedures.

Much of the awareness concerning toxicologic hazards has been derived from retrospective consideration of unfortunate events that have occurred; but it must be the objective of toxicologists to devise procedures which will be predictive. One might avoid future problems by retreating to the nihilistic position of abandonment of technological progress by withholding approval for the introduction of new agents of potential utility, but a more constructive approach must be to maximally utilize and build upon existing knowledge to define research programs which can provide a realistic assessment of the hazard which might be associated with the use of a new chemical agent. It must be recognized that no set of routine animal investigations will be universally applicable for definition of the toxicologic properties of all substances, and that all decisions regarding safety in use must be tentative and subject to subsequent reversal or restriction in the light of experience in use or with the development of more discriminating methodology for the study and interpretation of toxicologic hazard.

The urgent need is to recognize that chronic toxicity evaluations require a highly specific and sophisticated multidisciplinary approach that utilizes the best and most advanced scientific knowledge and techniques available. This serious challenge requires that no fixed course be charted based on regulations or past practices. A dynamic and evolutionary approach must be utilized on every problem of safety evaluation.

Traditional chronic toxicity studies have, for the most part, been conducted at dose levels selected to provide margins of safety over maximum anticipated exposure levels in man or in economically important animal species, assuming a qualitative and semiquantitative similarity in response of the chosen experimental species and those to whom the results are extrapolated. It must be an objective of toxicologists to define intrinsic toxicologic properties of a new material in a variety of systems, including environmental systems, without regard to in-use exposure levels. Only then can margins of safety be placed in proper perspective.

Pharmacologic studies have been largely ignored or have been limited to the pharmacologic parameter relevant to the proposed use. It is of extreme importance to understand the pharmacodynamic properties of a substance for selection of tolerable dose levels and to distinguish between expected pharmacodynamic responses and toxicologic effects. The differences may at times become obscure. For example, with a corticosteroid one expects to observe dose-related differences in mortality in rats from respiratory disease, or in rabbits from respiratory disease or coccidiosis because of suppression of immune responses by the agent being tested.

Metabolic studies have most commonly been conducted after chronic studies or concurrently with them. It is proposed that metabolic studies are vital to selection of species and dose levels for chronic studies. Such studies

should be conducted in several species, and in man, to be able to select appropriate species for chronic studies, species most like man with regard to metabolic pathways, blood levels, etc. Using tracer techniques, metabolic studies can be conducted in man with massive margins of safety based on the results of acute and subacute studies in animals.

Perhaps most importantly, target organ or target system studies should be conducted to provide a rational basis for the design of chronic studies. Frequently in a chronic study (at dose levels selected as multiples of use exposure levels), the target system is not apparent until the study is completed and neither a maximum no-effect level nor a minimal effect level may be defined. Unless the effect is functional, no knowledge of the rates of development of the effects or of the possible reversibility is achievable.

It is not a purpose of this presentation to define specific protocols but it is proposed that, after full consideration of all acute, subacute, pharmacologic, and metabolic data, target system studies in two species deemed to be most appropriate be conducted to determine:

1. The system most susceptible to the effects of the agent.
2. The rate of development of the effect as related to dose level.
3. Reversibility of effect upon withdrawal of the agent.
4. Relationship of blood or tissue levels of the agent or its metabolites to the appearance and development or persistence of an effect.
5. Possible equilibration of degree of effect at a fixed dose level.

It is recognized that such a study would require thoughtful and individualized consideration of each material investigated. It would require the use of sufficient numbers of animals to permit evaluation of all parameters considered to be toxicologically significant, including histopathologic changes, at all prescribed intervals during the study and would require continual exercise of judgment with regard to progressive adjustment of dosage. Having completed such a study, however, one would be in a position to design more meaningful chronic studies or perhaps even to eliminate the necessity for conducting certain traditional chronic studies: e.g., long-term cholinesterase inhibition studies at fixed dose levels. The results of a well designed target organ study would alert investigators to specific anatomical, physiological, and biochemical parameters requiring special attention.

It may be worthwhile to illustrate further the general concept of "target organ effects" in a context that may serve as examples of experimental approaches not always covered by routine toxicological methodology.

The liver is an important target organ for many substances that enter the body via the gut. The pioneer work of Sir Roy Cameron, later extended by others, on the effects of carbon tetrachloride illustrates the evolution of knowledge from the basic observation that this substance produces a non-specific pathologic change - fatty degeneration - to the elucidation of sophisticated mechanisms that attempt to explain the process, which involved more than routine histological examination of prepared liver slices under the light microscope. For example, in this specific instance the following were investigated: the accumulation of triglycerides in liver cells; the role of the ribosomes; the role of adenosine triphosphate; the role of inhibitors of protein synthesis; the problem of fat transport as well as other factors.

The kidney is another important target organ with mercury possibly serving as one of the better toxic agents for use in the study of this organ system. It is known that necrosis of the renal tubules occurs with some mercury compounds while others are useful as diuretics without tubular damage, and that certain alkyl mercury compounds affect the brain and not the kidney. This illustrates that different, and in some cases unrelated, tissue and/or organ responses to certain metals and their compounds may occur.

The extrapolation of animal data to prediction of safety in man is not certain. Some of the problems are:

1. Limitations due to various species.
2. Limitations due to differences with which the species metabolize drugs (chemicals).
3. Limitations of knowledge as to what is normal.
4. Limitations imposed by new tools and limited experience.
5. Limitations imposed by experimental design-exclusion.
6. Limitations imposed by the basic concept of toxicity testing as a measure of safety assessment - real versus relevant.

Regardless of one's personal views about the relevance of animal data to safety evaluation for man, it is obvious that much reliance must be placed on such data supplemented by limited, well-controlled toxicity and metabolic studies in man. Tests using man as the subject can be done safely, legally, morally proper, and ethically correct. Some examples are dermal irritation, sensitization, photosensitization, cholinesterase inhibition, and metabolic studies. Establishment of a degree of analogy of responses in experimental animals and man would enhance the confidence in predictive extrapolation, but absence of deleterious effects under conditions of use, or even misuse, must remain the ultimate test. Some of these are shown by results of epidemiological studies and industrial medical programs.

A very difficult problem is the assignment of relevance to certain measurable effects of chemical agents, particularly biochemical effects. What is the toxicologic significance of metabolic enzyme induction and liver hypertrophy which are associated with exposure to many agents? What is the significance of inhibition of erythrocyte delta-amino-levulinic acid hydrase by lead when this enzyme has no known function in the mature red cell? Perhaps such findings should encourage vigilance for detection of adverse functional or anatomic changes, but biochemical effects which cannot be associated with hazard should not be used as criteria for establishing safe levels of exposure.

The organization and deliberations of this Conference illustrate that there is much concern about chemical hazards in the human environment which may be manifest as recognizable clinical illness or poisoning at one extreme, and minimal biochemical or physiological alterations at the other end of the toxicological spectrum.

The effects produced may possibly occur from a single exposure of one individual to a chemical for a brief period or to repeated integrated variable doses over the lifetime of a community population. The evaluation of the latter situation is complicated by the heterogeneity of the large numbers of individuals with respect not only to temporal existence, innate anatomical, biological or physiological differences and, in some cases by deficiencies as well.

An additional complexity is the variability of exposures resulting from areas of discrete microenvironments sufficiently different to confuse and to complicate the prospective as well as retrospective evaluation of the medical effects of the hazards imposed (knowingly or inadvertently) by exposure to a single or to the total hierarchy of environmental chemicals.

In developing a set of principles for use as broad guidelines to evaluate the chronic effects of chemicals in the environment, one must first establish the criteria that dictate the need for definitive chronic toxicological information in one case and not in another. It is obvious that all present or future substances in the environment will not need chronic toxicological studies.

With variegated demands and limited resources, a rational, sequential deployment of test procedures is clearly indicated. Presently available testing procedures require critical reevaluation in terms of their predictability, information content, feasibility, and ultimately, benefit in terms of cost. Such a system must indicate each decision juncture whether further testing and development is indicated within the framework of the product's potential utility to society. When approached in this fashion, limited available resources can be deployed at optimum levels of efficiency consistent with maximum social safety.

The demands of society to live in a rich, but risk-free, environment cannot effectively be served in the present economic, social, and political milieu. However, it is not unreasonable for society to expect and receive assurance that it is not being subjected to undue hazard as the result of the use of substances that provide well-defined benefits in a specific or broad sense.

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PAPER NO. 4

PROBLEMS ASSOCIATED WITH DETERMINING SAFE LIMITS OF TOXIC SUBSTANCES

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INTRODUCTION

In preparing this article, I considered especially the language of the draft bill entitled, "Toxic Substances Control Act 1973" now before the Congress. The bill "is designed to regulate interstate commerce to protect health and the environment from hazardous chemical substances." The person responsible for the enforcement of the act is provided the following directives. "The administrator shall consider ALL relevant factors including: (1) the effects of the substance on health and the magnitude of human exposure; (2) the effects of the substance on the environment and the magnitude of environmental exposure; and (3) the benefits of the substance for various uses and the availability of less hazardous substances."

In the following sections, I discuss a few of the problems the administrator must confront in attempting to obtain a scientifically sound answer to the question, what is the safe limit of exposure to a particular toxicant?

Heavy metal examples are utilized in this paper to emphasize some of the problems one is likely to encounter. Factors to be discussed include:

1. Bio-amplification
2. Bio-transformation
3. Element-element interactions
4. Other factors modifying heavy metal tolerance.

BIO-AMPLIFICATION

Many toxicants are broken down rapidly into H_2O, CO_2, PO_4, etc., and thus rendered harmless. On the other hand, toxicants such as some chlorinated hydrocarbons are resistant to decomposition and have been shown to accumulate in certain organisms in concentrations many fold higher than ambient levels. This process, called bio-amplification, is an especially

important factor with heavy metals in aquatic environments. Algae concentrate Hg 10-50 fold above the concentration in the water. At the second trophic level, algae eaters concentrate the Hg another 10-20 fold. Further trophic levels continue the bio-amplification process until at the top of the aquatic food chain, pike have been found with concentrations of Hg in their muscle tissue that are 3000 fold greater than the Hg concentrations of the water. Terrestrial bio-amplification is usually not greater than 2-4 fold.

In determining the effect of a particular toxicant upon man and his environment, one should therefore obtain information about the substances' stability and bio-amplification patterns.

BIO-TRANSFORMATION

As heavy metals move through the various organisms, they are frequently transformed into compounds of very different toxicological properties from that of the original compound.

Evidence for such transformation of heavy metals has been obtained only recently. Prior to 1969 the Hg which had been discharged from industry into surface waters had been thought to remain in the bottom sediments, unavailable to fish and lower organisms. Jensen and Jernolov (1969), however, showed that this assumption was incorrect; they reported that inorganic Hg could be methylated by anaerobic microorganisms in the bottom sediment of fresh water to form volatile mono- and dimethylmercury. These conversions are the key to the biological concentration of Hg in the aquatic ecosystem (D'Itri, 1972).

The alkyl forms of Hg, particularly methyl and ethyl, are both significantly more toxic and biologically more mobile than the other forms of Hg (Friberg and Vostal, 1972). Dietary methylmercury as shown in Table 1 is absorbed almost completely (90-100%), is stable in the human body and has a longer biological half-life compared to other organo forms of Hg (aryl and alkoxy) which are poorly absorbed (2-20%), are transformed in the body to inorganic Hg, and are excreted more rapidly. The inorganic form is poorly absorbed from the diet and that which is absorbed is rapidly excreted, thereby largely accounting for its lower toxicity.

Much less is known about the biological transformations of Cd and Pb or the relative toxicity of different forms of these metals. Both Cd and Pb have extremely long biological half-lives in man (16-33 and 4 years, respectively); thus, these metals tend to accumulate in the body.

The possibility that a toxicant may accumulate in an organism over an extended period of time and that it may be transformed into forms with greatly altered toxicity must be considered.

TABLE 1. DIETARY INTAKE, ABSORPTION AND HALF-LIFE OF Cd, Hg, AND Pb IN MAN

	Average Daily Dietary Intake μg/day	Approximate % Absorbed	Whole Body Half-life in Years
Cd[1]	70	10	16-33
Hg[3]	25	90-100 ($MeHg^{+}$) 2-20 (Hg^{++})	0.2 ($MeHg^{+}$) 0.08 (Hg^{++})
Pb[3]	300	5	4

[1]Friberg et al., 1971
[2]D'Itri, 1972
[3]Committee on Biologic Effects of Atmospheric Pollutants, National Academy of Sciences, 1972.

ELEMENT-ELEMENT INTERACTIONS

Another factor modifying the toxicity of the heavy metals is their metabolic interaction with other elements. Methylmercury added to a tuna-corn-soya diet for Japanese quail was found to be considerably less toxic than an equivalent amount of methylmercury added to a corn-soya diet (Ganther et al., 1972). This suggested that factors other than the Hg content of the tuna may have modified the toxicity of the methylmercury. The protective factor in tuna was found to be Se. Selenium added to corn-soya diets in equivalent concentrations and fed either to quail or rats was found to decrease methylmercury toxicity (Ganther et al., 1973). Other species of predatory fish have been shown to contain similarly high levels of Se. This work indicates that both Se and Hg appear to accumulate in several predatory fish species and that the presence of Se reduces the toxicity of the methylmercury. Ganther in summarizing his own work on Hg and Se says: "The observation that individuals can have elevated concentrations of Hg in the blood and yet be free of symptoms of Hg poisoning suggests, as one possibility, that agents modifying the toxicity may indeed be operative" and "... that the danger for man of Hg in tuna may be somewhat less than anticipated, and that the total Hg content in the diet or even in the blood may not be valid criteria because of the presence of modifying factors." (Ganther et al., 1973).

These comments underscore the contention that data on the ratio of the concentrations of both of these elements are likely to provide information of more value in predicting toxicity than information on the concentration of either element alone.

The role of Se in protecting animals against the toxic effects of certain heavy metals, especially Hg and Cd, has been investigated in a number of toxicological and physiological studies over the past 17 years by Parizek's laboratory in Prague, Czechoslovakia. This work, recently reviewed by Parizek et al. (1971), shows that small amounts of injected selenite will protect animals against the effects of Cd-related damage to reproductive organs as well as against acute toxicity of larger doses of Cd. Although nutritional studies similar to those described with Hg and Se have not been done for dietary concentrations of Cd and Se, it is likely that a similar protective effect will be found.

The action of Hg, Cd and Pb not only may be modified by other elements such as Se but they also may interfere with normal metabolism of essential trace elements. Over the past decade Hill and Matrone (1970) have identified certain chemical properties or parameters of trace elements and heavy metals useful in predicting their interactions according to a hypothesis they have called "the chemical parameter concept."

In animal dietary studies, they have shown, for example, that Cd^{++} interferes with both Cu and Zn metabolism. This appears to be due to the fact that Cd^{++} has the same chemical parameters as Zn^{++} and Cu^{+}. But Hg^{++} does not interfere with either Cu or Zn metabolism since it differs in the chemical parameters of geometric configuration and coordination number (Bunn and Matrone, 1966; Hill et al., 1963). This interference of Cd with normal Zn metabolism is of particular concern in light of the important role of Zn in several enzymes as well as in wound healing (Pories et al., 1971). Currently, nutritionists are concerned about marginal trace element deficiencies, especially of Fe and Zn. Such marginal deficiencies should be evaluated with knowledge of the prevalent concentrations and effects of dietary intake of heavy metals on the metabolism of the essential trace elements.

In addition to having pronounced effects upon the metabolism of trace elements, heavy metals also interact with some major elements. For example, it is known that dietary concentrations of Ca and P influence the deposition of Pb in soft tissue and bone (Sobel et al., 1940). More recently, it has been reported that Ca and P affect the intestinal absorption of Pb (Weiss et al., 1971) and that lowering the dietary intake of Ca increases the toxicity of Pb in rats (Six and Goyer, 1970).

In the recent methylmercury-poisoning episode in the human population of Iraq, Bakir et al. (1973) found mortality more frequently among pregnant than among nonpregnant females of the same age. They also found wide variation in functional recovery of individuals after the ingestion of methylmercury had ceased. Similar observations with Pb and Cd susceptibility suggest that age, physiological status, and genetics may be important determinants of heavy metal toxicity. Interactions of heavy metals with nonmineral dietary ingredients also have been found. For example, Cd toxicity is decreased with dietary ascorbic acid supplements (Fox and Fry, 1970).

It is particularly important to keep these types of interactions in mind when setting and evaluating standards for safe exposure to heavy metals. The determination of where "normal" is on the diagram in Figure 1 cannot be determined for one element or compound alone but is interrelated with many other dietary factors and should be established accordingly.

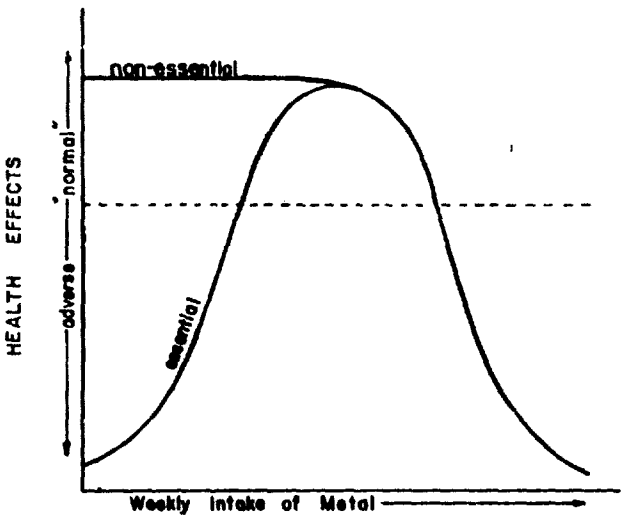

Figure 1. Hypothetical relationship between weekly intake of essential and nonessential elements and the health effects resulting from that intake.

In my estimation, "safe" exposure standards should be based upon ratios of concentrations of Hg:Se, Pb:Ca and P, Cd:Zn and Cd:Cu, and upon the form of the element and not solely upon the concentration of a single substance. More thorough elucidation of these types of interactions will be helpful in predicting and avoiding heavy metal toxicities. Such an understanding also would be of value in developing remedial procedures where the preventive procedures have not been developed or have failed.

OTHER FACTORS MODIFYING HEAVY METAL TOLERANCE

Defense Mechanisms

Since all organisms, including man, have been exposed to heavy metals throughout evolution, organisms may have developed mechanisms such as selective absorption and excretion to deal with these metals safely

(Ashida, 1965). Metallothionine appears to be an example of a protein produced by an animal to protect it against Cd. This protein contains unusually large amounts of both Zn and Cd and sometimes Hg (Margoshes and Vallee, 1957; Kagi and Vallee, 1961). Although it first was thought to be a Cd enzyme necessary to the cell, more recently metallothionine has been considered to play a protective role by sequestering Cd. Small initial doses of Cd have been shown to render rats and mice immune to doses of Cd which would have been toxic. This presumably is accomplished by inducing the synthesis of a Cd-binding protein similar to metallothionine which can then bind the large dose of Cd (Friberg et al., 1971; Flick et al., 1971). Other types of genetically controlled defense or detoxification mechanisms may be activated by a toxicant in an organism, or alternately as is discussed in the following section, the toxicant may predispose the organism to greater susceptibility to some other stress producing agent.

Altered Disease Susceptibility in Plants

An aspect of the heavy metals question deserving much more attention is the influence of heavy metals on disease susceptibility of plants. David et al. (1955) have shown that uptake of Pb by tobacco plants can induce symptoms resembling the early phases of frenching, a disease characterized by the development of narrow elongated leaves.

Foliar sprays of phenylmercuric acetate used to control the coffee berry disease have been shown by Bock et al. (1958) to induce zinc deficiency symptoms in coffee plants. Treated plants contained one-fourth the Zn concentration of nontreated control plants. Further, Ruschel and Costa (1966) have shown that the normal symbiotic nitrogen fixation pattern in bean is upset by low dosages of various insecticides and fungicides.

A limited amount of work with nematodes (Clarke and Sheperd, 1966; Zuckerman et al., 1971) has demonstrated that various inorganic ions commonly contained in fungicidal formulations stimulate increased hatch and support more rapid population increases and more severe disease losses result.

From these examples, we conclude that heavy metals applied advertently or inadvertently to plants may alter their trace-element metabolism and decrease their capacity of normal healthy development. An assessment of these types of interactions should become an integral part of all pesticide evaluation programs so that benefits from the control of one pathogen on one host are not nullified by concomitant accentuation of other disease problems. Care should also be taken to determine the effects of heavy metal pesticides on subsequent crops in a crop rotation sequence.

Altered Disease Susceptibility in Animals

Much of the concern about heavy metal toxicity has been due to the acute toxicity of these elements. Comparatively less attention has been given to chronic effects. Adamson (1973) suggests that the concentrations of Pb to which urban man is subjected may be predisposing him to enhanced susceptibility to infection. She concludes this on the basis of experiments with animals subjected to low concentrations of Pb that demonstrate an increase in susceptibility to bacterial infections and to bacterial endotoxins.

In Schroeder's experiments, groups of 50 or more rats of each sex were fed 5 ppm of Pb in their drinking water during their entire lifetimes (Schroeder et al., 1964). The rats receiving Pb showed a 16% increase in infant mortality, a 10% increase in the number that died from an epidemic of pneumonia, and a 26% reduction in longevity. Lead analyses performed on various organs of these rats at death, on wild rats, and on humans showed that in most cases the Pb content was lower in the treated rats than in wild rats or in adult humans, indicating that the wild rats and humans had been exposed to greater than 5 ppm Pb.

Hemphill et al. (1971), in some short-term experiments (30 days), preexposed mice to ca. 3 mg Pb/kg/day (I.P. as Pb-acetate). No signs of Pb toxicity were observed during this period. The treated and control mice were then exposed to a standard dose of Salmonella typhimurium. Within 7 days 54% of the Pb treated animals but only 13% of the controls died of the Salmonella infection. In terms of LD_{50} of Salmonella, the control animals were 10 times as resistant as the Pb treated ones.

Seyle et al. (1966) showed that a single dose of Pb-acetate caused up to a 100,000 fold increase in sensitivity of rats to endotoxins produced by several types of gram-negative bacteria. The largest dose of Pb used (27 mg/kg body wt. as a single dose) was well tolerated in the absence of endotoxin administration, with no mortality being observed with lead or purified endotoxin alone. If both Pb and endotoxin were administered, however, 80-100% mortality resulted within 24 hours. Even at doses as low as 5.5 mg/kg, 30% mortality resulted. Other workers (Trejo and DiLuzio, 1971; Trejo et al., 1972; Bertok, 1968; Filkins and Buchanan, 1973) have confirmed these findings.

What is the relevance of these data for humans? Are humans currently being predisposed to greater susceptibility to various types of infections due to their body burden of Pb? What are the differences in response to exposure of experimental animals to Pb for short periods and exposure of humans to low concentrations for a lifetime? No one has approached these questions directly but Holper et al. (1973), working with baboons, has recently found dramatic increases in sensitivity to endotoxins after exposure to Pb. There is also evidence that some human populations already contain body burdens

at which these types of effects can be expected, even though they do not have high enough concentrations to produce symptoms of Pb poisoning (Hall, 1972).

CONCLUSIONS AND QUESTIONS

These results raise the following questions: What parameters of an organism should be measured in order to determine when it is being affected by a particular toxic substance(s)? How can we develop sensitive behavorial or physiological tests that can be applied to a number of organisms in an ecosystem and from which we can derive information on "safe exposure limits"?

To date most of our maximum Safe Limits data for humans have been based upon extrapolations from data obtained from:

a. Short-term acute dosage effects of single or a small number of exposures of some inbred line of laboratory animal to a single toxicant, whereas in nature, organisms are frequently subjected to chronic low level dosages of several toxicants of biotic or abiotic origin for an entire lifetime. Adverse genetic effects of some substances may become evident only after organismal exposure for more than one generation. The toxicants may interact physiologically and evidence a pronounced reduction in toxicity or may interact synergistically and evidence an increased toxicity.

b. The experimental routes of exposure have often been different from those encountered in "nature."

c. Few dietary studies have been done, especially with Cd and Pb, in which comparisons have been made between the effects of the pure substance mechanically mixed in the diet just prior to consumption and the effects of the same concentrations of the substance after it has been biologically incorporated into the plant or animal. We do not know if the biotransformations associated with incorporation and accumulation in an organisms' tissues alter the spectrum of effects. What factors other than concentration affect the amount absorbed by an organism?

d. Almost exclusive reliance has been placed upon determinations of the concentrations of single toxicants with little or no reference to the concentrations of toxicity modifying substances.

The administrator responsible for enforcement of the provisions of the "Toxic Substances Control Act" will find that the following problems will be important factors to be considered:

- We must learn more about the movement, transformation, and accumulation patterns of the toxicants as they move through the environment.

- We must learn more about target species, age, sex, and organ of the toxicant.

- We must develop sensitive, continuous monitoring systems of representative natural ecosystems to provide us an "early warning" of unanticipated effects.

- We must make a distinction between a toxicant's toxicity and the hazard associated with it. A toxicant may have certain inherent toxic properties, but depending upon how it contacts the environment, the hazards or actual danger may be quite different.

- We must determine the effects of chronic low level dosages of single and combinations of toxicants over long periods of time.

- We must learn more about interactions of various toxicants and essential components of plant and animal metabolism. What levels of toxicants can be tolerated? What factors can be modified to reduce the hazard associated with a particular toxicant?

- We must do a realistic evaluation of the economic and environmental costs and the benefits of the use of a particular substance and the availability of safer alternative substances.

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PAPER NO. 5

INTERRELATIONSHIPS OF FEDERAL AGENCIES WITH RESPECT TO THE TOXIC SUBSTANCES ACT

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Ladies and gentlemen, let me first disclaim any expertise or insight as a political scientist or government bureau official. My remarks will be those of a chemist and toxicologist who, by the good wheel of fortune, is in a position to join each of you in service to our government and thus to the people of our country. I would like to acknowledge the financial support of several agencies acting through the Office of Naval Research Contract Number N00014-67-A-0244-0015.

With your indulgence, and apologies to John Donne, I would like to paraphrase a familiar quotation which he wrote 350 years ago.

"No Agency is an island, entire in itself, every Agency is a piece of the government, a part of the establishment; if an office be washed away by the sea of politics, the country is the less, as well as if a department were, as well as if a laboratory of thy friends or of thine own were; any man's RIF diminishes me, because I am involved in mankind; and therefore never ask to know for whom the phone rings; it rings for thee."

The converse is equally true, no new agency is independent; it is a piece of the government, and as it grows so do we all. When the phone rings in any office of the establishment it echoes throughout the country even into the ears of each of us. Each agency's business is every other agency's business and ours too.

Equilibrium Concepts:

Our socio-politico-economic system of government resembles, in many ways, a child's balloon. It contains a certain amount of energy which can be converted into mass or into dollars. It is highly flexible and has a certain amount of elasticity but its elastic limit can be exceeded if overstressed. Stresses applied in one position are relieved by alterations in

other areas of the system. It is a dynamic system but at any moment in time it can be considered as being in equilibrium. It can, therefore, be analyzed by the theorems of Le Chatelier.

His theorems can be simplified to two statements: (1) The forces, factors, and influences acting within a system will, in time, reach a state of balance or equilibrium, that is equal freedom or equal constraints on each of those factors. (2) When equilibrium has been reached, a change in any of those factors affecting the equilibrium will make changes in the other factors so as to return the system, in time, to equilibrium, which may be at some different point of balance. The rate of attainment of equilibrium can be increased by catalysts.

Let us consider some examples of equilibrium systems.

A. The reaction between carbonate and calcium ions to form calcium carbonate

$$CO_3^{=} + Ca^{++} \longleftrightarrow CaCO_3$$

$$\frac{(CO_3^{=})\ (Ca^{++})}{(CaCO_3)} = K$$

This is the process which has resulted in removing innumerable billions of tons of CO_2 from our atmosphere to form the white cliffs of Dover and the massive limestone beds underlying much of the earth's surface. The same reaction takes place in our own bodies. For example, Kenneth Schaefer has shown that submarine personnel exposed to a higher than normal partial pressure of CO_2 underwent a compensatory acidosis resulting in the deposition of excess $CaCO_3$ in their bones. This might be a way to overcome the calcium depletion observed in astronauts as a result of weightlessness.

B. (1) $TM \longleftrightarrow DF \qquad \frac{(TM)}{(DF)} = K_1$

(2) $TM \longleftrightarrow DDT \qquad \frac{(TM)}{(DDT)} = K_2$

In this pair of reactions TM, the tussock moth, is in equilibrium with the Douglas Firs of Oregon, and also in equilibrium with DDT. As TM approaches infinity DF approaches zero. As DDT approaches zero TM approaches infinity. The overall reaction is

(3) $DF \longleftrightarrow DDT \qquad \frac{(DF)}{(DDT)} = K_3$ as both approach zero.

Obviously, there are many other factors such as fish and eagles and EPA and USDA and the Sierra Club that influence this equilibrium. A few years ago this ecosystem was in near equilibrium. There were forests and streams for the birds and the fish while the tussock moth was counterbalanced by USDA's Forestry Service spraying DDT. However, the DDT and similar materials got into the fish and then into the eagles and made their eggshells so thin they broke in the nest and then no more eagles. EPA banned DDT and today there are more tussock moths than the eagles and other birds can eat. So far, 30 million dollars worth of Douglas fir have been killed in one forest of Oregon totaling 450,000 acres. Equilibrium has not yet been established and may not be until the forest lands are denuded and the streams fouled by erosion. It looks as though we desperately need a substitute for DDT so we can use another factor to shift the equilibrium. Obviously, there are many, many other factors in such a complex system and my oversimplification does not deal with all the causes and effects, but does serve to illustrate the interrelationship between the U. S. Forest Service and the EPA. The associated effects, such as the price of home construction lumber, demonstrate that these agency actions can touch each one of us. That phone rings for you and me.

Let me give you one more brief illustration of equilibrium.

$$B + P \rightleftharpoons \begin{matrix} U \\ C \end{matrix}$$

$$B = xP \qquad C = y\,U$$

In this system we have a fixed ratio of reactants and a fixed ratio of products including an undesirable byproduct formed by an irreversible side reaction. P and U are Product and Users. B and C are Beta naphthylamine and Cancer. Clearly, we can minimize the concentration of C by decreasing any of the others, number of Users, amount of Product, fraction y of exposed and susceptible Users, or fraction x of Beta naphthylamine in Products. In the past 20 years or so the major chemical companies have developed techniques to reduce the extent of exposure and the amount of Beta in the products. Recently, similar techniques have been adopted by the Occupational Safety and Health Administration in order to protect all workers. The EPA Office of Toxic Substances and the Consumer Products Safety Commission have a difficult task ahead in whether or how to regulate the theoretical, but undetectable, presence of a carcinogen in a product. If the FDA approach using the Mantell-Brian concepts is adopted the task will be easier. It should be remembered that their concepts are based on the extrapolation of measured data down to several orders lower of exposure levels. This may not be valid since different mechanisms may prevail.

Equilibrium among Agencies:

"No Agency is an island, entire in itself." Even the independent agencies such as the Consumer Products Safety Commission are far from being truly independent. They do not operate in either a political, economic, or scientific vacuum. It is true that no federal regulatory agency has any jurisdiction over another federal agency. This derives from the constitutional separation of the Legislative and Executive Branches of the federal government.

It is equally true that the President issues Executive Orders to all components of the Executive branch of the government directing them to voluntarily conform to such legislation as the Federal Insecticide, Fungicide and Rodenticide Act and the Environment Quality Act. The Executive Order relating to the Clean Air Act carries the phrase "shall be exemplary." Thus, in practice if not in legal theory, the mission oriented agencies such as the Department of Defense and USDA are subject to the regulations issued by EPA, FDA, and other regulatory bodies. The Le Chatelier equilibrium equation for all this is entirely too complex to be useful for our discussion today. However, Mr. Pope Lawrence is here today and he is responsible for encouraging federal agency compliance with EPA regulations.

Let us look first at the regulators then at the regulatees, and after that we'll look at some of the ways these interact.

Figure 1 shows those regulatory agencies requiring toxicology data.

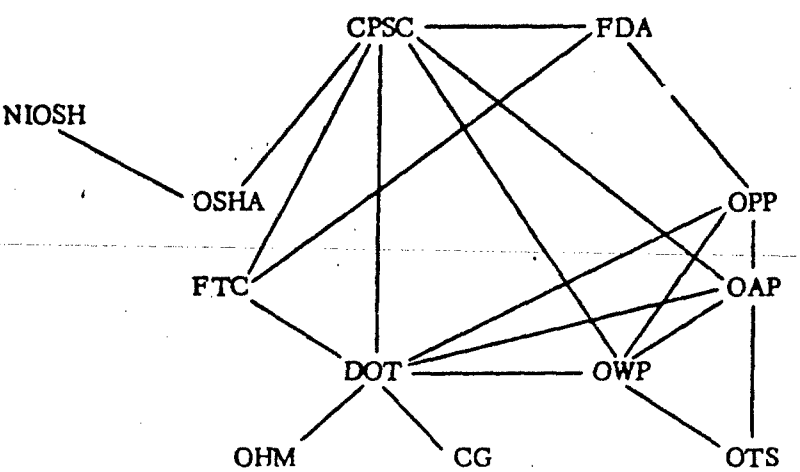

Figure 1. Federal Regulatory Agencies Requiring Toxicology Data.

There is also an extensive family of federal research agencies such as NCI, NIEHS, NCTR who generate basic toxicity information. Each of the regulatory and research agencies has a scientific staff and laboratory facilities but there is considerable difference among them in the size and kinds of staff and facilities. They all have scientific advisory boards and committees. In general the staff and advisory groups assist in the collection and evaluation of the required toxicity data. It is through these channels that the professional scientific community has a strong input to the regulatory process. In addition to such services by individuals there are many professional organizations assisting the regulatory agencies. Notable among these are ACGIH, ASTM, ANSI, AIHA, SOT. The National Academy of Sciences occupies a unique position which will be discussed later.

The interaction lines shown in this figure are only those that appear to be major. These arise from over-lapping or closely related responsibilities as assigned by various acts of Congress. Within the EPA side of the diagram the office of Toxic Substances is shown even though Congress has not yet passed its enabling legislation. For that reason its major interaction lines are presently within EPA. It seems certain to have an extensive interaction with other agencies, especially CPSC. These interactions are not always as clear-cut and straightforward as these lines indicate. Sometimes the individual with specific duties at the working level in these agencies is not fully informed of similar duties being carried out in other agencies or even within the same agency. These interactions are inevitably complicated by those human factors of ambition, selfprotection, jealousy that can occasionally become significant. For the most part there is a sincere effort at all responsible levels to cooperate as fully as possible for the sake of efficiency, cost reduction, and for a real concern for those being regulated. A specific case in point are the regulations pertaining to labelling and packaging of chemicals. These are based primarily on acute toxicity since they are intended to warn the user and shipper of hazards associated with spills. Over the last several years there has been a continuing series of informal working conferences between OHM, CG, OPP, CPSC, industry and independent scientists including staff from the Advisory Center on Toxicology. These have been aimed at standardizing the detailed methodology for such toxicity data as LD_{50}, LC_{50}, irritation, and corrosivity. Through the Coast Guard the results of these discussions have extended into the international arena. EPA is also involved in an international effort to develop uniform pollution standards on a global basis.

The next figure (Figure 2) shows some of the mission - oriented agencies and the regulations to which they are subject along with the kinds of data required. It is immediately clear that these are some of the same burdens placed upon the industrial community. It is also clear that many of the same kinds of data are required in each of these regulatory areas. The FDA regulations do not affect these agencies very much except the recent concern in USDA for naturally occurring toxicants in new varieties of food crops.

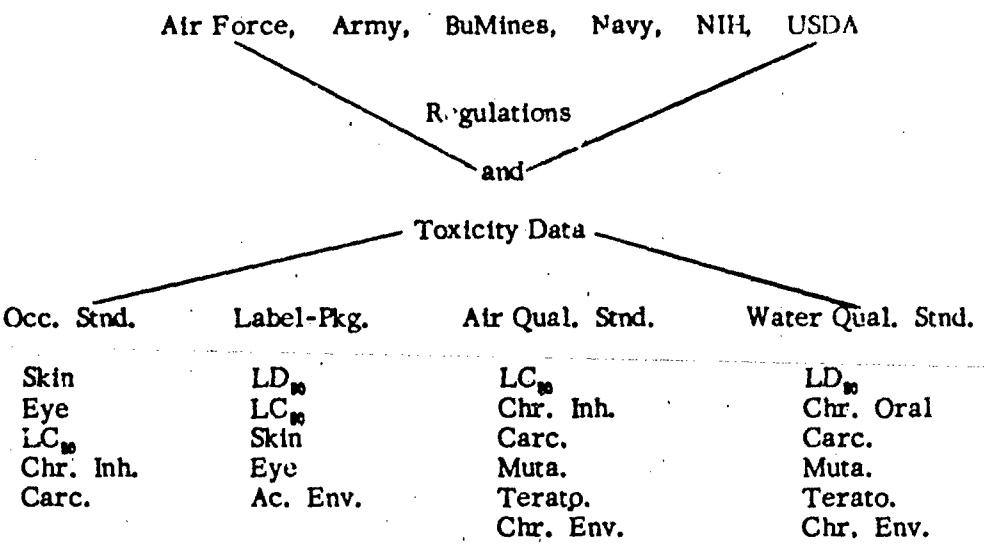

Figure 2. Regulations Applying to Mission-Oriented Agencies Along with Data Required to Set Standards.

Nevertheless, the FDA data requirements are similar to those for Water Quality Standards.

There are two important conclusions to be reached from this tabulation: I. The various regulations are using the same kinds of data and therefore it would be desirable that any given set of data could be used for meeting any of the regulatory requirements. II. The mission - oriented agencies use many of the same chemicals and therefore it would be desirable that they use the same set of data.

Agency Cooperation and Coordination:

This brings us to the two words which most clearly express the purpose of this discussion.

COOPERATION COORDINATION

In order to achieve the common goal of protecting the health and safety of the people of America, and to do so without impossible scientific and economic burdens, it behooves all Federal Agencies to cooperate to the fullest extent in coordinating their accumulation and evaluation of the necessary information. Repetition and duplication of research and testing must be avoided just as much as the acquisition of unnecessary data.

The first step is a willingness, or rather a desire, by all agencies, especially the regulatory ones, to submit to coordination. That's one definition of cooperation. The regulators must refine their data needs to minimize the amount of effort required and to maximize their commonality among themselves. The second step is the coordination of the data acquisition. For the regulators this may well involve a coordinated effort such as at NCTR to develop basic methodology.

The same burdens of cooperation and coordination fall upon the agencies being regulated. They must forgo some of their independence for the sake of efficiency and economy and join together in developing the data needed for meeting the regulations. There are some examples of this already such as the joint Army - Navy effort in assessing the environmental effects of munitions waste, and the joint Air Force - Navy effort on jet fuels. These two could benefit by incorporating private industry, producers, and consumers into the task. There was a joint study of air pollutants between EPA and two industry associations that yielded some good methodology and some very useful data. Unfortunately it seems that EPA may no longer be permitted to join in that kind of cooperative action.

Finally, let's look at some of the mechanisms of coordinating efforts. In the case of NCTR a scientific advisory group for HEW originated the idea and FDA began to implement it. At that time EPA was created and it became obvious to all that they needed the services of NCTR. An interagency memorandum of agreement was signed leading to the present arrangements. In addition to such agreements, agencies can make simple inter-departmental transfers of funds, enabling one agency to purchase services from another. The NASA related studies at AMRL are an example of this.

In addition to working directly with each other, agencies can go jointly or independently to a common private source. An example of a catalyst for these complex interactions is the Advisory Center on Toxicology of the National Academy of Sciences. In 1947 the Academy created a Committee on Toxicology. In the ensuing years several agencies began coming to the Committee for advice with increasing frequency. The correspondence and files got to be a little more than Harold Hodge, who was chairman at that time, could carry around in his hat. Shortly thereafter, in 1956 a memorandum of agreement was signed by the Air Force, Army, Atomic Energy Commission, and Navy, providing financial support for the Advisory Center on Toxicology at the Academy. All present sponsoring agencies are a party to that original agreement and they transfer funds to the Office of Naval Research which in turn contracts with the Academy.

Acting under the charter of that agreement, the Committee and the Center serve as a focal point for the cooperation and coordination of the toxicology interests among the sponsors and with non-sponsoring regulatory agencies. The success of this role of cooperation and coordination catalyst today speaks well for the essential goodwill of the agencies involved in achieving the present healthy state of toxicology and points towards a way of improving the present shortcomings. It serves as an enticing example to those agencies with similar toxicologic concerns to join in this cooperative venture. On behalf of the Academy's Committee and Center, as well as on behalf of the present sponsors a cordial invitation is extended to be coordinated. The phone is ringing. Is anybody listening? Our services are available to any federal facility and we constitute a common, non-combatant, meeting ground for scientific debate and recommendations.

OPEN FORUM

MAJOR MC CONNELL (Aerospace Medical Research Laboratory): I have a question of both Mr. Wands and Dr. Keplinger. Dr. Keplinger made the point that in determining whether a material has chronic toxicity or not, a rather broad outline should be used in setting up the scientific protocol to establish it. Mr. Wands showed us some very nice graphs of how different agencies interact with each other. When you get interaction of different agencies, doesn't this require that your methodology be spelled out exactly so that one agency can use the criteria of a toxic substance of another agency? We all differ in this. Won't you have to have specific guidelines as to what a specific substance must do to be considered a chronic toxicant?

DR. KEPLINGER (Industrial Bio-Test Laboratories, Inc.): Perhaps I was overemphasizing the specific protocol aspect. Yes, I agree, you are going to have to have cooperation, as Mr. Wands said, between the agencies to spell out some rather specific guidelines in many cases. What I was trying to emphasize is that requiring a very, very detailed protocol, where you put down the exact biochemical parameters and all of these details are spelled out in a regulation of some kind, ties the toxicologist's hands and doesn't allow him to make an evaluation. Perhaps I overemphasized leaving these requirements rather loose. I meant that to me there's a big difference between a guideline and a very specific protocol.

MR. WANDS (National Academy of Sciences): I'd like to add a comment to that. A standardized test method is necessary from the standpoint of legal enforcement. We find this particularly true in such things as labelling and packaging requirements. Again, most of these are based upon acute toxicity data where it is much easier to identify the LD_{50} as determined by a very minute cookbook type of procedure. I do think we have to forego, and are probably willing to forego, the freedom of scientific innovation for the sake of determining a simple straightforward LD_{50} or TLM_{96} in a fish or a minnow or whatever. At the same time, however, the question was focusing on chronic toxicology and here I think the only thing which can be said is that we need to investigate the toxic effects of long term, continuous exposures of a chemical substance to a variety of organisms and this means measuring such things as the obvious parameters - longevity, specific pathology, tumors, weight gain. Maybe such detailed tests as enzymatic assays on mitochondrial preparations are going to be the clue as to whether or not this material does indeed present an intolerable, unreasonable insult to the public health. We have to allow some freedom of selection of specific tests because it is not possible to predict in advance the kinds of data that are actually going to be meaningful for the ultimate decision as to whether or not to require some restriction on the introduction of a new

material for commercial use. This then places a very significant burden upon the regulatory agency to have within its own organization the expertise to evaluate all kinds of data and to give some guidance to the people collecting the data. Ultimately, however, the evaluation and decision to regulate or not, and if so, how much, rests with Mr. Schweitzer's organization. The administrator is the one who's going to have to make these decisions. He will make them not only on the basis of the scientific data which he will have evaluated by his staff but also the economic, political, and social impact such regulations will have.

DR. SHAFFER (American Cyanamid Company): I have a question for Mr. Schweitzer. Have you given thought to your possible responsibility under the National Environmental Policy Act? Will you be required to file Environmental Impact Statements on such measures, for example, as you may take in restricting use and distribution of chemicals?

MR. SCHWEITZER (Environmental Protection Agency): That question really transcends the issue of the Toxic Substances Control Act because there was language inserted in the EPA Appropriations Act last month calling upon the EPA to begin filing NEPA statements. In the past, EPA has contended, and this has been supported, that its regulatory actions were not the type of actions which would appropriately be handled by NEPA statements. However, the Congress disagreed with that interpretation and there now is a legislative requirement for EPA to begin filing NEPA statements. The situations in which the EPA will comply are currently a subject of debate within the agency. Regardless of what the Toxic Substances Act says, I'm sure that that will be subsumed in the broader agency policy on NEPA. But it's clear that very soon EPA is going to start issuing NEPA statements on regulatory actions. I can't be any more specific than that because the agency hasn't really made up its mind just how the act is going to be interpreted. There is, as you know, a provision in the one version of the law which calls upon EPA to file what they call Economic Impact Statements and we're not certain what that means and we are not certain how that would differ from the economic section of the Environmental Impact Statement but we are conscious of that requirement also.

DR. NETTESHEIM (Oak Ridge National Laboratory): I'll have a chance at a later session to talk about some of the particular aspects of chronic toxicology, namely carcinogenesis, but I'd like to bring up a topic which has been popping up today and I feel that the topic just keeps coming up rather than being dealt with. This is the common problem that we are all very familiar with except we keep avoiding it, and it is how to extrapolate laboratory experiments to the level where the data can be used for legislative and regulatory purposes. My question is, is there any really large scale effort going on to solve this problem? By large scale, I don't mean with a lot of dollars involved because I don't think that's going to do the trick. I

think it needs a lot of brains, that's what it's going to take to try to deal with this formidable problem. The major thing is not is the substance toxic or is it not toxic, or carcinogenic or not carcinogenic. That's relatively easy to find out. What is really the difficult issue is how toxic is it for man and how much risk is involved. I am very well aware, for example, about the efforts being started at Pine Bluff but from what I've seen, this is not going to do the job and is not going to give us the answers. I find that it is absolutely necessary that some of the researchers in chronic toxicology get together and start a long-term discussion, so that maybe ten years from now we can make a little bit more sense out of this formidable problem than we can presently. I wonder whether there are any plans along these lines. Anyone who wants to respond, please?

DR. HEHIR (Bureau of Biomedical Science): As I understand the burden of the question is the extrapolation from animal experiments to effect on man. Is that the burden of the question? I think the problem is that when you have a specific substance of concern, it's very difficult to get epidemiological data, it's easier to get animal data because you can subject animals to tests where it's not yet practical to subject humans to any risk. The approach we are trying to take is basically a targets of opportunity approach, such as the Iraqi mercury episode or the HCB contamination of people down in Louisiana or whatever. If you have an epidemiological study opportunity, and they are few and far between, try to parallel that with a toxicological experiment which you can do presumably without too much delay. Try to set both studies up so that you have comparable kinds of extrapolation opportunities. Being a newcomer to the field, the lack of this kind of research struck me almost immediately. I don't know how you do it except taking targets of opportunity in the epidemiological sense and then trying to have parallel toxicological studies so you can have measurable instead of theoretical extrapolations. That's one approach.

DR. NETTESHEIM: That sounds nice and I'm sorry to be harsh about it, but I want to start a little controversy anyway. I think that approach is completely useless because when we are dealing with organisms like mammals in our laboratory, we are dealing with a completely different set of circumstances no matter how hard we try to pattern our exposures to that experienced by man. It's just impossible to do chronic toxicology studies, and I'm in the business of carcinogenesis so I'm talking primarily about that field of toxicology, but I'm assuming there are similar problems in other fields of chronic toxicology. It's simply impossible when we are dealing with species that have a life span of two to three years to design an experiment whereby one can extrapolate from high dose level data down by a straight line to low dose level data. There is simply no way of extrapolating even if you quadruple or multiply by a factor of a thousand the efforts at Pine Bluff. You'll never find it out because it's impossible to design a laboratory experiment that simulates human exposure to respiratory tract carcinogens where you

are trying to imitate a situation where you would like to come up with a tumor cancer death rate of 40 in 100,000 per annum. It's impractical to do. I believe that we have to recognize the fact that for the next two generations of scientists, maybe longer, we will have to be satisfied in getting quantitative data in the laboratory that will be able to be used only in a qualitative fashion. I believe we cannot go far beyond saying a substance is toxic or carcinogenic at some dose level in the animal. I don't think that we can, in the foreseeable future, extrapolate from those levels used to produce acute responses in animals to those levels that may constitute a certain minimum risk in humans.

DR. SHAFFER: If Dr. Nettesheim wants some controversy, I'm willing to give it to him. I think you are asking the impossible. Probably a great many of you, and I'm sure you Dr. Nettesheim, will be familiar with the exchange of correspondence in Science that occurred last year. It was commenced by Dr. Weinberg in which he referred to the matter of extrapolation of animal results to human beings as being a trans-scientific judgment and which I, indeed, think it is. It's an act of faith, it's not a scientific process. Someone did write in later and criticize him and said that you could accumulate enough data in human beings to possibly establish some correlation. But basically, for those of us who work in toxicology and are charged with the responsibility of making judgments of safety, it is purely and simply an act of faith, and I think people ought to recognize it as such. Even before that comes the problem of deciding what is a safe dose for the experimental animal. We then get into the matter, after having detected some effective dosage of the compound in a particular experiment, of extrapolating a safe dose for that species. You can introduce a factor of safety which toxicologists have used for years and which has no real scientific basis or you can go through the motions of the parametric method and extrapolate out into the regions of extremely low responses. I think anybody familiar with the parametric method would recognize that it is not any more scientific than the arbitrary selection of a factor of safety; it just has the appearances of looking better. I really don't think that scientists are equipped or prepared to handle the projects which you place before us.

DR. CROCKER (University of California, Irvine): I do agree we'll have a chance to discuss this in greater detail at another time in the carcinogenesis session but two of our speakers referred to the question of a possible threshold limit for carcinogens. In view of the fact that a report of the working groups on criteria for toxicological testing, prepared at the San Antonio meeting, is about to be published, I'm wondering whether any rationale did emerge by which the kind of question that Dr. Nettesheim has asked can be answered. Will there be a suitable approach to the concept of a threshold limit for carcinogens?

DR. KEPLINGER: I know I used the term threshold limit value. What I was referring to was the industrial threshold limit value which we use in the industry as a guide for exposures. I wasn't talking about a threshold limit value of the public for a carcinogen. I would like to add to what Dr. Shaffer was saying. I agree that the matter of extrapolation is an insurmountable task. I don't know the answer, but once we determine the effect and apparent no adverse effect in the animal, then we as toxicologists and scientists have to make a judgment or decision. We can't just hide our heads in the sand. The threshold limit value that I was referring to is a value set for a chemical for occupational exposure and many of these have been set; we have used them and we have used different safety factors, but by and large, we have not injured people in industry by following those guides. The same can be said for the Food and Drug Administration's approach for setting safety factors in foods. By and large, it's a 100-fold factor and you can argue all day about whether or not this is the right factor. But I think our historical evidence indicates that FDA has a pretty good record in this area.

MR. SCHWEITZER: I hope that you haven't lost sight of Mr. Wands comments about the FDA publication this summer and their use of the Mantel-Bryant approach. I think we would particularly welcome alternative suggestions on how you go about these practical problems of determining the numerical limit that is to be used in specific situations. Maybe all of you aren't familiar with that FDA publication, but I think that Mr. Wands made a very significant statement when he said that if that survives the scrutiny of the scientific community in the next several months, it will be a significant landmark for toxicology. He may want to comment more on that, but as one who is involved in a very real way both in the kinds of research being done at NCTR and in the practical problems of establishing limits for carcinogens, I listened with considerable care to his comments.

MR. WANDS: I think I might respond to the question for Dr. Nettesheim in two ways. First of all, as Dr. Crocker pointed out, a large body of responsible scientists of all varieties of specialties did gather in San Antonio in February of this year. There were around 140 people altogether there working day and night, and I do mean day and night, for 7 days to come up with some test criteria. There are several items in the forthcoming report which bear upon the question of whether or not there is a threshold for any effect, whether there is a no adverse effect level, or whether there is a nondetectable adverse effect level. We get into very fine points of semantics here. Your point is quite well taken that our system is not perfect. It's going to be a long, long time before it is perfect, if ever, because the human body is such a terribly complex thing, such a highly adaptive and such a highly variable thing. There are large differences between you and me and everybody else here including sex differences. We may never be able to identify a precise level of a compound that will

truly be safe for everybody. I would remind you that one of the basic principles of toxicology is that no material is totally safe. Water is unsafe and people drown every year which was a favorite method of suicide in ancient China because it was so readily available and inexpensive. We can get too much oxygen, which has been well demonstrated here at Wright-Patterson in research experiments and has been duplicated in human experience. I think that the point that Mr. Schweitzer brings out that we have to conduct our laboratory animal work in such a way that it does parallel predicted human exposures as well as known human exposures where we have data, such as in the toxicological effects of hyperbaric oxygen, such as the known effects of beta naphthylamine in people and such as the recent studies on bis-chloromethylether. Here is a classic example of inhalation toxicity leading to carcinogenicity in both humans and in experimental animals, and it works very fast. This is going to be the route down which the profession of toxicology will proceed in the long range future and this is ultimately part of the program at the National Center for Toxicological Research. In the very early days of planning for that program, long before it came into existence, there was equal emphasis on the need not only for the "kilomouse" or "megamouse" or whatever other name you want to give their experimental approach there for studies using large numbers of animals but also on the need for a parallel set of epidemiological studies. In many cases, not necessarily all, the compounds that will be investigated at Pine Bluff are materials upon which we have a certain amount of epidemiolog' data available today. Dr. Cranmer has indicated that he recognizes the need for an expanded epidemiology effort to parallel his studies, but he just simply doesn't have sufficient funding. Epidemiologic studies cost a lot of money and take a lot of time. This is an area where we need the very best of our statisticians working hand in hand with toxicologists, epidemiologists and public health specialists to try to make the transfers and, hopefully, get enough points of transfer that will make this process of extrapolating animal data to human safety much more reliable than it is today. Everyone that's involved in this business of establishing a number for a limit that is "safe" for the public loses a great deal of sleep over it. No one feels completely comfortable with the limits because of the very shortcomings you pointed out, but it's the best we can do under the existing circumstances. I think concerning your comment that at least two more generations of scientists will be working in the dark and not having all the information they would like to have is true, but those scientists are going to have to continue to do the best they possibly can. In keeping with scientific methods, judgment is best achieved by a group of people rather than a single individual. This was the reason for my earlier comments here in the panel session when I said that Mr. Schweitzer and Dr. Hehir are going to have to have some real good in-house people to help them acquire and evaluate the experimental and epidemiologic data that are generated so that they can set reasonable limits.

DR. HODGE (University of California, San Francisco): Some of you may not have seen the proposal that Leo Friedman of the FDA made a year or more ago for a number which could be considered as acceptable for a known carcinogen. It's a very low number, in parts per trillion, but our analytical methods these days are so good that more and more substances or elements can be identified in parts per trillion in materials such as foods. This was the first instance that I know of anyone who actually was willing to stick his neck out and suggest an extremely low but real number that could serve as an acceptable intake for a carcinogen, or whatever compound you are worried about.

DR. SCHEEL (NIOSH): I should simply like to ask that the regulatory people who are involved at the Washington level recognize that the pattern of social response in the industry of this country has changed and the manufacturer is now willing to supply both in-house research data and human exposure data of industrial processes of manufacture so that we are not working in the dark anymore.

DR. FAIRCHILD (Environmental Protection Agency): My concern is that some of these biologic considerations on whether or not toxic substances are going to be permitted to be released in the environment at some controlled limit are going to be counterbalanced by economic considerations and the economic advantage of letting these things be used. I was wondering if you people would address yourselves then to the ultimate question of where one is going to strike the balance. It's a very complicated question but it seems to me that industry is deeply concerned with that question. It also seems to me, with respect to cigarette smoking, that a lot of people are very willing to tolerate this kind of toxicant because of the benefits that the individual believes he derives from it. Where are we going to strike the balance with these substances in the broad sense in the benefit-cost ratio?

MR. WANDS: I'll try to answer that question, Dr. Fairchild. You were at the San Antonio conference. You know that that subject was discussed by the group as I mentioned earlier. I think that what we're seeing here is a situation in which the public really is calling the tune to which we all must dance. And the public is a very fickle thing in many ways. It does tolerate the slaughter on our highways, it does tolerate the self-inflicted toxic effects of cigarettes and many other noxious materials, and these things get all wrapped up into emotional responses, every bit as emotional as whether one should use dogs or cats in research or not. And this is the reason why it is essential that our governmental agency administrative people must take into account what we call the social-political aspects of the problem as well as the scientific and economic aspects. The scientist, by and large, can give them some fairly firm numbers and information and so can the economist, but the administrators are the people who must be responsive to public pressures through our elected representatives

and thus whatever it is that the public will tolerate gets defined in some vague way in their mind, and they know where to strike this balance. There's no precise way of doing it. You can sit down and write equations. Some have been published in Science and there will be some equations in the Academy's publication of the proceedings of the San Antonio meeting. But it's art, it's witchcraft, or it's politics at its very best. Not in the sense that we've seen here lately, but in the real sense of statesmanship where this question gets answered, and it's a different answer for eve material and the answer will change from time to time.

DR. DOST (Oregon State University): I don't recall how long it took after it was first discovered that infectious disease could be transmitted from person to person until a communicable disease center started gathering epidemiologic information. But I think it probably is about time, it probably was time 20 years ago, that noninfectious disease was registered on a mandatory basis across the country. I think it would have been much easier to make some of the decisions about the pesticides that we've been talking about in the last few years. And it certainly would be much easier to make some of the decisions that are going to come up. Does any of the panel have any idea whether anyone plans to go to the burden and expense of establishing this kind of registry so that we'd have the real epidemiologic tools necessary to equate human disease with patterns of use of chemicals?

MR. WANDS: I think, Dr. Dost, you recognize just exactly how incomplete and useless the coroner's certificate of death is in terms of anything that would be meaningful in an epidemiologic sense.

DR. DOST: To be sure, I do recognize this, but it demands an entirely different system of reporting. In fact, it includes reporting circumstances all through the whole health history of citizens.

MR. WANDS: I want to add one thing here, Dr. Dost. There are some societies in the world who do have these kinds of data. I'm thinking particularly of the Japanese. They have very good data on morbidity and mortality from cradle to grave of individuals in their society. It's far from being a tightly controlled socialistic state there, but they have good records going back for many generations. That information was particularly useful to the Atomic Bomb Casualty Commission in their follow-up studies on what happens to succeeding generations when they were able to go back into previous generations and determine what the natural "background" incidence of cataracts, leukemia, and some of these other things had been in that population.

DR. DOST: I think this is of particular value. In fact, the easiest observations, of course, are in the area of carcinogenesis and in the area of teratogenesis.

DR. HENDERSON (Olin Chemical Corporation): I would remind you that the Communicable Disease Center has changed its name to the Center of Disease Control and it does acquire epidemiological data and not only on infectious diseases. Secondly, the Occupational Safety and Health Act requires reporting of occupational exposure effects.

DR. WILSON (Environmental Protection Agency): We toxicologists have made tremendous progress in developing methods for observing intrinsic toxicity of materials to biological functions. As Mr. Wands has stated here today, the extrapolation of these observations for purposes of safe handling and for setting tolerance limits should be done by a group of professionals. It is this point which I wish to address. There is an opportunity for us now to institute consensus approved values for these purposes. Unfortunately, the responsibility of setting tolerances by default and necessity has been assigned to the FDA and EPA. Certainly these agencies do not represent a consensus of our society. Consensus finding organizations, such as the American Standard Testing and Materials, may offer a model for such an organization. At this point, I wish to also address the question of extrapolating our observations on intrinsic toxicity to that of the use situation and to an evaluation of the hazards of use and human safety which will remain with us and problem us indefinitely. This question repeats itself in our endeavors to function as toxicologists in our society. Such repetition points to the constant need for unique observations in the area of associating intrinsic toxicology to the hazards of use. In the regulation of pesticides by EPA, there is an experimental permit system to allow for such observation prior to extensive use. EPA placed a particular value on wildlife in the environment of use as an indicator and as an opportunity to make these unique observations. The challenge for us is to learn new methods for making these unique observations.

SESSION II

TOXICOLOGY OF HALOGENATED SOLVENTS, AEROSOL PROPELLANTS, AND FIRE EXTINGUISHANTS

Chairman

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PAPER NO. 6

THE EFFECT OF 90-MINUTE EXPOSURES TO BROMOTRIFLUOROMETHANE ON MYOCARDIAL METABOLISM IN THE DOG*

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We have been engaged in the pharmacologic evaluation of low molecular weight fluoroalkanes for several years. These compounds are of interest primarily as fire extinguishing agents, but others are also of value as solvents, degreasers, refrigerants, and aerosol propellants.

The compound of primary interest is bromotrifluoromethane ($CBrF_3$), or Halon 1301. This agent has been proposed and tested for use in total flooding fire control systems on board aircraft as well as an ingredient in a new first aid fire extinguisher. Through the years the actions of this compound on the body during exposure by inhalation have been documented and a systematic elucidation of the mechanisms of action has been attempted. We provided evidence that among these actions, exposure to 1301 is accompanied by a decrease in myocardial contractility (Van Stee et al., in press). This report covers the results of some of the studies undertaken in an effort to examine the mechanisms that underlie this phenomenon.

We considered the inotropic effect from 2 different points of view, first in terms of the contractile machinery itself and second in terms of the energy supply to the process. Since the availability of energy to the contractile process is a function of the availability of metabolic substrates to the myocardium, we measured the availability of 4 different substrates and reported them in terms of myocardial uptake.

* This work was supported, in part, by the National Institute of Occupational Safety and Health, Cincinnati, Ohio.

METHODS

Twelve dogs were anesthetized with a combination of fentanyl-droperidol and α-chloralose in polyethylene glycol (Table 1). Eight of these were exposed to mixtures of $CBrF_3$ in O_2 and four were exposed to equivalent mixtures in which N_2 was substituted for $CBrF_3$. The ranges cited in Table 1 were computed from the means ± one standard deviation. The values cited were derived from 25 dogs used in similar studies (including the 12 used in this study) whose data are stored in our normal dog control data files. The dogs averaged 25 kg and the mass of the perfused left ventricular myocardium averaged about 50 grams.

TABLE 1. ANIMALS USED IN STUDIES OF THE EFFECTS OF FLUOROALKANES OF MYOCARDIAL METABOLISM

SUBJECTS:	Mongrel dogs, male or female
ANESTHESIA:	Innovar-Vet + α-chloralose
BODY WEIGHT (kg):	21.9 - 29.9
BODY TEMPERATURE (°C):	37.1 - 38.6
HEMOGLOBIN CONC (g/100 ml):	11.9 - 16.0
WEIGHT OF LEFT VENTRICLE (g):	43.9 - 58.3

Figure 1 is a schematic representation of the basic cardiovascular preparation. A pressure transducer was connected to a brachial artery for the determination of arterial blood pressure. A 13 gage stainless steel cannula was placed in the left ventricle through the left ventricular free wall. The cannula was connected to a pressure transducer to monitor left ventricular pressure (LVP) and left ventricular and diastolic pressure (LVEDP); dP/dt was derived electronically. Peak dP/dt + developed pressure (P) was determined on a vector oscilloscope from dP/dt and LVP according to a method described elsewhere (Van Stee et al., in press).

An electromagnetic flow probe was placed around the left coronary circumflex artery and a catheter placed in the great coronary vein just proximal to the coronary sinus. The mass of the ventricular myocardium perfused by the left coronary circumflex artery was determined at the conclusion of each experiment after injecting the artery with India ink at the site of placement of the flow probe and trimming away the unstained tissue.

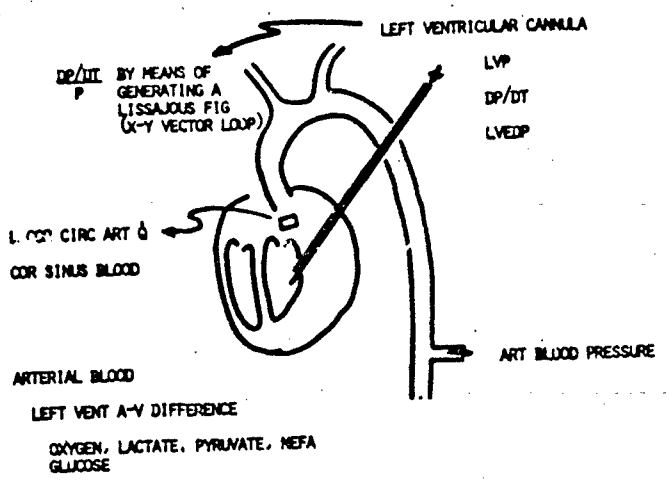

Figure 1. Schematic representation of basic cardiovascular preparation.

The extraction of oxygen, nonesterified fatty acids, glucose, lactate, and pyruvate were determined on the basis of the A-V concentration difference times the coronary flow rate divided by the mass of the perfused myocardium. Extractions were reported in terms of moles or ml per min per gram of tissue.

The concentration of $CBrF_3$ in oxygen to which the animals were exposed was determined by gas chromatography. Oxygen concentration was determined polarographically. Nitrogen (N_2) was determined by subtraction.

Blood pH, PO_2, and PCO_2 were determined by means of specific electrodes. Blood lactate and pyruvate levels were determined by a lactic dehydrogenase method (Marbach and Weil, 1967). Blood levels of nonesterified fatty acids (NEFA) were determined by a modified Dole technique (Tietz, 1970) and glucose levels by a hexokinase method (Slein, 1963).

Each time a sample of blood was withdrawn from the dog it was replaced by an equal volume of autologous, citrated whole blood obtained 2 weeks prior to the experiment.

Stewart-Hamilton dye dilution curves (Dow, 1955) were determined. Indocyanine green was injected into the right atrium while blood was withdrawn from the aortic arch through a cuvette densitometer at a rate of 24 ml/min by means of an infusion/withdrawal pump. Mean transit time (MTT) was the interval required for one-half of the injected dye to travel from the point of injection to the point of arterial blood sampling. Central venous volume (CVV) was computed from cardiac output (CO) and MTT. Total peripheral resistance was computed from mean arterial blood pressure (AP), left ventricular end diastolic pressure (LVEDP) and CO. The index of stroke work (SW) reflected

pressure-volume work only. This is commonly accepted as representing about 90% of the total stroke work (Ruch and Patton, 1966). All analog signals were recorded on a physiological recorder. Table 2 is a list of the 39 determinations made in this study.

TABLE 2. DETERMINATIONS

1301 Concentration	Arterial Lactate/Pyruvate
Arterial PO_2	Cor Sinus Lactate/Pyruvate
Cor Sinus PO_2	Arterial NEFA
Arterial O_2 Content	Cor Sinus NEFA
Cor Sinus O_2 Content	Myocardial NEFA Extraction
Myocardial O_2 Extraction	Arterial Glucose
Arterial PCO_2	Cor Sinus Glucose
Cor Sinus PCO_2	Myocardial Glucose Extraction
Arterial Bicarbonate	L Cor Circumflex Art Blood Flow
Cor Sinus Bicarbonate	Mean Arterial Blood Pressure
Arterial pH	Cardiac Output
Cor Sinus pH	Mean Transit Time
Arterial Lactate	Central Venous Vol (Pulm Blood Vol)
Cor Sinus Lactate	Total Peripheral Resistance
Myocardial Lactate Extraction	Heart Rate
Arterial Pyruvate	Stroke Volume
Cor Sinus Pyruvate	Stroke Work
Myocardial Pyruvate Extraction	Peak dP/dt
Arterial Base Excess	$\frac{\text{Peak } dP/dt}{P}$
Cor Sinus Base Excess	

The information in Table 3 illustrates the $CBrF_3$ (8 dogs) and control N_2 (4 dogs) exposure sequence and the first and second levels of data analysis. The animals were ventilated with 100% O_2 during the surgical preparation. Arterial PO_2, PCO_2, and pH were adjusted to accepted limits (see Tables 4 and 5) by adjustment of ventilation rate and tidal volume. Preexposure determinations were made. Exposure to $CBrF_3$ was begun at a 27% concentration and increased without interruption at 30-minute intervals to 51% and 75%, respectively. Determinations were made at the end of each 30-minute exposure. Final determinations were made after 30 minutes exposure to 100% O_2. The two rows of numbers following each measurement represent the data from the $CBrF_3$ exposure (above) and the N_2 control exposure (below).

TABLE 3. THE FORM OF THE TABULAR DATA TO BE PRESENTED

MEASUREMENT	ZERO	LOW	MED	HIGH	ZERO	REFERENCE VALUES
Art PO_2 torr	262±149	139±80	98±29	71±23	313±146	80-101
	322±77	196±65	106±28	61±8	254±126	50-312 (n=73)
Each of 39 variables measured either directly or computed from measured variables.	The 1st "ZERO" refers to pre-exposure measurements and the 2nd refers to 30-min post-exposure measurements. Animals were exposed to increasing concentrations of $CBrF_3$ in increments of 25%. Exposure was continued for 30 min at each level. Values cited, mean ± std dev. n = 8. Student's t-test for paired observations on each set of pre- and post-exposure values. [d] = sig ($p < .05$). The second set of entries represents corresponding values derived from controls in which N_2 replaced $CBrF_3$. n = 4.					Reference values from published sources in 1st line. Reference values from our lab in 2nd line. Ranges cited computed from mean ± std dev.

TABLE 4. $CBrF_3$ (1301) METABOLIC STUDY 90-MIN EXPOSURES

MEASUREMENT	ZERO	LOW	MED	HIGH	ZERO	REFERENCE VALUES[d]
% $CBrF_3$	0	27±2	51±3	75±3	0	NA
Art PO_2 torr	262±149[a]	139±80	98±29	71±23	313±146	80-101[c]
	322±77[b]	196±65	106±28	61±8	254±126	50-312 (n=73)
CS PO_2 torr	30±9	30±9	31±9	26±5	33±11	19-39[c]
	28±8	26±8	25±8	29±9	37±12	18-37 (n=73)
Art O_2 Content, ml/l	257±56	216±33	200±52	183±32	286±57	180-233[c]
	274±43	231±13	195±8	162±24	245±46	183-316 (n=70)
CS O_2, Content, ml/l	98±44	93±40	97±42	82±34	107±54	32-141[c]
	78±36	63±32	60±32	56±45	72±56	44-123 (n=73)
E O_2, µl/min per g	116±48	96±42	78±28	71±37	124±45	60-145[c]
	178±51	146±39	122±42	123±52	131±39	79-196 (n=73)
Art PCO_2 torr	29±7	31±6	32±6	29±5	31±4	34-53[c]
	31±4	32±5	30±7	31±6	33±6	26-43 (n=73)

a. $CBrF_3$ exposed
b. N_2 controls
c. Whereat and Chan, 1972; Pickrell and Schluter, 1973; Feigl and D'Alecy, 1972
d. Mean ± standard deviation

TABLE 5. $CBrF_3$ (1301) METABOLIC STUDY 90-MIN EXPOSURES

MEASUREMENT	ZERO	LOW	MED	HIGH	ZERO	REFERENCE VALUES[e]
CS PCO_2 torr	40±7[a]	41±7	42±8	38±8	44±7	44-64[c]
	43±6[b]	43±7	41±6	37±12	46±4	38-57 (n=73)
Art HCO_3^- mEq/l	18±3	18±2	18±2	17±3	17±2	15-29[c]
	17±1	17±1	16±3	16±3	16±4	15-22 (n=73)
CS HCO_3^- mEq/l	22±3	22±3	21±3	20±4	21±3	17-32[c]
	22±3	21±1	21±2	21±3	21±4	20-27 (n=72)
Art Base Excess mEq/l	-5.0±2.1[d]	-5.0±2.4	-6.3±2.6	-6.6±4.1	-7.5±3.7[d]	-11.5-3.8[c]
	-6.7 ±.5	-7.2±1.4	-8.4±1.8	-7.9±3.4	-8.6±3.6	-9.0-(-)2.3 (n=73)
CS Base Excess mEq/l	-2.7±2.0	-2.8±2.5	-4.0±3.3	-4.6±4.8	-5.2±3.7	-10.6-4.6[c]
	-3.7±1.9	-5.5±1.0	-4.9±2.3	-5.0±2.8	-6.5±5.0	-6.3-.77 (n=73)
Art pH	7.41±.06[d]	7.39±.06	7.36±.08	7.38±.09	7.34±.08[d]	7.23-7.38[c]
	7.36±.04[d]	7.34±.06	7.34±05	7.34±.04	7.31±.02[d]	7.28-7.42 (n=73)
CS pH	7.36±.04[d]	7.35±.04	7.33±.07	7.34±.09	7.30±.07[d]	7.19-7.34[c]
	7.32±.02	7.29±.05	7.32±.04	7.32±.03	7.25±.07	7.25-7.38 (n=72)

a. $CBrF_3$ exposed
b. N_2 controls
c. Whereat and Chan, 1972; Pickrell and Schluter, 1973; Feigl and D'Alecy, 1972
d. Sig, $p<.05$, see Table 3
e. Mean ± standard deviation

METHOD OF DEFINING REFERENCE VALUES

Reference values in the tables, representing estimates of expected normal ranges for the determinations made in this study, were adjusted to the dimensions used in this report to facilitate direct comparisons. The independent references are given in the first and/or second lines, when available. They are followed in the next line by accumulated normal, control values obtained from all similar experiments conducted in our laboratory.

The reference values for arterial and coronary sinus blood [PO_2, O_2 content (C), pH, PCO_2, HCO_3^-, and base excess (BE)] and myocardial oxygen extraction (EO_2) were derived in the following manner: The normal range of the mean ± one standard deviation (68%R) for dog blood Hb concentration (12.7-15.7 g/100 ml) was quoted from Robinson and Ziegler (1968). The 68%R for the ventricular weights, body temperature and coronary flow rate were derived from all of our experiments using 25 kg open-chested mongrels anesthetized with α-chloralose-opiate combinations.

An electronic calculator program was written based on the method of Pickrell and Schluter (1973): Blood hemoglobin concentration, ventricular weight, body temperature, arterial and coronary sinus blood PO_2, PCO_2, and pH were entered as program input (Fiegl and D'Alecy, 1972; Whereat and Chan, 1972). The output consisted of arterial and coronary sinus blood PO_2 (corrected [v. i.]), O_2 content, pH (corrected), HCO_3^-, and base excess. The method of computation includes correction for the pK' of carbonic acid and the solubility constant of CO_2 brought about by the difference in both body temperature and pH between canine and human blood, an equation for determining percentage of hemoglobin saturation with oxygen derived from canine measurements, and an equation for the calculation of base excess from pH and carbon dioxide tension (PCO_2). Temperature correction factors for oxygen tension (PO_2), PCO_2, pH, pK', and solubility coefficients of CO_2 and O_2 and a correction for hemoglobin unsaturation for PCO_2 derived by the Astrup technique (Pickrell and Schluter, 1973) are provided. The 68%R low and high extreme reference values were processed according to this program in appropriate combinations to provide an output representing corresponding corrected ranges.

Arterial and coronary sinus blood O_2 content were computed from data from Whereat and Chan (1972). For purposes of the calculations the assumptions were made that the mean hemoglobin concentration was 15 g/100 ml of blood; each gram of hemoglobin bound 1.34 ml of O_2; and each ml of blood dissolved 0.0031 ml of O_2 (Davenport, 1958). Blood PO_2 and PCO_2 were corrected to 38.6 C by the method of Severinghaus (Dittmer, 1961).

Values for arterial and coronary sinus blood nonesterified fatty acid levels (NEFA) and myocardial NEFA extraction were computed from the data of Kjekshus and Mjos (1972). Values for glucose were calculated from Ralston Purina Company (1973). The left coronary circumflex arterial blood flow reference was quoted from Koyama and Nakagawa (1972) assuming left ventricular weights of 50 grams. Mean arterial blood pressure, cardiac output, stroke volume, and stroke work (P-V) were derived from the data of O'Rourke and Bishop (1971). Central venous volume was quoted from Maseri et al. (1972). Peak dP/dt ÷ P was quoted from Gil-Rodriquez et al. (1971). Values for peak dP/dt were quoted from Horowitz and Bishop (1972).

DATA ANALYSIS

Pre- and postexposure values were compared using Student's t-test for paired observations (Sokal and Rohlf, 1969). Experimental values were compared with the reference values and N_2 control values by inspection, and by Student's t-test for independent samples (Sokal and Rohlf, 1969). The $CBrF_3$ exposure data were finally evaluated by 2-variable polynomial regression analysis or multiple linear regression analysis (Draper and Smith, 1966).

RESULTS AND DISCUSSION

The mean concentrations of $CBrF_3$ to which the animals were exposed are given at the top of Table 4.

The control values for arterial PO_2 were high because of the high inspired O_2 levels. It should be noted that the blood O_2 content varied less than PO_2. For example, the change from the 1st to 2nd periods in arterial PO_2 from 262 to 139 represented a decrease of 46%, whereas the corresponding change in arterial O_2 content from 257 to 216 represented a decrease of only 16%. This is so because most of the O_2 carrying capacity of the blood resides in the hemoglobin, which remained nearly 100% saturated throughout most of the experiments. Even at the lowest mean PO_2 of 61 torr, arterial hemoglobin was over 84% saturated. Values for arterial O_2 content greater than 150 ml/liter are not considered serious hypoxemia (Koyama and Nakagawa, 1972). That myocardial hypoxia was not a factor in these studies was reflected in the values for the O_2 content of the coronary sinus blood. If one accepted the view of Davis and Carlson (1973) that venous PO_2 reflects tissue oxygen tension more accurately than arterial PO_2, inspection of the values for coronary sinus PO_2 suggested that no degree of myocardial hypoxia existed throughout the course of the experiment, based on the reference cited in this table. On the other hand, it may be noted that during the period of the exposure to 75% $CBrF_3$ or N_2, the arterial PO_2 was lowest and differed significantly at the 95% level from the previous mean. The physiological significance of this difference is, however, questionable at best, since there was no significant difference between the corresponding values for coronary sinus blood oxygen content.

To summarize the discussion of the adequacy of myocardial oxygenation during these experiments, we concluded that tissue hypoxia was not present during the experiments at any time, with the possible exception of the period of exposure to the highest concentration of $CBrF_3$ or N_2 during which time a marginal hypoxia could be argued.

The values for PCO_2, HCO_3^-, base excess (or deficit), and pH reflect the myocardial tissue acid-base balance (Tables 4 and 5). Arterial base excess and arterial and venous pH were the only values in these studies that did not return to preexposure levels by 30 minutes postexposure. The data indicated the development of a slight metabolic acidosis. This may have been caused by the use of stored, citrated whole blood in the experiments. Although the tendency was there, it did not apparently progress to a significant degree when the values were compared with the references.

Arterial and venous lactate levels were somewhat higher than some references but agreed with others (Table 6).

TABLE 6. $CBrF_3$ (1301) METABOLIC STUDY 90-MIN EXPOSURES

MEASUREMENT	ZERO	LOW	MED	HIGH	ZERO	REFERENCE VALUES[g]
Art Lactate mM/l	3.1±1.5[a]	2.6±1.2	2.8±1.0	3.9±1.9	3.4±1.3	2.1-3.9[c,a] .58-1.56[e]
	1.8±0.6[b]	1.8±0.8	2.2±1.4	3.0±2.2	3.2±2.3	1.3-3.7(n=73)
CS Lactate mM/l	2.0±1.5	1.9±1.0	2.0±0.8	3.2±1.9	2.4±1.2	.35-.80[e]
	1.1±0.3	1.2±0.4	1.5±1.0	2.1±1.6	2.6±2.7	0.6-2.6(n=73)
E Lactate nM/min/g	721±587	585±796	599±410	485±311	738±438	583-752[e,f]
	687±373	573±355	655±335	897±545	502±319	263-985(n=72)
Art Pyruvate µM/l	170±56	164±58	179±44	221±70	216±64	100-200[c,d] 53-183 [e]
	129±23	127±25	125±20	149±17	137±25	106-210(n=73)
CS Pyruvate µM/l	133±67	135±63	143±50	182±75	187±107	41-89[e]
	73±31	132±90	96±44	119±43	126±69	49-162(n=73)
E Pyruvate nM/min/g	50±36	39±29	38±20	43±32	42±27	20-93[e,f]
	54±34	48±40	47±34	43±33	56±30	26-82(n=64)

a. $CBrF_3$ exposed
b. N_2 controls
c. Mixed venous blood
d. Dittmer, 1961
e. Whereat and Chan, 1972
f. Koyama and Nakagawa, 1972
g. Mean ± standard deviation

Under extreme conditions of reduced tissue oxygenation, the myocardium may add lactate to the blood rather than extract it. Shea and coworkers (1962) demonstrated that a progressive decrease of arterial blood oxygen content resulted in net lactate production when myocardial oxygen extraction increased to 85% of the total arterial blood oxygen. In our experiments, sufficient oxygen was delivered to the myocardium so that the oxygen extraction ranged from 52 to 63%. The fact that the extreme state was not reached does not, in itself, exclude the possibility that aerobic metabolism was slowed and anaerobic metabolism accelerated. On balance, however, we believe that the evidence supports the supposition that adquate oxygen was available to the myocardium at all times throughout the course of these determinations.

Pyruvate values were normal throughout and bore a normal relationship to lactate as expressed in the lactate/pyruvate ratios (Table 7). L/P ratios were higher than those of Whereat and Chan (1972). There was a slight, but statistically insignificant increase in the L/P ratios from the MED to the HIGH exposure periods.

TABLE 7. $CBrF_3$ (1301) METABOLIC STUDY 90-MIN EXPOSURES

MEASUREMENT	ZERO	LOW	MED	HIGH	ZERO	REFERENCE VALUES[g]
Art L/P	184.±6.8[a]	15.5±3.5	16.2±7.5	18.8±11.1	16.3±6.2	ca 20[c] 7.1-11.9[d]
	13.8±2.9[b]	14.1±4.1	17.1±7.4	20.1±13.5	21.6±15.7	10.0-22.2 (n = 72)
CS L/P	14.4±5.1	13.9±3.0	13.9±3.5	17.5±8.6	13.5±3.9	6.6-11.3[d]
	16.6±8.8	12.0±7.0	15.4±4.8	16.1±6.3	18.3±9.4	11.2-21.4 (n = 73)
Art NEFA μM/l	460±90	529±302	651±418	613±338	485±274	525-1025[e]
	404±178	400±69	500±192	508±163	496±119	25[illegible]-797 (n = 69)
CS NEFA μM/l	352±110	411±211	499±271	425±161	330±173	350-850[e]
	291±125	251±73	319±136	290±148	251±120	169-718 (n = 69)
E NEFA nM/min/g	78±54	82±113	115±122	161±168	135±97	54-174[e]
	103±61	131±35	163±65	120±55	199±91	179-734 (n = 65)
Art Glucose mM/l	6.8±1.9	6.8±1.2	7.1±1.0	7.8±1.6	8.1±1.5	1.4-6.9[f]
	5.9±0.9	5.9±0.4	5.9±0.7	6.0±1.1	6.4±1.5	5.6-8.4 (n = 55)

a. $CBrF_3$ exposed
b. N_2 controls
c. Öttmer, 1961
d. Whereat and Chan, 1972
e. Kjekshus and Mjos, 1972
f. Ralston Purina, 1973
g. Mean ± standard deviation

Arterial and coronary sinus blood NEFA levels rose modestly with increasing $CBrF_3$ concentration. Variations in myocardial NEFA extraction were irregular and bore no relationship to $CBrF_3$ concentration. Glucose values did not vary significantly. Variations in the cardiovascular dynamic data were unremarkable (Tables 8 and 9). Mean arterial blood pressure and the indices of the vigor of myocardial contraction decreased during exposure to $CBrF_3$ as expected.

TABLE 8. $CBrF_3$ (1301) METABOLIC STUDY 90-MIN EXPOSURES

MEASUREMENT	ZERO	LOW	MED	HIGH	ZERO	REFERENCE VALUES[f]
CS Glucose mM/l	7.1±4.0[a]	6.7±3.3	7.4±2.1	7.4±1.7	8.3±1.9	——
	5.4±0.6[b]	5.5±0.5	5.6±0.8	5.8±1.1	6.0±1.1	5.3-7.8 (n = 53)
E Glucose nM/min/g	310±240	519±275	331±244	339±188	312±103	——
	499±500	499±474	293±135	196±117	240±285	94-678 (n = 47)
Cor Art Q ml/min/g	.73±.22	.78±.25	.71±.18	.68±.20	.69±.18	0.60-1.01[c]
	.91±.20	.88±.23	.81±.29	.98±.22	.81±.27	0.56-1.14 (n = 71)
Mean Art BP torr	100±13	100±20	103±22	82±26	96±31	95-144[d]
	108±23	97±19	93±33	96±34	88±40	84-127 (n = 72)
Cardiac Output	38±15	39±15	42±17	38±23	41±24	36-59[d]
	41±22	41±20	39±21	37±18	31±17	21-57 (n = 26)
Mean Trans T sec	7±2	7±2	7±1	8±2	8±3	——
	7±1	7±1	7±1	7±2	9±2	6-10 (n = 26)
Cont Ven Vol ml	213±68	222±67	245±84	246±130	248±114	146-358[e]
	191±76	189±71	189±69	171±90	186±70	129-341 (n = 26)

a. $CBrF_3$ exposed
b. N_2 controls
c. Koyama and Nakagawa, 1972
d. O'Rourke and Bishop, 1971
e. Masori et al., 1972
f. Mean ± standard deviation

TABLE 9. $CBrF_3$ (1301) METABOLIC STUDY 90-MIN EXPOSURES

MEASUREMENT	ZERO	LOW	MED	HIGH	ZERO	REFERENCE VALUES[f]
Tot Perf R pr units	58±19[a]	57±26	54±23	51±28	54±23	——
	71±34[b]	64±28	61±15	64±13	67±13	39-81 (n = 26)
Heart Rate beats/min	179±25	177±25	175±32	175±53	171±39	——
	204±14	202±6	207±16	206±21	208±26	158-211 (n = 43)
Stroke Vol ml	11.7±5.9	12.1±6.5	13.6±7.9	13.7±11.1	14.4±11.3	17.6-27.8[c]
	9.0±5.1	9.0±5.0	8.6±5.4	8.2±4.9	6.9±4.1	3.9-19.2 (n = 26)
Stroke Work gm-m/g	.31±.15	.32±.17	.38±.24	.34±.34	.40±.38	.56-.72[c]
	.32±.23	.29±.19	.28±.22	.28±.22	.22±.19	.09-.59 (n = 26)
$\frac{dP/dt\ Peak}{P}$ sec^{-1}	31±5	32±6	31±7	28±6	33±11	21-53[d]
	30±2	31±2	30±2	31±3	31±6	26-53 (n = 73)
dP/dt Peak torr/sec	2222±305	2310±470	2269±486	1721±389	2254±434	2179-3071[e] 2494-3602[e]
	2650±387	2650±342	2550±733	2650±794	2375±814	1412-2563 (n = 53)

a. $CBrF_3$ exposed
b. N_2 controls
c. O'Rourke and Bishop, 1971
d. Gil-Rodrigues et al., 1971
e. Horowitz and Bishop, 1972
f. Mean ± standard deviation

The values for arterial blood glucose were consistently higher than the reference values cited which represented determinations made on unanesthetized animals not subjected to stresses greater than those accompanying phlebotomy. Heart rate was constantly high following the vagolytic doses of atropine administered. A reference value was not cited because of the special conditions of the experiment. The values for stroke volume and stroke work were lower than the reference values because of the high heart rates. The reference values (O'Rourke and Bishop, 1971) were determined on conscious dogs with heart rates of 90-124 beats/minute. Expressed in terms of minute work there was no significant difference between our mean control value (62.7 g-m/g of left ventricular myocardium) and that computed from O'Rourke and Bishop (68.0 g-m/g assuming that the mean left ventricular weight equalled 50 g). Likewise, there was no significant difference between our mean control value for cardiac output (39.0 ml/min/g left ventricular myocardium) and that computed from O'Rourke and Bishop (47.5 ml/min/g assuming that the mean left ventricular weight equalled 50 g). The lower mean CO in our experiments, while not statistically significant, was considered to be physiologically significant and the result of thoracotomy.

The direction of the changes of peak dP/dt + P and peak dP/dt corresponded to those documented earlier (Van Stee et al., in press), suggesting a negative inotropic effect of $CBrF_3$.

A correlation network (Figure 2) was constructed from product-moment and multiple correlation coefficients obtained from the regression analyses. Such a network provides a graphical representation of possible interactions among measured variables.

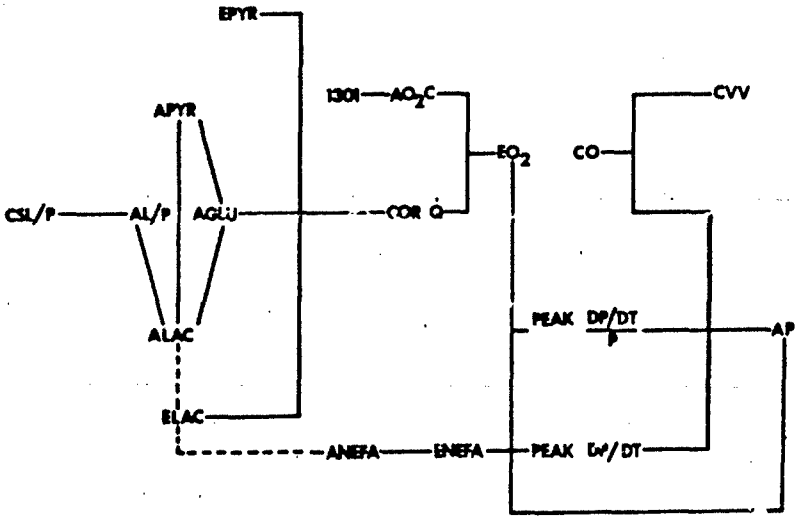

Figure 2. A correlation network was prepared that was based on multiple linear repression and polynomial regression analyses of some combinations of the 39 variables that were measured. The solid lines represent F-ratios and correlation coefficients significant at the 1% level and the broken lines represent significance at the 5% level.

A note of caution should be recognized when making inferences from correlation data. Correlations do not prove causal relationships. We note later in this discussion, however, that many of the correlations determined in this study represented causal relationships that have been established by others.

Two criteria were required for admission of data to the network: the F-ratio for regression had to be significant ($p<0.5$) and the Student's t for significance of the correlation had to exceed the critical value ($p<0.5$). Solid lines in Figure 2 represent correlations significant at the 5% level. All correlations represented in the network were linear with the exception of the relationship between EO_2 and peak dP/dt ÷ P which was parabolic.

All possible permutations of the variables were not tested for correlations. Certain combinations were selected based on previously established causal relationships and what might be expected intuitively to be significant combinations of variables. Furthermore, some correlations were rejected as insignificant because the linear model was inappropriate. We recognize that "conclusions obtained by minimizing variance error only and assuming the postulated model to be correct are likely to be wrong in many practical design situations" (Draper and Smith, 1966, p. 19).

A consideration of the position of the inspired $CBrF_3$ (1301) concentration in Figure 2 and the cardiovascular dynamic changes measured during $CBrF_3$ exposure (Tables 8 and 9) revealed an unanticipated finding. Although peak dP/dt + P, and peak dP/dt decreased with increasing $CBrF_3$ concentration, the changes were not statistically significant. This is in contrast to studies performed in this laboratory using beagles (Van Stee et al., 1974). There are at least two possible explanations. Beagles and large mongrels may differ in responsiveness to the cardiodynamic actions of $CBrF_3$. Another explanation may be referable to the anesthetics used. Alpha-chloralose with fentanyl-droperidol was used in this study whereas ethanol-morphine was used in the former study.

The attenuation of the hypotensive response that was also observed was attributed to the α-adrenergic blocking action of droperidol (Yelnosky et al., 1964) since the hypotensive response to $CBrF_3$ is a function of a decrease in vasoconstrictor tone (Van Stee and Back, 1973). However, the attenuation of the negative inotropic response measured by peak dP/dt + P is less easily explained. The total dose of droperidol (5.5 mg/kg) given over a period of 4 hours to the dogs in our study could have been enough to affect myocardial contractility (Yelnosky et al., 1964) and the control values for peak dP/dt + P in the present study were significantly lower than those determined on dogs anesthetized with ethanol-morphine in the earlier study (Van Stee et al., in press). On the other hand, control values from a series of mongrel dogs prepared for metabolic studies of another fluoroalkane according to the present method, except for the substitution of morphine (4 mg/kg) for fentanyl-droperidol, were not significantly different from those obtained in this study. This would seem to exclude droperidol as the source of the apparent discrepancy. It is considered not likely that the common anesthetic denominator, alpha-chloralose, was responsible, since the total doses (70 mg/kg) were not excessive and a significant negative inotropic action has not been attributed to α-chloralose at these levels. The lack of a satisfactory explanation for the differences observed in peak dP/dt + P between beagles under one anesthetic regimen and large mongrels under another underscores the necessity of viewing such indices of myocardial contractility only in terms of relative changes within the context of any given experiment rather than in absolute terms.

The concentration of $CBrF_3$ to which the animals were exposed was correlated inversely with the arterial blood oxygen content (1301 - AO_2C, Figure 2). Variations in coronary arterial blood flow (COR Q) and AO_2C were correlated with oxygen extraction (EO_2). EO_2 correlated significantly with contractility. The coronary flow - contractility - oxygen extraction relationship has been defined by Abel and Reis (1970) and Fisher et al. (1969).

Myocardial extraction of O_2 and NEFA, and mean arterial pressure (AP) were positively correlated with peak dP/dt. The direct relationship that we observed between cardiac output (CO) and central venous volume (CVV) has been documented by Maseri et al. (1972).

The myocardial extractions of lactate and NEFA were positively correlated with the arterial levels of the respective substrates. Lactate and pyruvate extraction and arterial glucose level were positively correlated with coronary flow. The determination of an inverse relationship between arterial NEFA levels and lactate extraction was consistent with the observations of Carlson et al. (1972). The observations made in the present study suggested the possibility that the availability and balance of lipid and carbohydrate substrates to the myocardium may have been related to the coronary flow as well as oxygen extraction and myocardial contractility.

The relationships among the lactate and pyruvate variables were consistent with the observations of Whereat and Chan (1972) except that the ratios were higher. The coronary sinus L/P ratio was consistently lower than the arterial L/P ratio reflecting a preferential extraction of lactate by the myocardium.

Neither blood levels and myocardial extraction of energy-yielding substrates and oxygen, nor their interactions (to the extent that such interactions may be inferred from correlations) differed significantly from the expected values. This led to the conclusion that the negative inotropic effect of exposure to $CBrF_3$ probably was not the result of a diminished myocardial supply or uptake of these substrates.

Figure 3 summarizes our view of the problem by determining the mechanism of the negative inotropic effect of fluoroalkanes.

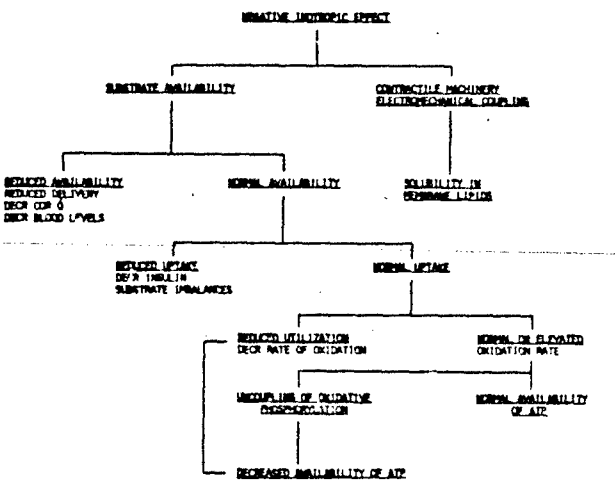

Figure 3. The problem of the approach to the study of mechanisms of inotropic action.

A negative inotropic action could result through the action of a fluoroalkane on either or both of 2 systems necessary for the conversion of the chemical energy of reduced substrates to the mechanical energy of myocardial contraction: (1) the provision of ATP to the contractile mechanism, and (2) the coupled electro-mechanical event. Implicit in these concepts is the requirement for adenosine triphosphatase (ATPase) activity to modulate the rate of ATP hydrolysis associated with the coupled processes resulting in muscle shortening.

Substrate is made available to the myocardium via the coronary blood flow. The rate of delivery of substrates and oxygen is the product of the blood levels and the coronary flow rate. Reduced delivery of metabolites could be the consequence of reduced blood levels and/or reduced rate of coronary perfusion.

If delivery to the myocardium were normal, uptake of individual substrates may have been altered. Decreased insulin activity, for example, would reduce glucose uptake. Alterations of the ratios of nonesterified fatty acids and the carbohydrate substrates could affect the uptake of the individual substrates (Carlson et al., 1972).

The results of the present study suggested that substrate availability and uptake were not altered by $CBrF_3$. Clues to the elucidation of the mechanism of the negative inotropic effect therefore should be sought at the subcellular level of substrate utilization and metabolism. If the uptake of substrates is reasonable, actual utilization may be affected and the rate of oxidative metabolism reduced. Such a reduction would result in a reduction in the rate of synthesis of ATP (or creatine phosphate) by aerobic metabolism. If the rate of oxidative metabolism is normal (or elevated) it still may not result in the synthesis of maximal amounts of ATP. If oxidation becomes uncoupled from phosphorylation, oxygen consumption and energy conversion continue at normal or elevated rates, but chemical energy is degraded to heat at the expense of ATP synthesis. Thus, either a reduced rate of oxidative metabolism or uncoupling of oxidative phosphorylation would result in a decreased availability of ATP to the contractile apparatus.

Consider further the branch of the diagram in Figure 3 at the upper right: contractile machinery and electromechanical coupling. Myofibrillar activation and contraction are preceded by electrical depolarization of the plasma membrane, represented by the action potential. The wave of depolarization may be conducted through the T-tubular system to the interior of the myocardial cell where it is coupled to the release of calcium ions from the sarcoplasmic reticulum. Thus, factors that alter the properties of the action potential are followed by alterations in myocardial contractility (Miller and Gilmore, 1972). The current theories of excitation-contraction

and the contractile process are reviewed at regular intervals (Page and Katz, 1973; Kones, 1973; Langer, 1973; Tonomura and Oosawa, 1972).

A final consideration is that of the ATPase activity necessary in the transfer of the ATP terminal phosphate bond energy to various endergonic processes such as the accumulation of ions against electromechanical gradients. Factors that influence ATPase activity therefore have a secondary effect on contractile processes.

We suggest a working hypothesis based on the lipid solubility of fluoroalkanes related to the Mullins and Meyer-Overton theories of anesthesia (Miller et al., 1972) as a general, unifying hypothesis that may underlie many of the actions of fluoroalkanes. Since the fluoroalkanes are lipid-soluble, they can be expected to become dissolved in the lipid phases of plasma and subcellular membranes. Sequences of enzymatic reactions such as aerobic oxidation, the processes that maintain the plasma transmembrane sodium and potassium gradients, and probably the reactions involved in the release and re-uptake of calcium ions by the sarcoplasmic reticulum, that are dependent on the integrity of membrane structure, might be expected to be altered by the presence of fluoroalkane molecules in the membrane lipid phase. Indeed, the presence of fluoroalkane molecules within the lipid phase could be expected to alter the primary relationship among the phospholipid molecules of a given leaflet as well as the secondary relationship between the bilayers and phospholipid-protein structural relationships. Such a presence may be demonstrated to constitute an alteration of the structural integrity of the membranes incompatible with the normal functioning of the membrane-bound enzymatic systems (Green, 1972).

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PAPER NO. 7

THE EFFECTS OF FLUOROCARBONS ON HEPATIC MICROSOMAL ENZYMES

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INTRODUCTION

Many drugs and foreign compounds are biotransformed in the body by enzymes located in the microsomal fraction of the endoplasmic reticulum. Remmer (1965) and others have reported on a number of drugs and compounds that affect enzymes of the hepatic smooth endoplasmic reticulum (SER). Numerous compounds stimulate this common enzyme system to metabolize drugs more readily while many other compounds inhibit the system (Mannering, 1967, p. 106). Evaluation of the effect of a drug or foreign compound on drug metabolism in an organism requires that a sequence of steps be performed. These steps were discussed in detail by Van Stee (1971) at a previous conference. The approach as applied in this study is outlined in Figures 1 and 2.

Figure 1 illustrates two main categories, in vivo studies and in vitro studies. Studies in vivo encompass a qualitative change in the rate of drug metabolism, measurement of the duration of hexobarbital sleeping and/or zoxazolamine paralysis times. After exposure to a test compound for several days, the mice are injected intraperitoneally with hexobarbital or zoxazolamine. The pharmacological action of hexobarbital is terminated in part through its biotransformation to the inactive ketoisomers. The active zoxazolamine is hydroxylated to the inactive 6-hydroxy-zoxazolamine. The interval between the loss and return of the righting reflex is considered the duration of sleeping or paralysis time.

Studies in vitro are more definitive than sleep time studies and measure quantitative changes in the rate of substrate disappearance or product formation, which reflects the effect of a test compound on the drug metabolizing enzymes.

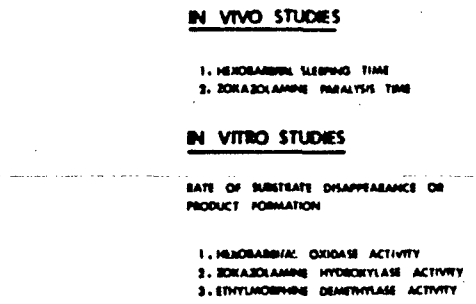

Figure 1. In vivo and in vitro studies - drug metabolism.

Figure 2 illustrates the reactions involved for substrate metabolism both in vivo and in vitro. The first reaction represents substrate metabolism in the mouse during hexobarbital sleeping time. The active form of hexobarbital is oxidized to the inactive ketoisomers. The second reaction represents substrate metabolism during zoxazolamine paralysis time. The active zoxazolamine is hydroxylated to the inactive 6-hydroxy-zoxazolamine. For in vitro studies, side chain oxidation of hexobarbital, hydroxylation of zoxazolamine and N-demethylation of ethylmorphine are the reactions of choice.

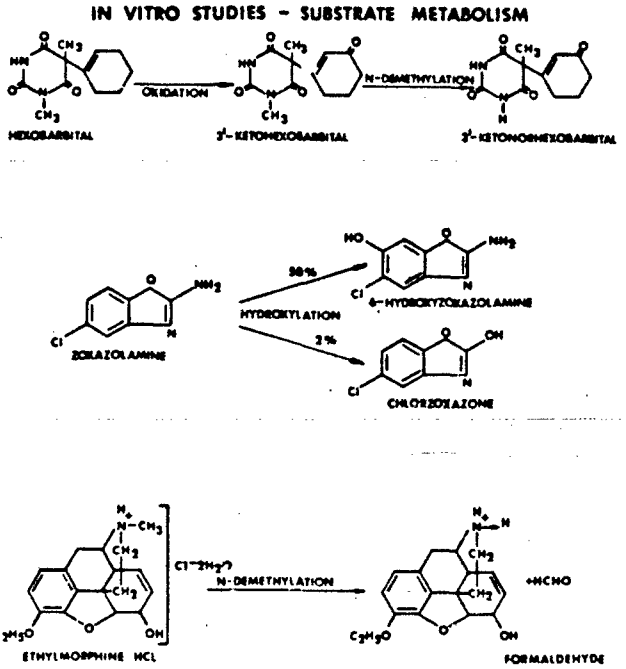

Figure 2. In vivo and in vitro studies - substrate metabolism.

Halogenated alkanes as a class are noted for their hepatotoxicity. Outstanding are the chlorinated compounds, carbon tetrachloride, chloroform, dichloromethane, and tetrachloromethane. Lal (1970a) showed that continuous inhalation exposure at low concentrations of carbon tetrachloride prolonged hexobarbital-induced narcosis in rats. Studies in vitro showed that hepatic microsomal preparations from rats exposed to carbon tetrachloride metabolized hexobarbital at a slower rate (inhibition) than similar preparations from untreated rats.

Other short chain halogenated hydrocarbons, such as methylchloroform, stimulate hepatic drug metabolism (Lal, 1970b). Fuller (1970) showed that short term inhalation of methylchloroform decreased the duration of action of hexobarbital and zoxazolamine in rats. Metabolism in vitro by hepatic microsomes of hexobarbital and zoxazolamine was also increased.

Fluoroalkanes are of interest to the Air Force. Generally, fluorinated alkanes would not be considered significant hepatotoxins, owing to firm C-F bonds which, according to Clayton (1967, p. 197), do not easily dissociate. The presence of fluorine in the molecule tends to exert an influence by stabilizing the adjacent carbon-halogen bonds.

Figure 3 represents the fluoroalkanes that have come under investigation in this laboratory. They are: bromotrifluoromethane (H-1301), a fire extinguishing agent and propellant; trichlorotrifluoroethane (H-2330), a solvent and refrigerant; dibromotetrafluoroethane (H-2402) and dibromotrifluoroethane (H-2302), fire control agents. Interest in the latter two stems from Air Force use of H-2402 for engine nacelle fire protection systems. Dibromotrifluoroethane (H-2302) has similar engineering properties and could become a contender for such an application.

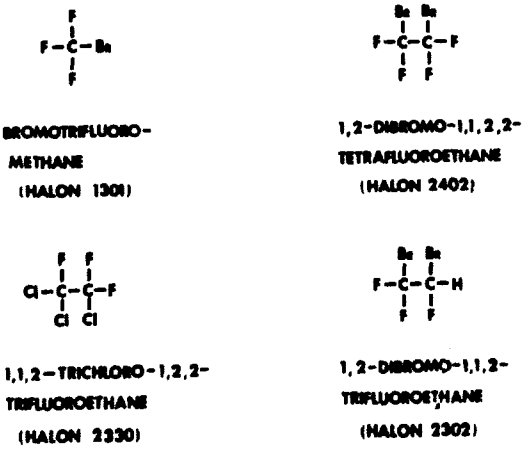

Figure 3. The fluoroalkanes investigated, structural formula and nomenclature.

Due to the low boiling points of these compounds, exposure by inhalation is most likely. Preliminary screening of these fluorocarbons based on hexobarbital sleeping and zoxazolamine paralysis times has revealed no significant effect with either bromctrifluoroethane (H-1301) or with trichlorotrifluoroethane (H-2330). Dibromotetrafluoroethane (H-2402) inhalation by mice has been reported to reduce hexobarbital sleeping and zoxazolamine paralysis times and increase the in vitro metabolism of hexobarbital and zoxazolamine (Murphy, 1973, p. 2843). Preliminary screening of the compound dibromotrifluoroethane (H-2302) has also shown reduction of sleep time activity.

The effect of exposure to these compounds on the duration of hexobarbital sleep and zoxazolamine paralysis times suggested the possibility that the compounds induced hepatic microsomal enzymes. The present study was conducted to test further this hypothesis.

MATERIALS AND METHODS

Animals

Male CF-1 albino mice, 18-22g, obtained from Carworth Laboratories Midwest (Portage, Michigan) were maintained on a balanced commercial laboratory chow and water ad libitum. All sleep time studies and chamber exposures for control and treated animals were carried out at the same time periods each morning to eliminate the daily rhythmic variation reported by Radziałowski (1968).

Inhalation Exposure

The animals were exposed in a plexiglas chamber to a mixture of fluorocarbon in air at ambient temperature for 5 hours daily, 1 through 4 days. The exposure chamber unit included a dual syringe feeder, vaporizer column, and air flow meter. Chamber concentration was maintained by controlled delivery of liquid fluorocarbon into the vaporizing column utilizing the dual syringe feeder. The oxygen and carbon dioxide levels of the atmosphere in the exposure chamber were determined periodically using the micro gas analyzing technique of Scholander (1947). The fluorocarbon concentrations in the exposure chamber were monitored by a Perkin-Elmer Vapor Fractometer Model 154, equipped with a thermal conductivity detector.

In Vivo Studies

The duration of hexobarbital sleeping time and zoxazolamine paralysis time was determined on groups of 30 and 60 mice, using half the groups as controls. Intraperitoneal injections of hexobarbital (120 mg/kg) or

zoxazolamine (100 mg/kg) were made 18 hours postexposure. Hexobarbital sleeping time or zoxazolamine paralysis time was defined as that interval between the loss and return of the righting reflex. Reasonable efforts were made throughout the study to hold constant extraneous factors that might alter mouse drug responsiveness (Vesell, 1968, p. 81). All in vivo and in vitro experiments were carried out on separate groups of animals.

In Vitro Studies

Hexobarbital oxidase and zoxazolamine hydroxylase activities were determined 18 hours postexposure on the hepatic 9000g microsomal suspension. Mice were killed by cervical dislocation, the gall bladder was excised and the liver was removed and placed immediately in cold isotonic KCl. All tissue and homogenate handling was carried out at 0^{o} to 4^{o}. Homogenates were centrifuged at 9000g for 20-30 minutes in a Sorvall RC-2 refrigerated centrifuge. The supernatant fraction was aspirated and used for the determination of enzyme activity.

Enzyme Assays

The microsomal suspension was incubated at 37°C in phosphate buffer, pH 7.35 containing glucose-6-phosphate, $MgSO_4$, nicotinamide, NADP, and the appropriate substrate. Conditions of incubation cofactors used and concentrations were optimal and similar to those described by Gram and Fouts (1966). Hexobarbital metabolism was determined by the method of Cooper and Brodie (1955); zoxazolamine metabolism was determined by the method of Juchau (1965), both following substrate disappearance. The substrate metabolized was the difference between zero time and 30-minute incubation. Linear relationships were demonstrated for hexobarbital oxidase activity up to and beyond 30-minutes incubation of hepatic 9000g supernatant for control and fluorocarbon exposed animals. Linear response was demonstrated for hexobarbital and zoxazolamine activity relative to the protein concentration of the incubate. Protein content of the hepatic 9000g supernatant was determined by the method of Lowry (1951). Data are expressed as nanomoles of substrate metabolized per milligram of 9000g supernatant protein per incubation period.

Light and Electron Microscopy

Livers from mice exposed to 1.0% dibromotetrafluoroethane and control mice were examined by light and electron microscopy according to the technique of Weinstein (1971, p. 147). Livers from mice exposed to 0.63% dibromotrifluoroethane and control mice were examined by light microscopy.

Statistical Analysis

Sleep and paralysis time data were tested by use of Student's t-test. Hexobarbital oxidase and zoxazolamine hydroxylase specific activities were each treated as a mixed model, nested analysis of variance. Significant differences among means in the analysis of variance were determined using Duncan's multiple range test.

RESULTS AND DISCUSSION

The results and data are presented in two parts, the first part dealing with dibromotetrafluoroethane (H-2402), the second part with dibromotrifluoroethane (H-2302).

Hexobarbital Sleeping Time and Duration of Zoxazolamine Paralysis

The inhalation of dibromotetrafluoroethane in mice, 5 hours daily for 3 days reduced up to 50% the hexobarbital sleeping time and duration of zoxazolamine paralysis time. Figure 4 illustrates the effect of dibromotetrafluoroethane exposure on the duration of hexobarbital sleeping in mice. Sleeping time is represented along the ordinate as percentage of control, and concentration is represented along the abscissa on a logarithmic scale ranging from 0.25 to 2.5%. The parabola plotted through the points represents a curve determined by the method of least squares and has an R^2 of 0.83. Each point on the curve represents 13-16 mice. Exposure to increasing concentrations of dibromotetrafluoroethane (up to 1.0%) reduced sleeping time and increased sleeping time with concentrations from 1.0 to 2.5%.

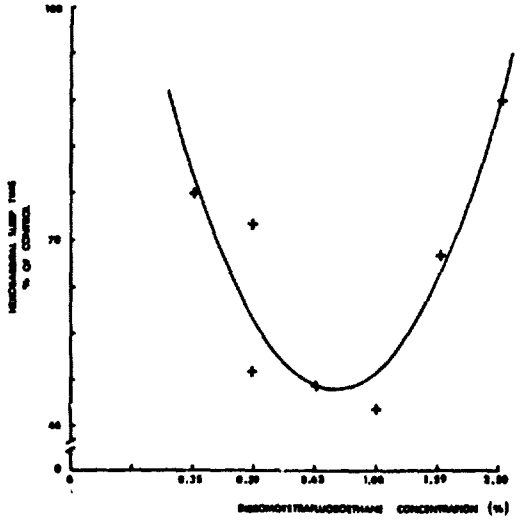

Figure 4. The effect of dibromotetrafluoroethane (H-2402) exposure, 5 hours daily for 3 days on the duration of hexobarbital sleeping time in mice.

Figure 5 illustrates the effect of dibromotetrafluoroethane exposure on the duration of zoxazolamine paralysis time. Paralysis time is represented along the ordinate as percentage of control and concentration is represented along the abscissa on a logarithmic scale ranging from 0.25 to 3.9%. Zoxazolamine paralysis time decreased to the 1.0% concentration and increased from 1.0 to 3 9%.

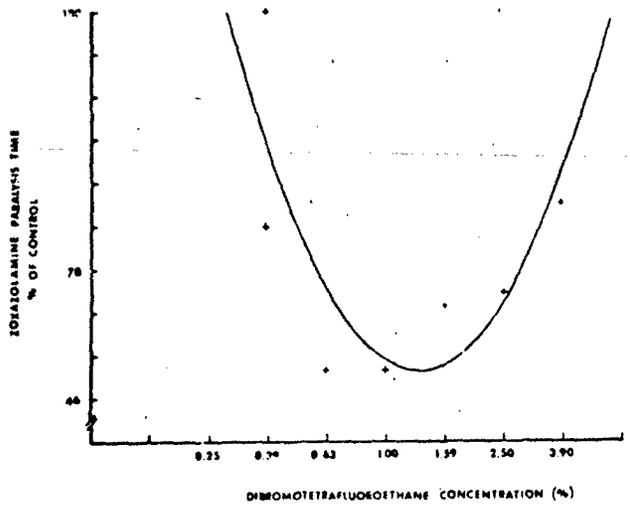

Figure 5. The effect of dibromotetrafluoroethane (H-2402) exposure, 5 hours daily for 3 days on the duration of zoxazolamine paralysis in mice.

The duration of hexobarbital and zoxazolamine sleep times expressed as percentage of controls showed similar time courses and appeared to be concentration dependent up to 1.0%. The parabolic relationship suggests a phasic effect, with increased metabolic rate (induction) up to 1.0%, and a possible hepatotoxic effect beyond that concentration (Kutob, 1962, p. 354). The pharmacological effects of exposure were convulsions at levels of 2-3%, narcosis at 4-5%, and death at 5-6%. Similar signs observed in rats at somewhat higher concentrations were reported by Rainalde (1969, p. 3).

Study In Vitro

Definitive metabolic studies were conducted in vitro with the 9000g supernatant fraction from control animals and animals exposed to those concentrations that had displayed maximum changes in vivo, that is at the 0.63 and 1.0% level.

Data presented in Tables 1 and 2 indicate that at the 0.63 and 1.0% concentrations the livers from the dibromotetrafluoroethane-exposed mice metabolized hexobarbital and zoxazolamine at rates significantly higher than those from the control animals.

TABLE 1. EFFECT OF H-2402 INHALATION ON THE METABOLISM OF HEXOBARBITAL* IN MOUSE LIVER

% H-2402	Number of Exposures**	Hexobarbital Oxidase		
		Control	Treated	Sig.
0.63	3	15.89 ± 3.72 (8)***	22.59 ± 3.92 (8)	$p < 0.05$
1.0	3	18.58 ± 1.14 (8)	24.10 ± 2.17 (8)	$p < 0.05$
1.0	4	14.64 ± 0.94 (16)	23.04 ± 1.43 (16)	$p < 0.01$

* Values are mean ± S.E.M. expressed as nanomoles of hexobarbital metabolized per milligram of 9000g supernatant protein per 30 minutes at 37°C.
** 5 hour exposure/day.
*** Number of groups tested in parenthesis.

TABLE 2. EFFECT OF H-2402 INHALATION ON THE METABOLISM OF ZOXAZOLAMINE* IN MOUSE LIVER

% H-2402	Number of Exposures**	Zoxazolamine Hydroxylase		
		Control	Treated	Sig.
0.63	3	12.53 ± 0.63 (8)***	16.30 ± 0.47 (8)	$p < 0.01$
1.0	3	13.23 ± 0.74 (8)	17.34 ± 1.11 (8)	$p < 0.05$

* Values are mean ± S.E.M. expressed as nanomoles of zoxazolamine metabolized per milligram of 9000g supernatant protein per 30 minutes at 37°C.
** 5 hour exposure/day.
*** Number of groups tested in parenthesis.

Table 1 illustrates the effect of dibromotetrafluoroethane inhalation on the metabolism of hexobarbital by the 9000g supernatant fraction of mouse livers. The microsomal 9000g supernatant fraction was prepared from livers of mice exposed to dibromotetrafluoroethane 5 hours daily. Livers were removed 18 hours postexposure and pooled, five livers per group. The number of group observations is in the parenthesis. Values quoted are the mean ± S.E.M. expressed as nanomoles of hexobarbital metabolized per milligram of 9000g supernatant protein per 30 minutes. Data presented in this table suggest that at 0.63 and 1.0% the livers from dibromotetrafluoroethane exposed animals metabolized hexobarbital at rates significantly higher than those of control animals.

We observed that a single 5-hour exposure and a 2-day exposure had no apparent effect upon hexobarbital sleeping or zoxazolamine paralysis time at 1% but extending the exposure period from 3 to 4 days further increased the hexobarbital oxidase activity of exposed mice over the control mice.

Data presented in Table 2 illustrate the effect of dibromotetrafluoroethane inhalation on the metabolism of zoxazolamine. Values quoted are the mean ± S.E.M. expressed as nanomoles of zoxazolamine metabolized per milligram of 9000g supernatant protein per 30 minutes. The data indicate that at 0.63 and 1.0% the livers from exposed animals metabolized zoxazolamine at rates significantly higher than control animals.

The next part of this paper is concerned with the effect of inhalation of dibromotrifluoroethane in mice, 5 hours daily for varying periods. Animals were treated in a manner similar to the dibromotetrafluoroethane study.

Hexobarbital sleeping time was reduced by 40% following the 3-day exposure to dibromotrifluoroethane. This is illustrated in Figure 6. Sleep time is represented along the ordinate as percent of control, and concentration is represented along the abscissa on a logarithmic scale ranging from 0.10 to 0.63%. The line plotted through the points represents a curve determined by the method of least squares and has an R^2 of 0.98. The curve indicates a decrease in sleep time with increased concentration. The pharmacological effects of exposure were narcosis at 0.39 to 0.63% with death at 1.0%; therefore, exposures could not be carried out at higher concentrations.

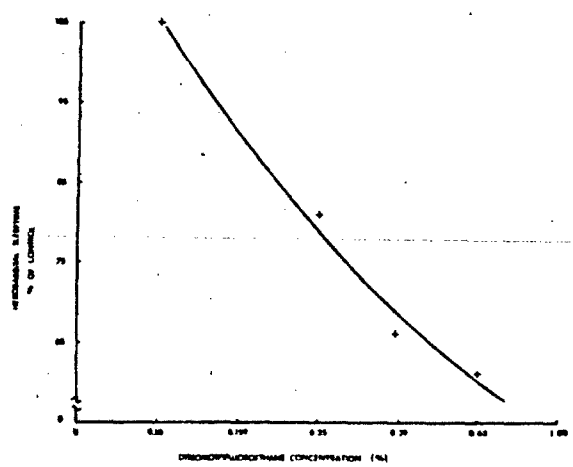

Figure 6. The effect of dibromotrifluoroethane (H-2302) exposure, 5 hours daily for 3 days on the duration of hexobarbital sleeping time in mice.

Exposures were made for 1 through 4 days to test further the effect of dibromotrifluoroethane at the highest concentration. Figure 7 illustrates the effect of 1, 2, 3 and 4 days of exposure to 0.63% dibromotrifluoroethane. A line plotted through the points represents a curve determined by the method of least squares. The curve shows an increased metabolic rate (induction effect) up to the third day of exposure. Increasing the exposure to four days suggested a possible trend toward hepatotoxic effect.

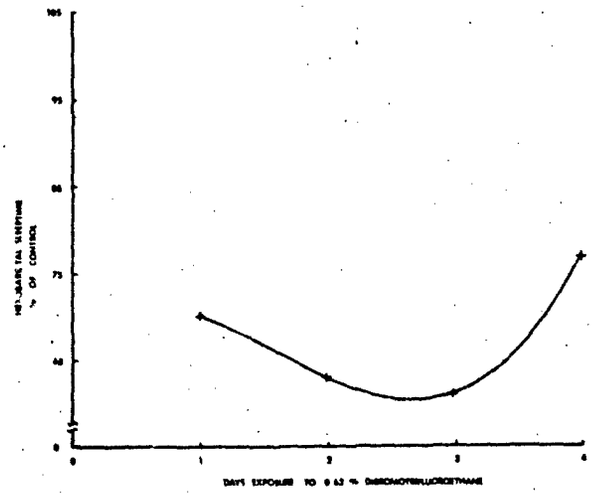

Figure 7. The effect of dibromotrifluoroethane (H-2302) exposure, 5 hours daily for 1-4 days on the duration of hexobarbital sleeping time in mice.

No significant effect of dibromotrifluoroethane exposure on the duration of zoxazolamine paralysis was detected in this study.

Definitive metabolic studies conducted in vitro with the 9000g supernatant fraction from control animals and animals exposed to dibromotrifluoroethane paralleled the decreased hexobarbital sleep time studies in vivo.

Tables 3, 4 and 5 illustrate the effect of exposure to 0.25 to 0.63% dibromotrifluoroethane on the metabolism of hexobarbital and zoxazolamine for 1 to 4 days. The numbers of group observations are in parentheses. Values quoted are the mean $\pm$ S.E.M. nanomoles of hexobarbital or zoxazolamine metabolized per milligram of 9000g supernatant protein per 30 minutes at 37°C. Data presented in Table 3 illustrate the effect of exposure to 0.25 to 0.63% dibromotrifluoroethane on the metabolism of hexobarbital for three days by the 9000g supernatant fraction of liver. Data indicate that at 0.63% the livers of the exposed animals metabolized hexobarbital at rates significantly higher than those of control animals. At the 0.25 and 0.39% concentrations the differences were not as great, but were significantly different at the 95% level.

TABLE 3. EFFECT OF H-2302 INHALATION ON THE METABOLISM OF HEXOBARBITAL* IN MOUSE LIVER

% H-2302	Number of Exposures**	Hexobarbital Oxidase		
		Control	Treated	Sig.
0.63	3	16.12 ± 0.74 (21)***	24.63 ± 1.23 (21)	$p < 0.05$
0.39	3	12.86 ± 0.77 (16)	13.71 ± 0.47 (16)	$p < 0.05$
0.25	3	14.67 ± 0.68 (4)	15.64 ± 0.50 (4)	$p < 0.05$

* Values are mean ± S.E.M. expressed as nanomoles of hexobarbital metabolized per milligram of 9000g supernatant protein per 30 minutes at 37°C.
** 5 hour exposure/day.
*** Number of groups tested in parenthesis.

Table 4 illustrates the effect of dibromotrifluoroethane inhalation on the metabolism of zoxazolamine at concentrations ranging from 0.39 to 0.63% for 3 days duration. No significant differences were detected between the exposed and control animals at these concentrations. This is in agreement with the in vivo studies.

TABLE 4. EFFECT OF H-2302 INHALATION ON THE METABOLISM OF ZOXAZOLAMINE* IN MOUSE LIVER

% H-2302	Number of Exposures**	Zoxazolamine Hydroxylase		
		Control	Treated	Sig.
0.63	3	17.59 ± 1.35 (6)***	18.67 ± 1.39 (6)	N.S.
0.39	3	14.53 ± 0.50 (12)	13.42 ± 0.31 (12)	N.S.

* Values are mean ± S.E.M. expressed as nanomoles of zoxazolamine metabolized per milligram of 9000g supernatant protein per 30 minutes at 37°C.
** 5 hour exposure/day.
*** Number of groups tested in parenthesis.

Exposures were made for 1 to 4 days to test further the effect of dibromotrifluoroethane inhalation on the metabolism on hexobarbital at the high concentration. The results of 0.63% dibromotrifluoroethane inhalation

on the metabolism of hexobarbital for 1, 2, 3 and 4 days duration are in Table 5. Values are mean ± S.E.M. nanomoles of hexobarbital metabolized per milligram of 9000g supernatant protein per 30 minutes at 37°C. The livers of treated animals metabolized hexobarbital at rates significantly higher than those of control animals at all exposure periods.

TABLE 5. EFFECT OF H-2302 INHALATION ON THE METABOLISM OF HEXOBARBITAL* IN MOUSE LIVER

% H-2302	Number of Exposures**	Hexobarbital Oxidase		
		Control	Treated	Sig.
0.63	1	13.84 ± 0.29 (6)***	18.33 ± 1.10 (6)	$p < 0.05$
0.63	2	13.35 ± 0.58 (6)	22.11 ± 0.16 (6)	$p < 0.05$
0.63	3	16.13 ± 0.38 (4)	19.39 ± 0.19 (4)	$p < 0.05$
0.63	4	16.75 ± 0.37 (6)	22.13 ± 0.38 (6)	$p < 0.05$

* Values are mean ± S.E.M. expressed as nanomoles of hexobarbital metabolized per milligram of 9000g supernatant protein per 30 minutes at 37°C.
** 5 hour exposure/day.
*** Number of groups tested in parenthesis.

The observation was made in the present study that inhalation of 0.25 to 1.0% dibromotetrafluoroethane reduced the duration of hexobarbital sleeping time and zoxazolamine paralysis time, while hexobarbital oxidase and zoxazolamine hydroxylase activities were increased. The inhalation of dibromotrifluoroethane at concentrations lower than 0.10 to 0.63% reduced the duration of hexobarbital sleeping time while hexobarbital oxidase activities were increased. Preliminary studies have shown dibromotrifluoroethane to have no significant effect upon zoxazolamine metabolism either in vivo or vitro.

Inability to demonstrate alterations in the smooth endoplasmic reticulum (SER) of dibromotetrafluoroethane exposed mice suggests 2 possibilities: (1) there is no proliferation of SER membrane with exposure to dibromotetrafluoroethane, (2) SER membrane surface area could have been mildly increased, yet not be apparent with the technique used. The induction of the microsomal enzyme system as demonstrated favors the second possibility. Electron micrographs of livers exposed to dibromotrifluoroethane were not made because it was felt that the subtle changes in SER membrane might be impossible to detect properly in the absence of morphometric analysis.

Conclusion

A structure-activity relationship may be inferred from a comparison of the effects of the compounds dibromotetrafluoroethane and dibromotrifluoroethane on hexobarbital sleep time in vivo, and hexobarbital oxidase activity in vitro.

Dibromotetrafluoroethane is a per-halogenated compound, whereas dibromotrifluoroethane has a hydrogen atom substituted for a fluorine atom. The enzyme-inductive capacity of both compounds was similar, but the toxic response of the mice differed dramatically.

The toxic response following induction (Figure 4) in the case of dibromotetrafluoroethane may be based upon the increase in sleep time, or decrease of enzyme activity at concentrations greater than 1.0% (Kutob, 1962).

The toxic response in the case of dibromotrifluoroethane may be based upon the inability of mice to survive at concentrations greater than those required to cause maximal enzyme induction (> 0.63%).

The response to exposure may be divided into 3 categories:

1. Hepatic microsomal induction
2. Hepatic microsomal inhibition
3. A lethal response presumably a function of cardiopulmonary failure.

Both compounds caused similar responses.

A further increase in the concentration of dibromotetrafluoroethane (> 1.0%) resulted in decreased sleep time and finally death.

A further increase in the concentration of dibromotrifluoroethane (> 0.63%) resulted in death before enzyme inhibition could be detected.

The evidence supports the hypothesis that the compounds possibly induce hepatic microsomal enzymes. Further evidence supports the hypothesis that the first defluorination of dibromotetrafluoroethane would not affect the capacity of the compound to induce hepatic microsomal enzymes, but rather increase its capability to activate lethal mechanisms within the body.

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DISCUSSION

DR. DREW (National Institute for Environmental Health Sciences): In your in vivo data, you demonstrated a depression down to 1% of initial sleeptime which is rather drastic. Based on those findings, I would expect a rather dramatic induction of enzymes, yet you didn't have much induction, only by a factor of 2. Do you have any comment relative to that?

MR. MURPHY (Aerospace Medical Research Laboratory): I agree with you. It's not a factor of 2; approximately 60% of the controls are decreased. I don't know why. It is dependent upon the mouse strain used to get a particular induction effect, and if you happen to use the wrong strain of mouse, you won't get any effect at all. Certain mouse strains will give a greater induction effect or larger change that would be comparable to the phenobarbital effect which you get, and others won't give any effect.

DR. DREW: It just looks to me like there might be something else going on in addition to the induction effect which you've shown.

DR. GEHRING (Dow Chemical Company): In your in vivo work, you exposed animals to higher concentrations of fluorocarbon than 1% and then subsequently measured the reduction in hexobarbital sleeping time and also zoxazolamine paralysis time. I was wondering, did you expose any animals to those higher concentrations and subsequently measure hexobarbital oxidase and also the zoxazolamine hydroxylase?

MR. MURPHY: Did I expose them to higher concentrations?

DR. GEHRING: Yes, in your in vivo data, you have the parabolic curve that goes back up, but I didn't see any data for the higher exposure concentration.

MR. MURPHY: They were exposed to concentrations up to 3.9%. Initially, we were looking for the fluorocarbon concentration that would give the greatest induction effect, so we went to the 0.63% and the 1% levels. We wanted to achieve a maximum response from the animals and then after getting that, we proceeded to this other aspect of attempting to show whether hepatotoxicity had increased.

DR. GEHRING: My question is were you getting breakdown of the endoplasmic reticulum, or were you actually getting additional induction of the enzymes which wasn't compensated?

MR. MURPHY: We don't know.

AMRL-TR-73-125

PAPER NO. 8

ULTRASTRUCTURE OF GUINEA PIG HEART AFTER EXPOSURE TO FLUOROCARBON 1301

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INTRODUCTION

Interest in the possible toxicologic effects of fluoroalkanes has been generated because of their widespread use as aerosol propellants, refrigerants, fire extinguishing agents, and anesthetics (Egle et al., 1972; Flower and Horan, 1972). Bromotrifluoromethane (fluorocarbon 1301) is well established as an excellent fire-extinguishing agent (Proceeding of Fire Hazards and Extinguishment Conference, 1967; Botteri, 1968). A striking aspect of the toxicology of $CBrF_3$ as well as several related fluorinated alkanes is that they depress myocardial contractility; examples are $CBrF_3$ and $CBrClF_2$ (Van Stee et al., 1974), halothane (Goldberg and Ullrick, 1967; Goldberg and Phear, 1968), and CCl_2F_2 (Kilen and Harris, 1972). Although this depression of contractility, i.e., negative inotropic effect, has been clearly demonstrated the intracellular targets of the fluoroalkane toxicity are not known.

Another, perhaps related, phenomenon is that a large number of hydrocarbons and halogenated hydrocarbons are known to increase myocardial sensitivity to epinephrine-induced arrhythmias (Bulletin, 1969; Burgiston et al., 1955; Carr, 1949; Chenoweth, 1946; Garb and Chenoweth, 1948; Levy, 1911; Levy and Lewis, 1911-12; Levy, 1912; Levy, 1913-14; Meek et al., 1937; Meek, 1940-41; Reinhardt, 1971). Like other members of the congeneric series, $CBrF_3$ may induce spontaneous cardiac arrhythmias whose severity varies as a function of arterial blood pressure and respiratory acid-base balance, as well as the level of circulating epinephrine (Van Stee and Back, 1971). Halogenated hydrocarbon effects on cardiac electrical activity are generally interpreted in terms of changes in the plasma membrane action potential and sodium and potassium flux at the plasma membrane (Seeman, 1972). However, little is known about the exact mechanism of these effects by such a wide variety of unsubstituted and halogenated alkanes.

In an effort to determine if there are ultrastructural correlates to the inotropic and arrhythmogenic effects of myocardial exposure to fluorocarbon, we have exposed guinea pigs to $CBrF_3$, fixed the hearts, and processed them for quantitative electron microscopy.

MATERIALS AND METHODS

Twenty-one adult guinea pigs weighing from 500 to 1100 grams were divided into two groups, a control group of twelve and an experimental group of nine. The control guinea pigs were anesthetized with an intraperitoneal injection of 20-30 mg of sodium pentobarbital.

In three of the controls, the heart was rapidly excised and biopsies of left ventricles were placed in fixative B (Table 2) overnight. The tissues were washed briefly in buffer, fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours, dehydrated in a graded series of ethanol solutions, and embedded in standard epoxy plastic medium.

In the other nine control guinea pigs, the abdominal aorta was isolated and cannulated cephalad below the renal arteries. The inferior vena cava was cut and the coronary arteries were perfused via the aortic catheter. As soon as the perfusion was begun, the intestinal arteries and veins were clamped with a large hemostat. The brachial arteries and veins, the carotid arteries, and the jugular veins were compressed with heavy ligatures. The first minute of perfusion was with Hanks physiologic salt solution (Table 1). The following 20 minutes of perfusion were with fixative A (Table 2). The thoracic cavity was not opened until the perfusion was complete. The heart was carefully dissected free and sliced into 1-2 mm slabs with a razor blade. Coagulated blood was removed from the ventricular cavities and small 1-2 mm square blocks were cut at random from the slices of left ventricular wall and septum. These blocks were fixed overnight in fixative C (Table 2). The tissues were washed in buffer, fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer pH 7.4 for 2 hours, dehydrated in graded ethanol solutions, and embedded in epoxy plastic.

The experimental group of nine guinea pigs were exposed to $CBrF_3$ and oxygen mixtures. Freon 1301® concentrations were intermittently monitored by gas chromatography with a Barber-Coleman Model 61-C gas chromatograph. Oxygen concentrations were constantly monitored with an oxygen probe [Beckman Process Oxygen Analyzer; Beckman Company]. The animals were exposed in a large stainless steel box that was open at the top so that the animals could be observed. Overflow gases were exhausted. After 45-60 minutes exposure, the guinea pigs were individually anesthetized with an intraperitoneal injection of 20-30 mg sodium pentobarbital. For three of the guinea pigs, the hearts were fixed by perfusion after exposure to 70 (± 10%) $CBrF_3$/30% oxygen.

TABLE 1. HANKS PHYSIOLOGIC SALT SOLUTION

Compound	Grams/liter
NaCl	8.00
K Cl	0.40
$MgSO_4 . 7\ H_2O$	0.10
$MgCl_2 . 6\ H_2O$	0.10
$NaHPO_4 . 2\ H_2O$	0.06
KH_2PO_4	0.06
Glucose	1.00
$NaHCO_3$	0.35
$CaCl_2$	0.14

TABLE 2. ELECTRON MICROSCOPY FIXATIVES

Fixative A (initial perfusion fixative)

1% formaldehyde (from paraformaldehyde)
1.25% glutaraldehyde
0.0013% picric acid
Hanks physiologic salt solution

Fixative B (initial immersion fixative)

2% formaldehyde
2.5% glutaraldehyde
0.0013% picric acid
0.1 M sodium cacodylate buffer pH7.4

Fixative C (immersion fixative for perfused specimens)

4% formaldehyde
5% glutaraldehyde
0.0026% picric acid
0.1 M sodium cacodylate buffer pH 7.4

At the onset of stage 3 anesthesia, each guinea pig was placed in a smaller open-top stainless steel tank which had been equilibrated with the $CBrF_3/O_2$ mixture. This small tank was moved to a dissecting table equipped with a downdraft exhaust system. The dissection and perfusion was completed in this container with the animal still breathing the $CBrF_3/O_2$ mixture until the glutaraldehyde perfusate entered the aorta. The glutaraldehyde perfusate often triggered several deep respirations of the gas mixture. During movement of the small tank and the dissection, the overall tank concentration of $CBrF_3$ dropped to approximately 50% $CBrF_3$, but the 70% $CBrF_3$/30% oxygen was delivered to a tube at the guinea pig's nose throughout the procedure. The dissection took from 4-7 minutes. The fixation and embedding were performed exactly as for the control animals. Six of the guinea pigs were exposed to 20% $CBrF_3$/30% oxygen. After the onset of stage 3 anesthesia, their hearts were rapidly excised, within one minute. The heart was quickly sliced into 1-2 mm slices, and the slices were fixed by immersion in fixative B (Table 2) and prepared as described for the control hearts that were fixed primarily by immersion.

ELECTRON MICROSCOPY

One micron sections, cut from all tissue blocks, were stained with 1% toluidine blue in 1% sodium borate pH 10.0. Blocks were chosen at random to provide three types of samples: longitudinal, cross, and oblique sections. Thin sections, 400-600 Å, were cut from these blocks using diamond knives and a Porter-Blum MT-2 microtome. The sections were mounted on uncoated grids, stained with uranyl acetate and lead citrate, and examined in a RCA EMU-4 electron microscope or in a JEOL Company JEM-100B high resolution electron microscope. The sections were photographed systematically, as suggested by Weibel (1969), by photographing at 7,000 times magnification, each corner of each grid square. The negatives were printed at 3x magnification on Kodabromide paper. Prints were selected with a random numbers table for further processing. The randomly selected prints were overlayed with a square lattice of lines spaced 1 cm apart. The intersection points of the lines were used as sampling points for point-count planimetry, according to Weibel (1969). The morphometric data was put on punched cards and statistical analysis was performed on a Control Data Corporation 6600 computer.

RESULTS

Gross Appearance and Behavior of the Animals

The animals exposed to 20% $CBrF_3$ -80% oxygen showed no grossly observable differences from control guinea pigs. With 70% $CBrF_3$ and 30% oxygen, the guinea pigs appeared ataxic but in no particular distress. Ear vessels appeared dilated but were not cyanotic. Animals exposed to $CBrF_3$ were more readily anesthetized by intraperitoneal pentobarbital than the control animals. This difference between control and exposed animals was reduced in significance since the heart perfusions or excisions were begun at the onset of stage 3 anesthesia and were rapid procedures.

Electron Microscopy

The best results were obtained by perfusion fixation and only the results on perfused hearts were taken for quantitative morphometric analysis. Results obtained on tissues fixed primarily by immersion were more variable since the depth of fixative penetration and trauma due to razor blade trimming produced variable preservation of cellular architecture. However, some qualitative observations were possible on such tissues.

The Control Myocardium

Heart muscle cells are approximately cylindrical cells that branch and are attached end-to-end at intercalated discs. A fusiform nucleus usually lies near the center of the cell. The contractile system of proteins is arranged in lattices of interdigitating filaments that show prominent cross striations. The muscle filaments, i.e., myofilaments, form a continuous mass throughout the cytoplasm in adult cells. Clefts and lacunae in the myofilament mass contain the other cytoplasmic organelles. Large mitochondria lie within the clefts and are closely applied to the surface of the myofilaments. The plasma membrane has long tubular invaginations that course within the clefts, often near the Z band of the myofilaments. These tubules are called transverse tubules and together are referred to as the T-system. The endoplasmic reticulum of the myocardial cell is called the sarcoplasmic reticulum. It also runs through minute clefts and is closely applied to the surface of the myofilament mass. Other components within the clefts are glycogen granules and ribosomes. Occasional lipid droplets are present near the mitochondria in the larger clefts in the myofilament mass. The detailed arrangement of these structures has been recently reviewed (Fawcett and McNutt, 1969; McNutt and Fawcett, 1973).

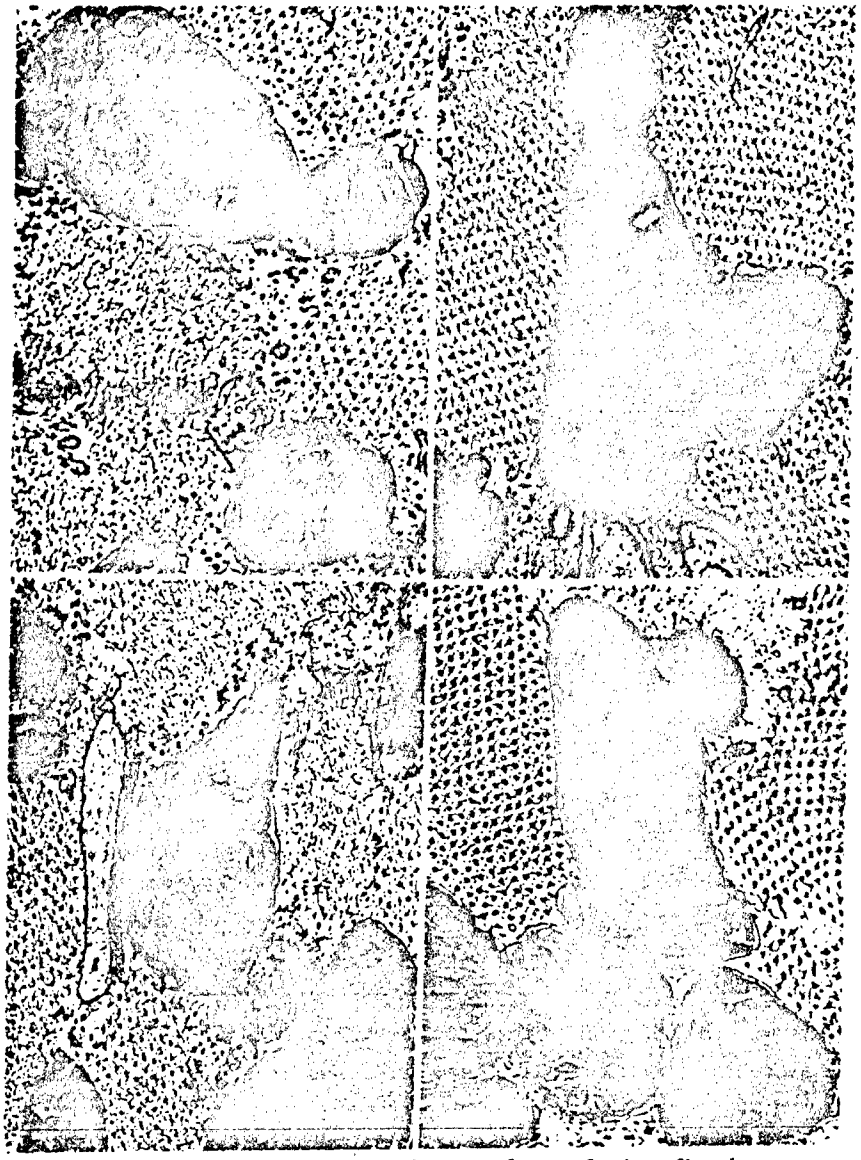
The mitochondria are of particular interest in this study. Heart mitochondria are frequently approximately 0.2-0.5 μ in diameter in 1-4 μ in length. Each mitochondrion has two membranes, the outer limiting membrane and the inner membrane. The inner membrane is folded into thin, flattened sheets called cristae. In many mitochondria the cristae are broad transverse septae that incompletely divide the mitochrondria. In heart muscle cells that are well preserved, many mitochondria have cristae that form a complex labyrinthine network due to angulations, fenestrations, and interconnections between adjacent cristae. This configuration of the cristae has been termed the energized configuration (Harris et al., 1969). Some mitochondria have flattened sheet-like cristae, a configuration called the orthodox configuration. The orthodox configuration is the one usually seen in electron micrographs since many fixatives do not contain substrates for mitochondrial oxidation and do not supply the cells with adequate oxygen. If the fixative solution contains substrate and is applied rapidly, many of the mitochondria are stabilized by glutaraldehyde while still in an energized configuration (Figures 1, 2, 3, 4, 6). Some mitochondria are in an orthodox configuration in these cells and some individual mitochondria have both orthodox and energized cristae.

The inner mitochondrial membrane encloses the inner compartment or matrix compartment of the mitochondrion. The matrix of heart mitochondria is moderately electron opaque and has small dense granules approximately 200-300 Å in diameter that are thought to represent calcium deposits. The compartment between the inner and outer mitochondrial membranes is called the intermembrane space or the outer compartment. The outer compartment is usually electron lucent and includes not only the space between outer and inner membranes of each crista. In the energized configuration, the matrix is contracted and has an increased density and the outer compartment is enlarged compared to their volumes in the orthodox configuration.

The energized configuration of mitochondria is frequently seen in conductive cells of the left and right bundle branches in the control perfused hearts (Figure 6).

The Exposed Myocardium

The hearts of animals exposed to 70% $CBrF_3$/30% oxygen appeared slightly more dilated than control hearts when the perfusion was stopped and the thoracic cavity was opened. Hearts exposed to 20% $CBrF_3$/80% oxygen and fixed by immersion were qualitatively indistinguishable from control hearts.



Figures 1-4. Four regions of control, perfusion-fixed guinea pig heart showing mitochondria at moderately high magnification. The mitochondrial cristae are not flat sheets of inner membrane but rather are complexly folded sheets that occasionally interconnect (at arrows). The folds give the cristae a prominent "zig-zag" appearance often called the energized configuration of heart mitochondria. (Figure 1: 50,000x; Figure 2: 40,000x; Figure 3: 35,000x; Figure 4: 50,000x)

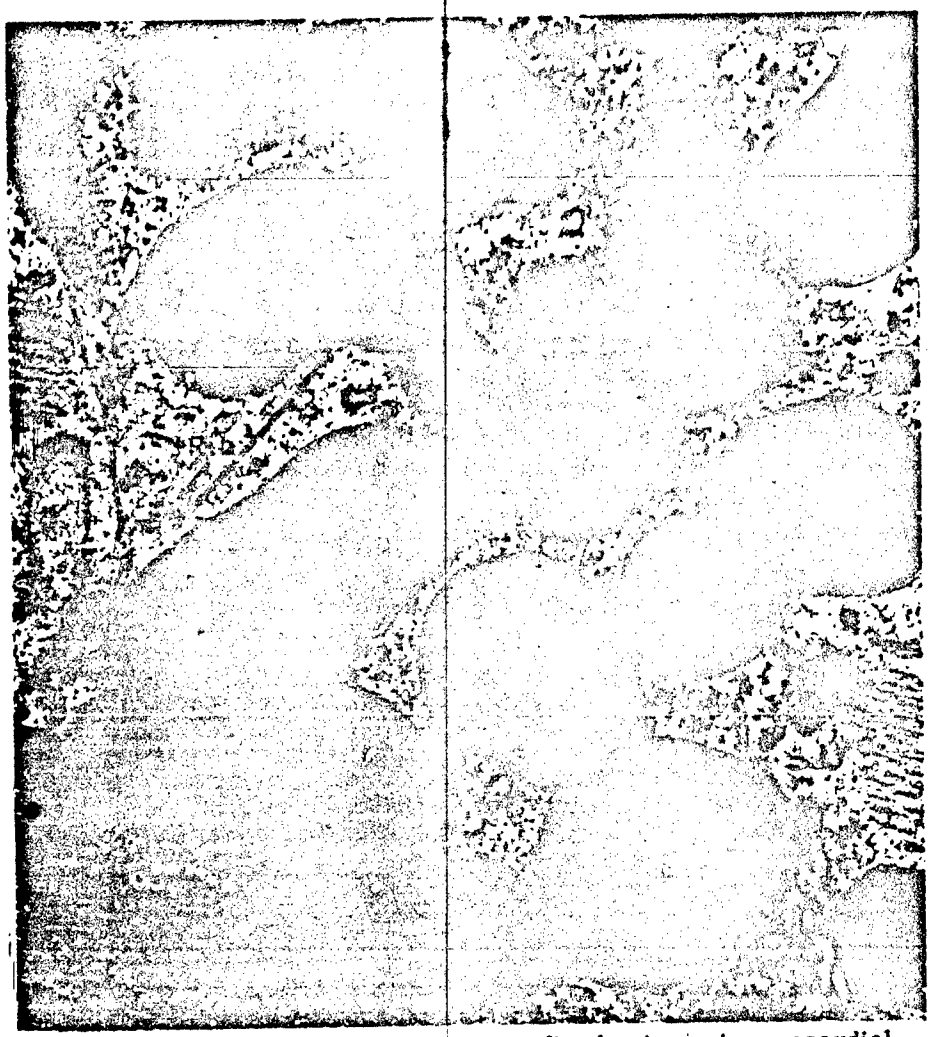


Figure 5. $CBrF_3$ exposed, perfusion-fixed guinea pig myocardial cell cytoplasm at moderately high magnification. This rather typical region shows mitochondria with many cristae that are relatively flat sheets (at arrows). This configuration, often called orthodox, implies some degree of impaired mitochondrial function. The small dark cytoplasmic granules are ribosomes, (Rib); glycogen is depleted. (40,000x)

Figure 6. Control unexposed conductive system, Purkinje cell cytoplasm at moderately high magnification. The mitochondrial diameters are smaller in Purkinje cells than in the ordinary ventricular cells. The cristae are small, rather tubular, and form prominent ring shapes. These complex cristae are similar to the energized cristae in Figures 1-4. (40,000x)

Figure 7. $CBrF_3$ exposed, perfusion-fixed guinea pig Purkinje cell cytoplasm. The mitochondrial cristae are rather flat tubes suggestive of the orthodox configuration, also seen in Figure 5. (48,000x)

Morphometric data indicated that the volume fraction (of the total cellular volume) devoted to myofilament, mitochondria, T-system, and sarcoplasmic reticulum was the same in control hearts and in hearts exposed to 70% $CBrF_3$ (Figure 8). There was a statistically significant decrease in the volume fraction of glycogen and an increase in the volume of lipid droplets ($P<0.01$).

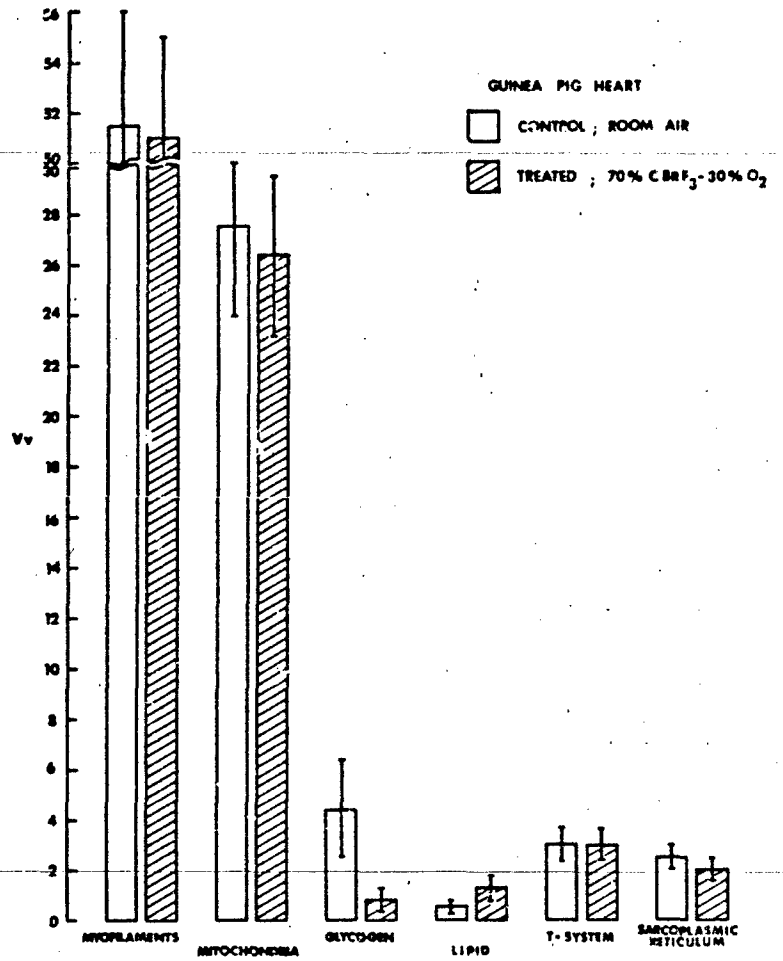


Figure 8. Morphometric data from perfusion-fixed control and $CBrF_3$ exposed hearts. The ordinate is the volume of selected organelles, i.e., that fraction of the total cell volume occupied by an organelle, expressed as a percent of total cell volume. The abscissa has plotted control and treated myofilaments, mitochondria, glycogen, lipid, T-system and sarcoplasmic reticulum. Ranges indicated are ± 1 standard deviation. Statistically significant ($P<0.01$) decreases in glycogen and increases in lipid are present without significant changes in other organellar volumes.

The hearts exposed to 70% $CBrF_3$ had mitochondria predominately with the orthodox configuration (Figure 5), whereas control hearts contained more mitochondria with the energized configuration. This difference has not been quantitated and was not apparent in hearts exposed to 20% $CBrF_3$/80% O_2 and fixed by immersion.

In tissue blocks taken from the high interventricular septum, the conductive cells of the left and right bundle branches were occasionally sampled. The conductive cells of 70% $CBrF_3$ exposed hearts tended to have mitochondria in the orthodox configuration (Figure 7).

DISCUSSION

The principal findings in this study are that, in animals exposed to 70% $CBrF_3$/30% O_2, the hearts were slightly dilated and the myocardial cells had decreased glycogen and increased lipid droplets. The mitochrondria of exposed hearts tended to be in the orthodox configuration.

Since this study was performed on whole animals, a fine dissection of the mechanism of these effects is impossible. However, the data themselves do suggest several possible mechanisms that are of interest even if we are unable to choose between them on the basis of this experiment.

The most interesting possibility is that the fluorocarbons, being lipid-soluble, dissolve in cell membranes, affect their fluidity, and tend to interfere with highly coordinated membrane processes. One such highly coordinated process is oxidative phosphorylation by the inner mitochondrial membrane and its sequence of cytochromes and ATPase components. $CBrF_3$ interference with inner mitochondrial membrane function is suggested by the predominance of the orthodox configuration of the mitochrondria. The orthodox configuration of heart mitochondria can be induced by dinitrophenol, a known uncoupler of oxidative phophorylation (Harris et al., 1969). Low substrate and low oxygen tensions also induce the orthodox configuration of mitochondria (Harris et al., 1969; Tandler and Hoppel, 1972). Interference with mitochrondrial function could explain the glycogen depletion and lipid accumulation observed in the myocardial cells exposed to 70% $CBrF_3$. Sluggish production of ATP by mitochondria would be expected to trigger increased glycogenolysis and glycolytic production of ATP.

An outer mitochrondrial membrane is often closely applied to lipid droplets. The outer membrane has fatty acid - Coenzyme A synthetases which begin fatty acid oxidation to CO_2 and H_2O. Thus it is possible to speculate that interference with outer mitochondrial membrane function could lead to accumulation of lipid droplets. Mitochondrial impairment would have a negative inotropic effect on myocardial contractility via a diminished ATP supply.

There are several trivial explanations for the observations that cannot be rigorously excluded. These explanations may seem trivial in terms of new information, but are not trivial if indeed they represent the mechanism of myocardial changes induced by $CBrF_3$.

One possible explanation could be that $CBrF_3$ induced a profound hypotension, decreased coronary perfusion, and the changes observed in the heart are ischemic alterations. Blood pressures and heart rates were not monitored. Bromotrifluoromethane induces a reversible, concentration dependent drop in mean arterial blood pressure in dogs and monkeys (Van Stee and Back, 1969). The mechanism of this hypotension appears to be related, at least in part, to ganglionic blockage (Van Stee and Back, 1972). This explanation based on hypotension appears unlikely to account for the electron microscopic observations. Examination of the data of Van Stee and Back (1972) would suggest that the degree of hypotension is insufficient to seriously restrict coronary perfusion since the diastolic pressures did not drop below 70 mmHg after exposure to 70% $CBrF_3$ and total ganglionic blockage with hexamethonium only reduced the diastolic pressure to 40 mmHg. Another consideration is that the exposed guinea pigs were ataxic but were alert enough to resist handling and did not appear to be in profound hypotensive shock. In shock, focal myocyte necrosis would be expected and this was not observed in our specimens.

Another possible explanation might be that the $CBrF_3$ is such a heavy gas that the work of respiration becomes enormous and the animals become hypoxic. Arterial blood gasses were not determined in this study. In hypoxic myocardium, glycogen depletion and mitochondrial swelling have been observed. Glycogen depletion was observed in this study but there was no mitochondrial swelling as quantitatively indicated by the morphometric data. This absence of mitochondrial swelling is strong evidence against a purely hypoxic basis for the morphological observations. However, minimal hypoxia cannot be excluded as an explanation without measuring arterial PO_2 and testing the effects of various PO_2 levels on mitochondrial configuration.

Our results are somewhat at odds with the results obtained by Rhoden and Gabriel (1972) on rat heart exposed to 79% $CBrF_3$/21% O_2. They observed a 298% increase in myocardial glycogen after exposure to $CBrF_3$ compared to control values of myocardial glycogen. A totally inexplicable aspect of their data is that they report 1644.8 ± 70.1 mg of glycogen per gram (i.e., 1000 mg) of wet weight of tissue. This is obviously an impossible level of glycogen accumulation. Therefore, these data have to be disregarded until clarified by the authors. The studies of Ross and Cardell (1972) support our observations. These investigators found that halothane caused decreased glycogen in rat liver cells.

The experiment as performed cannot exclude the possibility that exposure to $CBrF_3$ plus the pentobarbital anesthesia cooperated in producing the morphological observations. This possibility has a diminished impact because the hearts of control and exposed guinea pigs were perfused as quickly as possible after the onset of stage 3 anesthesia. The perfusions cannot be performed on unanesthetized animals.

Halothane chemically resembles $CBrF_3$ in that halothane also has one bromine and three fluorine atoms. Halothane has been reported to depress mitochondrial function as measured by decreased rate of oxygen uptake by isolated rat liver mitochondria (Schumer et al., 1971) and rabbit brain mitochondria (Smith, 1973). At high concentrations of halothane, e.g., 4%, some uncoupling of oxidative phosphorylation occurs in isolated rabbit brain mitochondria (Smith, 1973). These studies on halothane-induced changes in mitochondrial function support the working hypothesis that $CBrF_3$ impairs mitochondrial function, thereby causing a negative inotropic effect on the heart.

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PAPER NO. 9

CARDIOVASCULAR EFFECTS OF CHRONIC AND ACUTE FLUOROCARBON 12 EXPOSURES ON RABBITS

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The fluoroalkane gases used as aerosol propellants have been shown to produce cardiac depression and arrhythmias (Harris, 1973). In rabbits, a previously untested species, we have defined acute and chronic inhalation effects of a common aerosol propellant, Fluorocarbon 12, dichlorodifluoromethane.

METHODS

Endotracheal tubes were used in pentobarbital-anesthetized, male rabbits. Saline filled catheters were placed in the jugular vein and femoral artery. The arterial catheter was used both for pressure measurement and blood sampling. A third catheter, equipped with a high fidelity pressure transducer at its tip, was advanced from the carotid artery into the left ventricle (LV). Electronic differentiation of the LV pressure curve yielded its first derivative, LV dP/dt. This is a commonly used empirical index of myocardial contractility (Parmley et al., 1973; Covell et al , 1973). Contractility, as opposed to the hemodynamic or "pump" function of the heart, can be thought of as the mechanical reflection of biochemical and thermodynamic events in muscle cells during contraction.

With the rabbits thus prepared, we were able to record the electrocardiogram (ECG), mean arterial pressure (MAP), left ventricular pressure (LVP), and LV dP/dt. Cardiac output (CO) was measured using the indocyanine dye dilution technique, with dye injection in the jugular vein and sampling from the abdominal aorta (Vollm and Rolett, 1969).

Gas mixtures were administered through a "T" tube placed over the endotracheal tube.

Before acute exposures, control cardiac output was measured in duplicate with the animal breathing 80% oxygen. Then, with continuous physiologic monitoring, Fluorocarbon 12 mixed with oxygen or air was administered. At one minute of the acute exposures, blood for determination of arterial blood gases and Fluorocarbon 12 concentrations was drawn, the indocyanine dye injected and the cardiac output withdrawal pump started. All data except for CO were recorded just before arterial blood sampling, at one minute of exposure. The CO dye dilution curve appeared by 75 seconds of exposure, and the 8-10 ml blood withdrawn was reinfused with the appearance of this curve. The acute exposure was stopped by 1-1/2 minutes.

The inhalation protocol is outlined in Table 1. Five rabbits in Group I breathed first 1%, then 10%, then 20% Fluorocarbon 12 mixed with air. These one-minute exposures were separated by 30-minute rest periods. CO was not measured in this group. Group II breathed 10% and Group III, 20% Fluorocarbon 12 in oxygen for 1-1/2 minutes. Six rabbits in Group IV breathed 10% Fluorocarbon 12 in oxygen for 30 minutes. Four rabbits in either of Groups V or VI received a 6 hour/day exposure to room air (Group V) or 10% Fluorocarbon 12 in room air. This 29 consecutive day exposure was done blind. On the morning following the 29th day, these animals were studied like those in Group III. After determination of baseline cardiac outputs, rabbits in Groups V and VI were exposed acutely to 20% Fluorocarbon 12 in oxygen.

TABLE 1. INHALATION PROTOCOL

Group	N	
I	5	Successive 1 min exposures to 1, 10, 20% Fluorocarbon 12 (F_{12}) in air
II	6	10% F_{12} in oxygen, 1-1/2 min
III	6	20% F_{12} in oxygen, 1-1/2 min
IV	6	10% F_{12} in oxygen, 30 min
V	4	Sham exposure - room air - 6 hrs/day for 29 days
VI	4	10% F_{12} in air - 6 hrs/day for 29 days

RESULTS AND DISCUSSION

The preexposure control values of all the groups studied were statistically similar. None of the acute exposures to Fluorocarbon 12 mixed with oxygen or air resulted in changes in arterial blood gases or pH. Arterial Fluorocarbon 12 concentrations measured at 1 minute of exposure by gas chromatography (Taylor et al., 1971) reached about 0.4 mg/100 ml for each percent inspired Fluorocarbon 12. Of the parameters measured, acute Fluorocarbon 12 inhalations did not alter heart rate or left LV end-diastolic pressure (LVEDP), and caused only small, nonsignificant falls in MAP and LVP. In contrast, LV dP/dt and CO were depressed by the acute inhalation of Fluorocarbon 12 (Figure 1). The depression in dP/dt was a beat by beat phenomenon, progressing throughout the exposure. Cessation of exposure resulted in rapid recovery; control dP/dt levels were generally reached within 30 seconds after exposure.

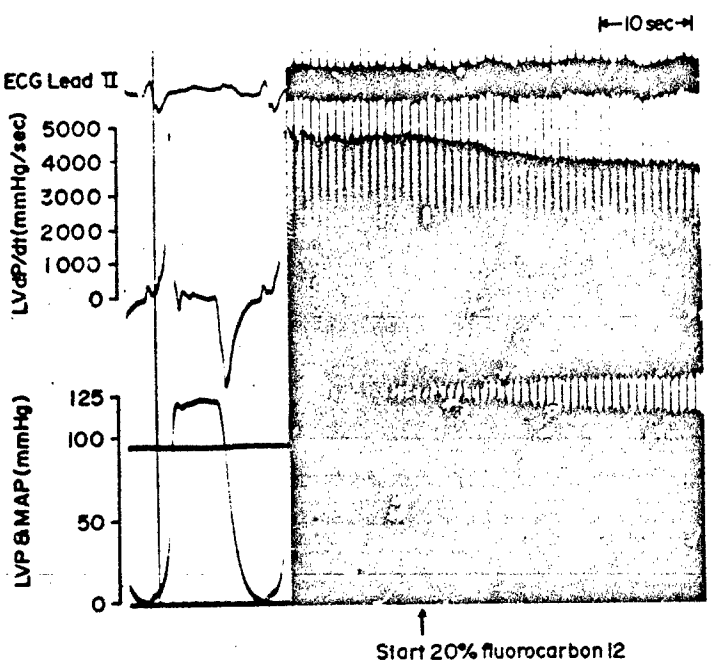

Figure 1. The effect of an acute inhalation of fluorocarbon 12 on left ventricular function.

Successively breathing 1%, 10%, then 20% Fluorocarbon 12 resulted in a dose related fall in dP/dt with a corresponding rise in arterial Fluorocarbon 12 when both dP/dt and Fluorocarbon concentrations were measured at 1 minute of exposure (Figure 2).

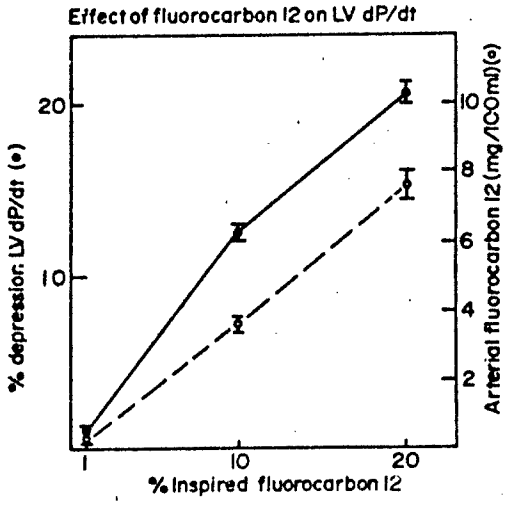

Figure 2. Effect of Fluorocarbon 12 on LV dP/dt and arterial Fluorocarbon concentrations (N = 5).

With repeated inhalations of Fluorocarbon in the same animal, there is a possibility of a residual effect between exposures. We therefore studied separate groups of rabbits inhaling varying concentrations of Fluorocarbon 12 (Groups 2 and 3). The acute inhalation of 10% or 20% Fluorocarbon depressed LV dP/dt 15% and 19%, respectively. As seen in Figure 3, depression of CO was dose related, with 10% and 20% Fluorocarbon inhalation causing a 14% and 20% depression, respectively.

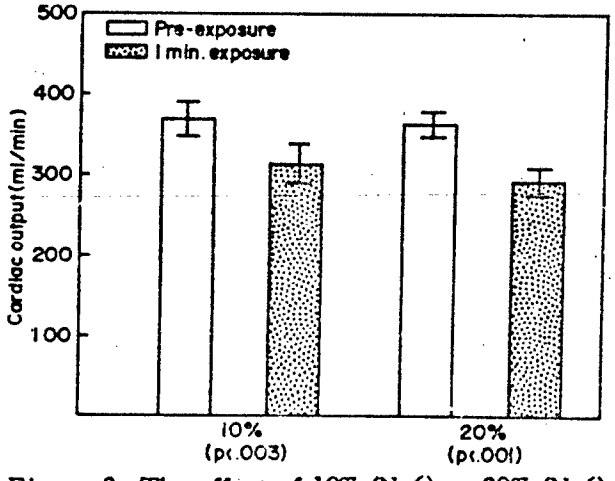

Figure 3. The effect of 10% (N=6) or 20% (N=6) Fluorocarbon 12 inhalation on cardiac output.

To answer the question of whether these changes could be transient during exposure, we exposed 6 rabbits to 10% Fluorocarbon 12 in oxygen for 30 minutes. Figure 4 shows that during this prolonged exposure, LV dP/dt progressively fell and CO, at the end of exposure, was depressed more than when exposures were limited to a minute. Knowing that we were observing effects that lasted at least 30 minutes, we proceeded to a chronic inhalation experiment.

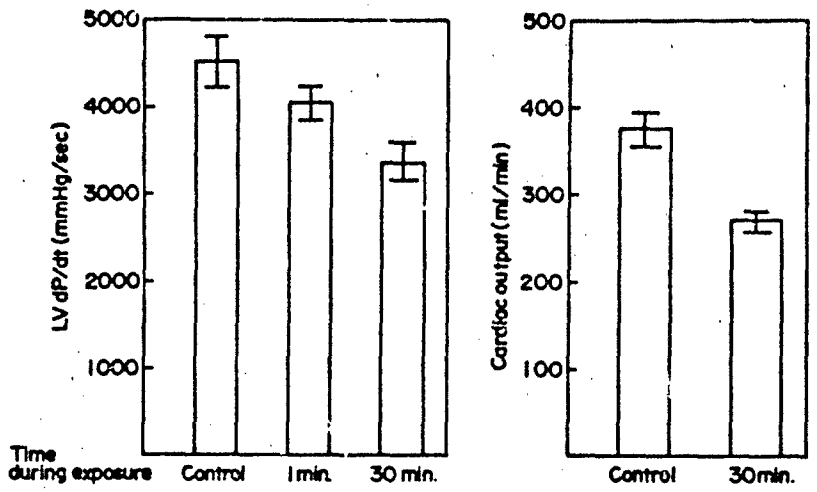

Figure 4. Effect of a 30-minute exposure to 10% Fluorocarbon 12 in oxygen (N = 6).

We first asked whether chronic, intermittent 10% Fluorocarbon 12 challenge caused permanent changes in the parameters measured. Heart rate, MAP, LVP, LVEDP and ECG's were similar in the sham and exposed groups. As seen in Figure 5, LV dP/dt and CO also were similar in the two groups.

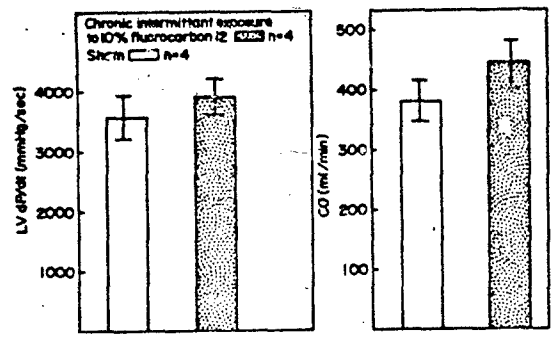

Figure 5. The effect of chronic, intermittent inhalation of Fluorocarbon 12 on resting cardiac output and LV dP/dt.

We next asked whether chronic exposure would alter the animal's response to an acute Fluorocarbon 12 load; is there adaptation? Exposure of the rabbits in Groups 5 and 6 to 20% Fluorocarbon 12 resulted in the acute depression of LV dP/dt and CO. The degree of depression was similar to that seen in Group 3, suggesting that there was no adaptation.

The absence of clear cut chronic effects in this study does not rule out their existence. We looked only for hemodynamic and mechanical changes in normal, intact animals. It is possible that there are changes in myocardial metabolism and physiology which we were not looking for. Recent studies, for example, have shown that ejection indices of LV function might be more sensitive than such isovolumic indices as LV dP/dt, even though the isovolumic indices are useful in monitoring acute interventions (Kreulen et al., 1973).

While care should be exercised in applying animal data to man, these results suggest that those deliberately inhaling propellant gases may experience cardiovascular depression. One must also ask whether people with heart disease might deplete their slim cardiac reserves during the use or misuse of cosmetic, household or medical aerosols.

ACKNOWLEDGEMENT

We gratefully acknowledge the technical assistance of Messrs. F. W. Harrington, M. T. Riley, and E. M. Lores and the advice of Dr. J. R. Fouts.

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DISCUSSION

MAJOR VAN STEE (Aerospace Medical Research Laboratory): Dr. Taylor, when you used the term left ventricular dP/dt, did you mean maximum ventricular dP/dt?

DR. TAYLOR: Yes.

MAJOR VAN STEE: Well, I cannot accept this as an index of myocardial contractility. One must make the distinction between contractility and something less specific that might be referred to as vigor of myocardial contraction. As long as your results indicate a decrease in peak dP/dt accompanied by an elevation in end diastolic pressure, you're probably on firm ground in inferring that a contractility decrease did take place. In some of your latter experiments, you inferred that there was no difference in contractility because of an absence in the difference in peak dP/dt; however, peak dP/dt is sensitive to changes in myocardial wall tension, and these may be affected by end diastolic volume. Through the mechanism of heterotrophic autoregulation, the peak dP/dt may be compensated in those experiments in which you thought you were unable to detect any difference.

DR. TAYLOR: Right, by changing compliance.

MAJOR VAN STEE: Yes, heterometric autoregulation. When selecting an index of myocardial contractility, it is absolutely necessary that one select an index that is independent of end diastolic volume, such as peak dP/dt divided by the developed pressure, or perhaps by using a Brody strain gauge which is not too good on a small heart like you were using. There are other indices that are more accurate and you could have derived these from the transducers that you had implanted in your animals.

DR. TAYLOR: As a matter of fact, we did. The answer to your question comes in several parts. The first is that I was quite surprised when you mentioned in your paper that peak left ventricular dP/dt is not a good index of contractility. At the American College of Cardiology sessions in San Francisco last year, this question was brought up because of the recent papers by Fowler et al. published in Circulation Research which challenged the whole concept of left ventricular V_{max} as a result of changes in wall tension and afterload. Prior to that, Dr. Grossman and his group at Peter Brent Brigham and Sonnenblick's group had questioned these same concepts because of changes in preload. The entire Hill model for the myocardium has been questioned severely. As a result of these questions, a great deal of time was spent discussing these problems at that meeting. Probably the most definitive paper was presented there by Dr. Parmley, and another paper was presented by one of Dr. Langer's groups which discussed and compared a variety of methods of measuring contractility. The

methods discussed peak dP/dt, peak dP/dt divided by developed pressure, dP/dt chosen at a common left ventricular pressure and divided by that pressure, as well as left ventricular V_{max}. They determined that probably the least useful of these was V_{max}. They determined among the other three, in terms of ranking, possibly the most useful was peak dP/dt, the second most useful was dP/dt divided by 40 mm of mercury developed pressure, and finally by the index which you use, peak dP/dt divided by left ventricular pressure developed at that point. The point in these studies was that there really wasn't much difference between the 3 useful indices he described. All I can say is, I just question the statement that peak dP/dt is not useful when it is being commonly employed by researchers in muscle physiology around the country right now, and it is highly regarded by them. That's all I can say about that. As to your other point; this is a gross science and we would be misleading the people in the audience if we gave the impression that any of these numbers were magic, they're all empirical indices. For that reason, it's usually better to rely on more than one, and so with that in mind, I went ahead and divided by left ventricular dP/dt's at a common left ventricular pressure, and I did choose 60 mm Hg and found that these results followed the findings that I described in terms of left ventricular dP/dt depression during acute inhalation exposure to fluorocarbon 12.

DR. BACK (Aerospace Medical Research Laboratory): Did you arbitrarily use 60 mm Hg pressure rather than developed pressure at the point in time at which you measured dP/dt? That doesn't make sense to me.

DR. TAYLOR: No, what I did was I chose 60 mm Hg, measured dP/dt at 60 mm Hg, and divided dP/dt by 60.

MAJOR VAN STEE: Did you read Roger Taylor's paper published in 1970 or 1971?

DR. TAYLOR: That's an old paper, and Taylor's work has really been brought into question.

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PAPER NO. 10

THE EFFECTS OF FREON® 11 AND CERTAIN DRUGS ON ISOLATED AURICLES

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Last year, I reported that 6 fluorinated halocarbon compounds that we had studied, Freons® 11, 12, 116, and C-318 and Halons® 1301 and 2402, had been found to sensitize the myocardium to the arrhythmogenic action of epinephrine. As an extension of that work, we undertook a study of the effect of Freon® 11, one of the most potent sensitizing compounds, on isolated atrial tissue.

We used the auricles of the heart of the rat, which were suspended between the bendable member of a bonded strain-gauge transducer and a fixed point and immersed in a both of Locke's solution. This bath was aerated through a fritted glass disk with a gas mixture containing 95% O_2 and 5% CO_2 pumped by a diaphragm pump. The same pump was arranged to transfer the same mixture of gases containing a small amount of vaporized Freon® 11 from a spirometer through the organ bath. The set-up is represented in Figure 1.

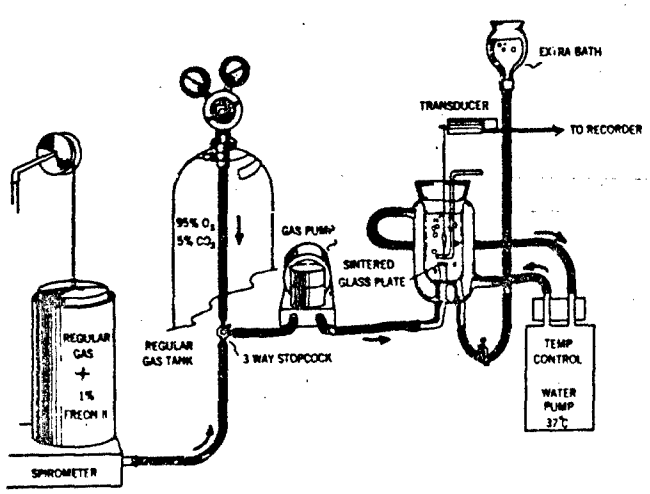

Figure 1.
Diagram of Apparatus

Figure 2 shows the beginning of an exposure of a pair of auricles to the gas mixture containing 1% (v/v) of vaporized Freon® 11. It is obvious that there was no immediate effect After a period of exposure of the auricles to the gas mixture containing Freon® 11, there was a marked decrease in their rate of repetitive contraction (Figure 3). This was followed fairly promptly by complete cessation of contraction (Figure 4).

Upon replacement of the gas mixture containing Freon® 11 by the uncontaminated gas, rhythmic contraction by the auricles was resumed rapidly (Figure 5) and continued for long periods of time thereafter. This recovery of normal rhythmic contraction indicates to us that the exposure to vaporized Freon® 11, even though it was pressed to the point of stopping the spontaneous contractions of the auricles, did not injure the auricular muscles.

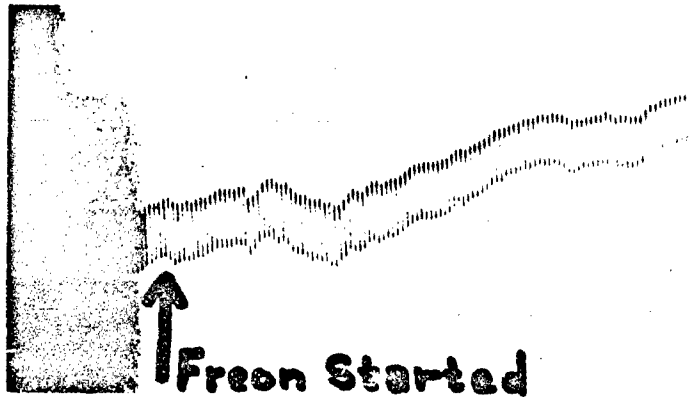

Figure 2. Beginning Exposure of Auricles to Aerating Gas Containing Freon® 11.

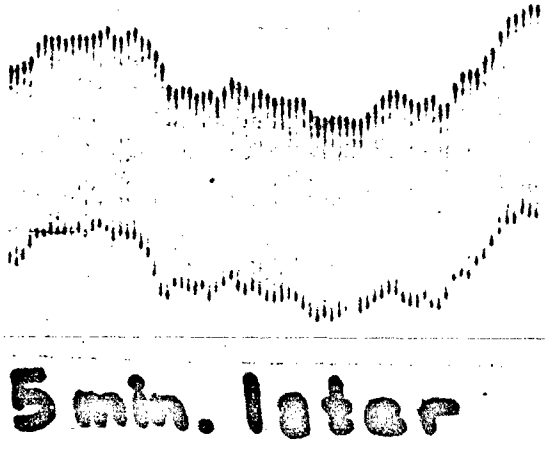

Figure 3. Decrease in Rate of Beating of Auricles Exposed to Vaporized Freon® 11.

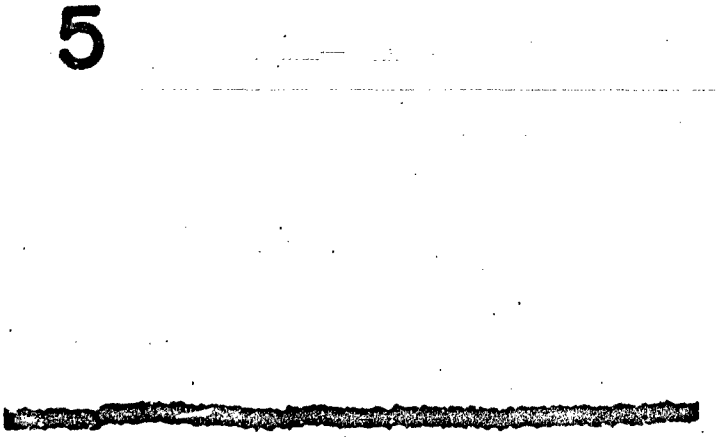

Figure 4. Cessation of Contraction of Auricles Exposed to Vaporized Freon® 11.

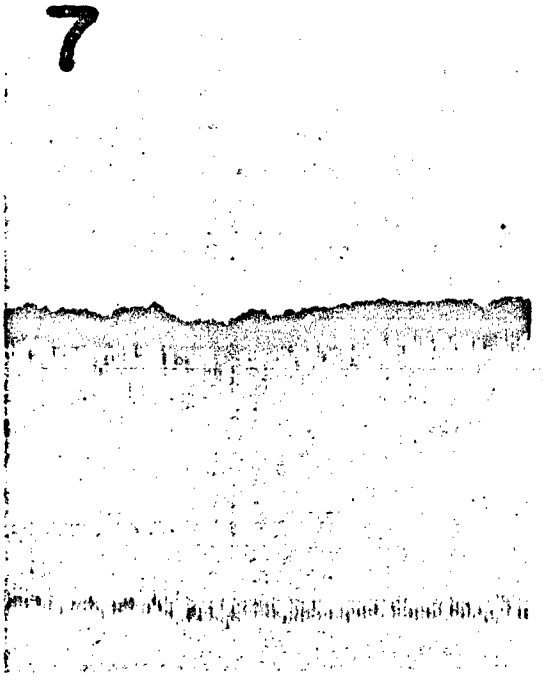

Figure 5. Resumption of Beating by Auricles after Aeration with Gas Containing no Freon® 11.

Last year, we presented evidence that the sensitization of the heart to extrinsic catecholamines by inhalation of vapors of Freon® 11 was not related to an increased concentration of norepinephrine within the myocardium, to enhanced formation of either DOPA or DOPamine, or to altered permeability of the myocardial cells to K. Because of the well-known ability of calcium to link the excitatory and contractile processes in the myocardial muscle (Langer, 1968; Bailey and Dresel, 1968; Little and Sleator, 1969; Sanborn and Langer, 1970; Saari and Johnson, 1971: Shine, Serena, and Langer, 1971), we decided to use the isolated auricles to study the effect of a low external concentration of calcium on the response of the heart to catecholamines as well as on spontaneous activity by the auricles. We found that lowering the concentration of calcium in the Locke's solution to 1/10 its usual value decreased the force of contraction of the auricles with only a slight, immediate effect to decrease their rate of beating, but eventually did stop the contractions; the auricles did not respond to addition of epinephrine to the fluid within the bath either while they continued to beat or after they became quiescent (Figure 6). Addition of $CaCl_2$ to the fluid within the tissue bath restored both the force of contraction of the auricles (Figure 7) and their responsiveness to added catecholamines (Figure 8). The threshold concentration of calcium in the Locke's solution to render the auricles responsive to epinephrine was found to be about 0.225 of the usual concentration (or about 2.7 mg% instead of the standard 12 mg%).

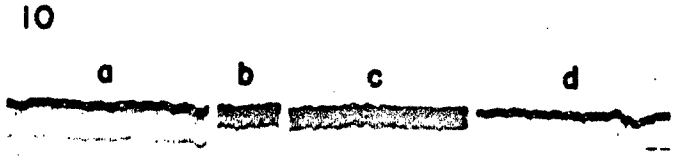

Figure 6. Decrease in force of contraction and eventual cessation of beating of auricles suspended in Locke's solution containing only 1/10 the usual concentration of Ca; lack of responsiveness of these auricles to epinephrine.

a = normal

b = after 0.2 normal Ca^{++}

c = after epinephrine

d = later, second addition of epinephrine

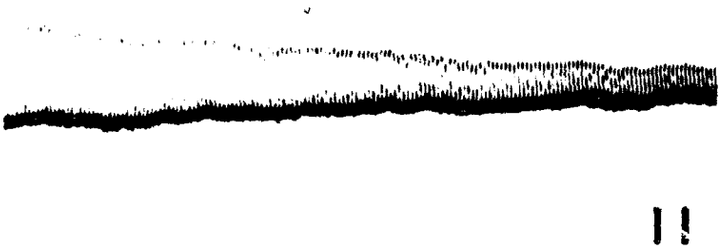

Figure 7. Restoration of contractile force by addition of $CaCl_2$ to the bath.

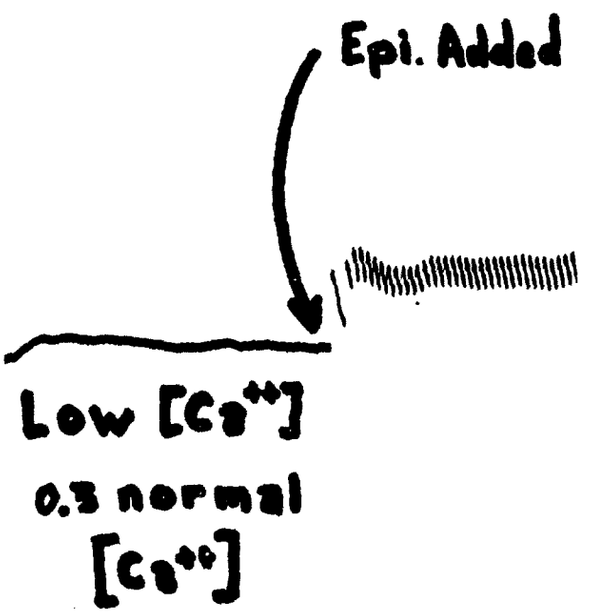

Figure 8. Restoration of responsiveness to epinephrine after addition of $CaCl_2$ to the bath.

Because we were interested in providing to NASA, if possible, guidance on ways for preventing the sensitization of the heart by the fluorinated halocarbon compounds, we thought that Verapamil (or Iproveratril or Isoptin) might be of use. When we applied this drug, which is held by some to act by preventing the migration of calcium through the excited membrane of the muscle fiber to the contractile elements, minimizing thereby the activation of ca-dependent ATPase (Fleckenstein et al., 1967; Fleckenstein, 1970), we found that its addition to the bath in a concentration of 0.48 mg/100 ml had effects quite different from those of lowered external concentration of calcium. Instead of decreasing the force of contraction, as did a low external concentration of calcium, Verapamil increased the force of contraction, but did slow the rate of beating somewhat (Figure 9.) In this figure, the rate had been decreased by only about 7%. With longer exposure to the drug, the rate fell further (to about 33% of its original value), but the force of contraction increased even more (Figure 10).

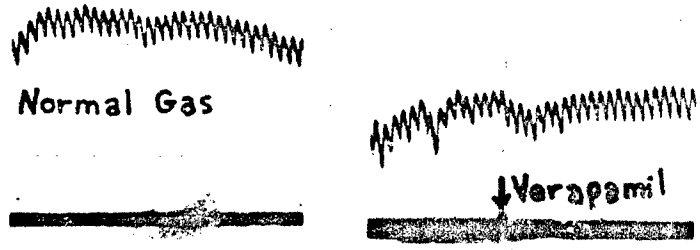

Figure 9. Decrease in rate of beating and increase in force of contraction of auricles by addition of Verapamil to the bath.

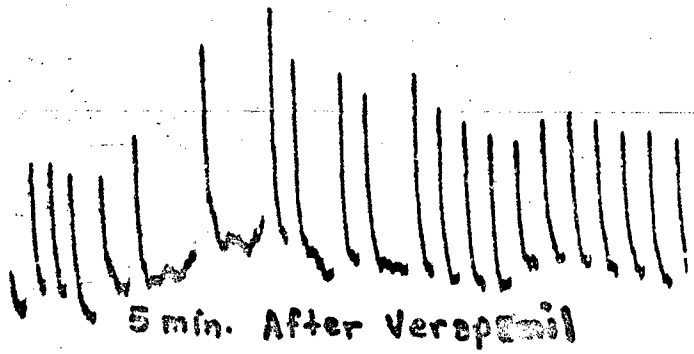

Figure 10. Further slowing and increase in force of contraction of auricles by longer contact with Verapamil.

Another drug that we have studied with our isolation auricle preparation is Na capobenate. This drug had been found to prevent the induction by epinephrine of arrhythmias in anesthetized dogs, cats and guinea pigs that were breathing vapors of halocarbon compounds. This compound, added to the bath after periods of exposure to Freon® and of recovery thereafter, induced an apparent increase in the strength of contraction of the auricular muscle (Figure 11).

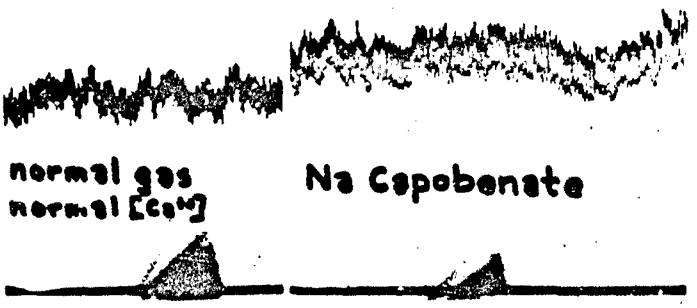

Figure 11. Increase in strength of contraction of auricles by Na capobenate added to the bath after periods of exposure to Freon® 11 and of recovery therefrom.

From this work, we derive the following conclusions:

1. Vaporized Freon® 11 apparently has enough solubility in water to be able to affect the contractile properties of auricular muscle, decreasing the rate of beating after an initial heightening of tension development.

2. Exposure to vaporized Freon® 11, although it may stop spontaneous beating of the auricles, seems not to damage the tissue, so that spontaneous beating will reappear when the Freon® has been washed out of the tissue.

3. A lowered external concentration of calcium and addition of Verapamil to the Locke's solution in which the auricles were suspended had quite different effects on the beating of the auricles, so that we tend to be somewhat skeptical about the theory that Verapamil operates entirely through preventing the ingress of calcium to the cardiac muscle cell.

4. A compound that has β-adrenergic blocking activity and also some α-adrenergic blocking activity, Na capobenate, seems to be able to improve tension development by auricular muscle after a period of exposure to vaporized Freon 11.

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DISCUSSION

DR. STOKINGER (National Institute of Occupational Safety and Health): What's the chemical nature of those drugs? Are they amines or what?

DR. WILLS (Albany Medical College): Sodium capobenate is a fairly long chain fatty acid ester. Verapamil has an amine structure, but I can't give you its constitution in detail now. I have it in my briefcase.

DR. BACK (Aerospace Medical Research Laboratory): I looked it up. It's a dimethoxy compound that would look like a steroid if it were bent around. It has 2 benzene rings on it with 2 methoxy groups on each ring. If you could bend it around, it would look like a veratrum alkaloid but it isn't. It's in the Merck Index. I never heard of the compound before, frankly. I think it's a German drug, but I don't know whether it's ever been marketed. I'm really not familiar with it.

MR. VERNOT (University of California, Irvine): Dr. Wills, was it your intention to use this preparation as an in vitro model of the heart to try to obtain some insight into the arrythmic potential of these freons in the intact animal?

DR. WILLS: That's right. Unfortunately, however, we've not been able to demonstrate sensitization with this preparation.

MR. VERNOT: You didn't show that epinephrine, itself, had any action on the system, did you?

DR. WILLS: Yes, it does but we haven't been able to show that freon increases its action.

AMRL-TR-73-125

PAPER NO. 11

BLOOD LEVELS OF FLUOROCARBON DURING AND AFTER ACUTE INHALATION

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INTRODUCTION

Previous studies at our laboratory (Reinhardt, 1971 and 1972) and others (Clark, 1971) have shown that a certain, critical, inspired concentration of fluorocarbon can sensitize the dog heart to the arrhythmic actions of an epinephrine challenge. In the present study, we measured blood concentrations of trichlorotrifluoroethane (fluorocarbon 113), dichlorotetrafluoroethane (fluorocarbon 114), and chloropentafluoroethane (fluorocarbon 115), respectively, that are associated with these sensitizing inspired levels. For comparison, blood fluorocarbon concentrations at nonsensitizing inspired levels were also determined. In an earlier study by Azar (1973), we reported similar data on trichlorofluoromethane (fluorocarbon 11) and dichlorodifluoromethane (fluorocarbon 12).

METHODS

Indwelling catheters were surgically implanted into the external jugular vein and common carotid artery of beagle dogs and positioned near the major vessels entering and leaving the heart, respectively (Azar, 1973). Following recovery from surgery (> 72 hours), unanesthetized dogs were exposed to known inspired fluorocarbon concentrations and blood samples withdrawn for fluorocarbon analyses both during and after exposure. In contrast to our previous cardiac sensitization studies (Reinhardt, 1971 and 1972), no challenge epinephrine was administered to these test animals.

Fluorocarbon Exposures

Four dogs were exposed via face mask for 10 minutes to 0.1, 0.5, and 1.0% fluorocarbon 113; 0.1, 2.5, and 5.0% fluorocarbon 114; and 10.0, 15.0, and 25.0% (V/V in air) fluorocarbon 115. Reinhardt et al. have

previously reported details on the method of exposure (1971, and on the generation and analysis in air of fluorocarbon 113 (1972) and fluorocarbons 114 and 115 (1971). A minimum of 48 hours was allowed before a dog was used for a subsequent fluorocarbon exposure.

Fluorocarbon Analyses in Blood

Samples of arterial and venous blood were simultaneously withdrawn from catheters (Figure 1) at 0, 1, 3, 5, 7, 10, 12.5, 15, 20, and 25 minutes after the start of each exposure. For fluorocarbons 113 and 114, 2 ml aliquots were extracted with hexane and analyzed by gas chromatography using electron capture detection; for fluorocarbon 115, 4 ml samples were withdrawn and analyzed by a headspace-electron capture detection procedure (Terrill, 1972). The limits of detectability for these analytical procedures were: fluorocarbon 113, 0.015 μg/ml at 0.1% and 0.08 μg/ml at 0.5 and 1.0% exposures; fluorocarbon 114, 0.014 μg/ml at 0.1% and 0.29 μg/ml at 2.5 and 5.0% exposures; and fluorocarbon 115, 0.06 μg/ml. The change in limits at higher concentrations of fluorocarbons 113 and 114 resulted when a microsplitter was inserted in the gas chromatographic system.

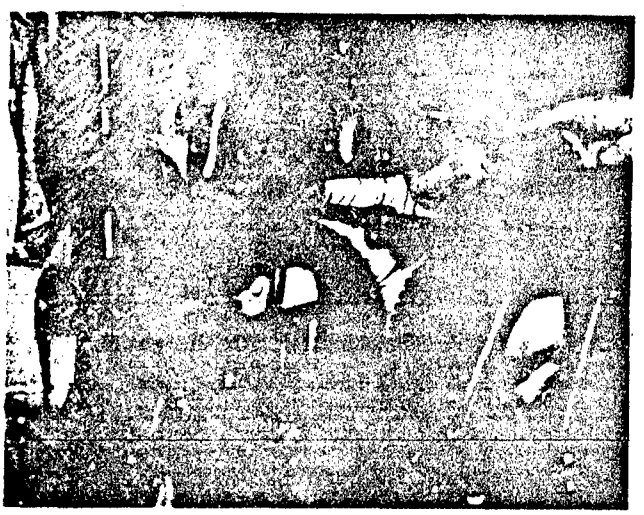

Figure 1. Experimental run showing position of dog with mask in place and the simultaneous withdrawal of arterial and venous blood for fluorocarbon analysis.

The fluorocarbon concentration (V/V in air) during exposure as measured by gas chromatography agreed closely with the nominal concentration as seen in Table 1.

TABLE 1. COMPARISON OF MEASURED AND NOMINAL FLUOROCARBON CONCENTRATIONS FOR 10-MINUTE EXPOSURES (V/V IN AIR)

Fluorocarbon	Nominal (ppm)	Measured (Mean* ppm ± 1 S.D.)
113	1,000	963 ± 87
	5,000	4,969 ± 107
	10,000	10,090 ± 84
114	1,000	1,020 ± 37
	25,000	24,825 ± 299
	50,000	49,525 ± 780
115	100,000	100,383 ± 531
	150,000	149,750 ± 1700
	250,000	251,500 ± 6240

*Mean of four dog exposures/concentration level/compound, except for fluorocarbon 115 at 100,000 ppm which involved 6 exposures.

In Tables 2, 3, and 4, the average concentration ± 1 standard deviation (s.d.) and range of fluorocarbon in blood at the various sampling times are tabulated for fluorocarbons 113, 114, and 115, respectively. The difference in blood concentration between dogs at the same inspired level is probably attributable to differences in rate and depth of breathing in these unanesthetized animals.

TABLE 2. FLUOROCARBON 113 AVERAGE BLOOD CONCENTRATION ± 1 S.D.: μg/ml DURING AND AFTER EXPOSURE

	EXPERIMENTAL MINUTE								
	FLUOROCARBON 113					AIR			
	1	3	5	7	10	12-1/2	15	20	25
0.10 (V/V)									
A	2.6 ± 0.4	2.6 ± 0.1	2.6 ± 0.3	2.8 ± 0.4	2.7 ± 0.1	0.8 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.1
R	1.6 - 2.9	2.4 - 2.7	2.3 - 3.3	2.5 - 3.2	2.6 - 2.8	0.6 - 1.0	0.3 - 0.5	0.2 - 0.3	0.1
V	0.5 ± 0.2	0.9 ± 0.3	1.5 ± 0.1	1.7 ± 0.3	1.9 ± 0.3	1.2 ± 0.2	0.7 ± 0.3	0.4 ± 0.3	0.2
R	0.4 - 0.8	0.6 - 1.2	1.0 - 1.6	1.3 - 2.0	1.6 - 2.2	1.0 - 1.4	0.4 - 1.0	0.1 - 0.7	0.1 - 0.3
0.50 (V/V)									
A	9.8 ± 3.1	12.7 ± 2.4	14.3 ± 4.1	10.3 ± 2.4	11.3 ± 2.4	2.6 ± 1.3	1.4 ± 0.4	1.0 ± 0.4	<0.2
R	7.8 - 12.7	9.8 - 14.2	7.8 - 17.5	8.8 - 13.8	9.8 - 14.8	1.1 - 3.5	1.0 - 2.0	0.8 - 1.5	<0.2
V	1.3 ± 0.2	5.5 ± 3.2	6.9 ± 2.4	7.0 ± 3.3	7.6 ± 3.9	6.7 ± 3.7	3.7 ± 1.1	1.8 ± 0.7	1.0
R	1.1 - 1.6	2.0 - 9.3	2.9 - 8.4	4.5 - 11.7	3.1 - 11.7	4.3 - 11.0	2.3 - 4.9	1.4 - 2.9	1.0
1.00 (V/V)									
A	5.8 ± 2.3	11.4 ± 1.6	18.0 ± 3.7	19.7 ± 3.5	16.8 ± 2.1	6.6 ± 1.7	3.7 ± 0.6	0.8 ± 0.3	0.5 ± 0.2
R	3.1 - 8.6	9.2 - 12.7	15.0 - 23.0	16.0 - 23.0	14.3 - 19.0	5.2 - 8.6	1.7 - 3.1	0.6 - 1.1	0.3 - 0.7
V	1.8 ± 1.4	4.9 ± 1.0	12.1 ± 3.3	18.3 ± 0.1	19.3 ± 4.3	9.3 ± 0.9	7.2 ± 3.6	3.6 ± 0.7	3.3 ± 1.0
R	1.1 - 4.0	4.0 - 5.8	9.2 - 15.0	18.3 - 18.4	15.5 - 24.0	8.6 - 10.5	4.6 - 12.5	2.9 - 4.6	2.3 - 4.6

A = Arterial V = Venous R = Range

TABLE 3. FLUOROCARBON 114 AVERAGE BLOOD CONCENTRATION ± 1 S.D.: μg/ml DURING AND AFTER EXPOSURE

	EXPERIMENTAL MINUTE								
	FLUOROCARBON 114					AIR			
	1	3	5	7	10	12-1/2	15	20	25
0.1% (V/V)									
A	0.3 ± 0.06	0.4 ± 0.02	0.4 ± 0.03	0.4 ± 0.0*	0.4 ± 0.05	0.07 ± 0.02	<0.03	<0.014	N.D.
R	0.2 - 0.3	0.3 - 0.4	0.3 - 0.4	0.3 - 0.5	0.3 - 0.4	0.05 - 0.09	<0.014-0.06	<0.014	N.D.
V	0.2 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.06	0.2	0.1 ± 0.06	<0.07	<0.04	N.D.
R	0.07 - 0.4	0.1 - 0.4	0.1 - 0.4	0.2 - 0.3	0.2	0.014 - 0.14	0.05 -0.09	<0.03-0.07	N.D.
2.5% (V/V)									
A	10.6 ± 1.2	13.9 ± 1.5	13.0 ± 1.6	12.7 ± 1.9	9.9 ± 6.5	<1.9 ± 1.4	<0.7	<0.3	<0.3
R	9.6 - 12.0	11.1 - 14.0	12.6 - 16.0	11.0 - 15.5	4.0 - 14.8	<0.3 - 3.0	<0.3 - 1.1	<0.3 -0.4	<0.3
V	2.5 ± 1.0	4.5 ± 1.3	7.2 ± 2.2	6.5 ± 1.7	8.4 ± 1.2	2.9 ± 2.5	2.0 ± 0.8	0.7 ±0.4	0.5
R	1.8 - 3.6	3.6 - 5.4	6.8 - 9.0	7.2 - 10.8	7.2 - 9.6	0.4 - 3.0	1.2 - 2.7	0.4 -1.2	0.4-0.7
5.0% (V/V)									
A	16.7 ± 1.8	21.5 ± 4.0	23.6 ± 4.0	24.6 ± 6.7	21.8 ± 4.0	4.6 ± 1.1	<0.6	<0.4	<0.5
R	14.4 - 18.3	14.4 - 25.0	18.5 - 28.0	16.2 - 30.0	16.5 - 28.0	3.6 - 5.8	<0.3 - 0.9	<0.3 -0.7	<0.3-<0.7
V	4.0 ± 1.0	8.0 ± 0.8	10.0 ± 0.9	12.4 ± 3.0	12.3 ± 2.7	6.6 ± 1.8	4.3 ± 0.5	2.4 ±0.6	1.6
R	2.7 - 5.0	7.2 - 10.8	9.3 - 11.2	9.0 - 15.2	10.0 - 16.2	5.4 - 9.3	3.6 - 4.7	1.8 -2.9	1.4-1.8

A = Arterial V = Venous R = Range N.D. = Not Determined

TABLE 4. FLUOROCARBON 115 AVERAGE BLOOD CONCENTRATION ± 1 S.D.: μg/ml DURING AND AFTER EXPOSURE

	EXPERIMENTAL MINUTE								
	FLUOROCARBON 115					AIR			
	1	3	5	7	10	12-1/2	15	20	25
10.0% (V/V)									
A	2.4 ± 0.4	2.6 ± 0.6	2.8 ± 0.3	2.6 ± 0.5	2.9 ± 0.6	<0.2 ± 0.1	<0.06 ± 0.01	<0.06	N.D.
R	1.8 - 2.9	1.8 - 3.2	2.3 - 3.8	1.9 - 3.0	2.0 - 3.7	<0.06 - 0.4	<0.06 - 0.08	<0.06	N.D.
V	0.9 ± 0.2	1.6 ± 0.4	1.9 ± 0.7	1.9 ± 0.5	2.3 ± 0.5	1.2 ± 0.2	0.8 ± 0.3	0.4 ± 0.1	N.D.
R	0.4 - 1.2	0.9 - 2.0	1.1 - 3.0	1.2 - 2.5	1.4 - 2.7	0.8 - 1.5	0.5 - 1.3	0.3 - 0.6	N.D.
15.0% (V/V)									
A	5.7 ± 1.2	5.8 ± 2.5	5.8 ± 2.3	5.6 ± 2.8	5.9 ± 1.9	1.1 ± 1.0	<0.06	N.D.	N.D.
R	4.8 - 7.3	3.9 - 9.5	4.2 - 9.0	3.2 - 9.6	4.4 - 8.8	0.5 - 2.2	<0.06	N.D.	N.D.
V	1.6 ± 0.6	2.8 ± 1.0	3.9 ± 1.0	3.6 ± 1.2	3.7 ± 1.1	2.6 ± 0.9	1.5 ± 0.3	1.2 ± 0.7	0.7
R	1.0 - 2.2	2.1 - 3.9	2.8 - 4.7	2.4 - 4.9	2.7 - 4.7	1.6 - 3.8	1.1 - 1.8	0.8 - 2.2	0.7
25.0% (V/V)									
A	7.5 ± 0.7	9.4 ± 0.7	11.4 ± 1.8	8.1 ± 0.9	8.7 ± 2.6	<0.1 ± 0.08	<0.06	N.D.	N.D.
R	6.8 - 8.2	8.5 - 10.2	9.7 - 14.0	7.5 - 9.4	5.0 - 11.8	<0.06 - 0.2	<0.06	N.D.	N.D.
V	2.7 ± 2.5	4.3 ± 1.0	5.9 ± 0.6	4.7 ± 0.5	5.2 ± 1.2	2.0 ± 1.3	1.3 ± 1.0	0.5 ± 0.2	N.D.
R	1.4 - 6.4	3.8 - 5.1	5.3 - 6.4	4.3 - 5.8	3.8 - 6.1	1.0 - 3.5	0.5 - 2.6	0.3 - 1.8	N.D.

A = Arterial V = Venous R = Range N.D. = Not Determined

Blood fluorocarbon concentration increases rapidly during the first five minutes and then more slowly or not at all for the remaining minutes of exposure (Figures 2, 3, and 4). At the end of the 10-minute exposure, there is a sharp decrease during the first few minutes followed by a slower decline thereafter.

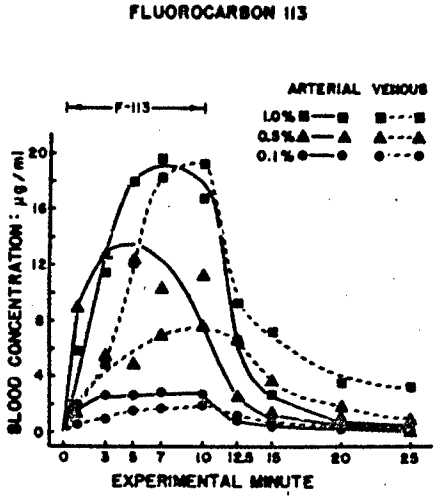

Figure 2. Fluorocarbon 113 concentration in arterial and venous blood of beagle dogs during and after 10-minute exposures to three inspired levels (4 dogs/level).

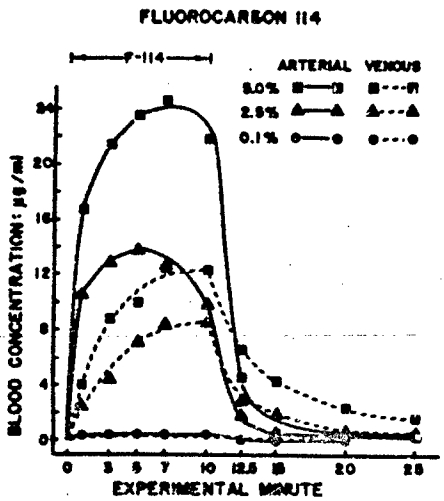

Figure 3. Fluorocarbon 114 concentration in arterial and venous blood of beagle dogs during and after 10-minute exposures to three inspired levels (4 dogs/level).

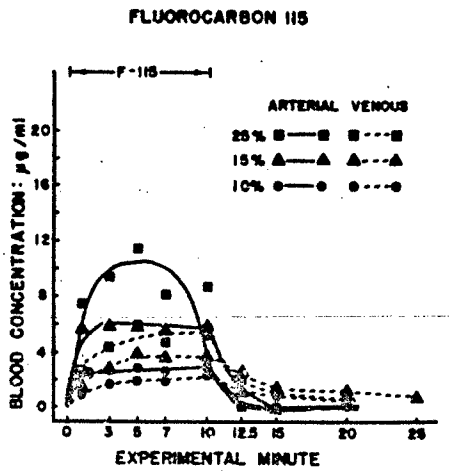

Figure 4. Fluorocarbon 115 concentration in arterial and venous blood of beagle dogs during and after 10-minute exposures to three inspired levels (4 dogs/level at 15.0 and 25.0%, 6 dogs at 10.0%).

When blood fluorocarbon concentrations are plotted in terms of arterial-venous difference (Figures 5, 6, and 7), it is apparent that arterial concentration is generally higher than that of venous blood during exposure and that the trend is reversed when the exposure is terminated. This finding, which has been previously reported for fluorocarbons 11 and 12 (Azar, 1973) and for many anesthetic agents (Chenoweth, 1962), suggests a tissue uptake of fluorocarbon during exposure followed by a release into the venous blood after the exposure is terminated.

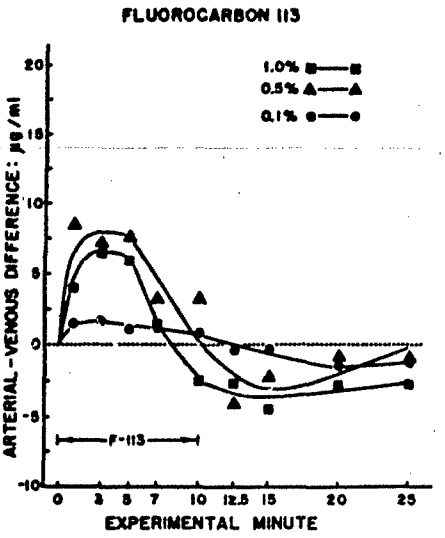

Figure 5. Difference between arterial and venous fluorocarbon 113 concentration in beagle dogs during and after 10-minute exposures to three inspired levels (4 dogs/level).

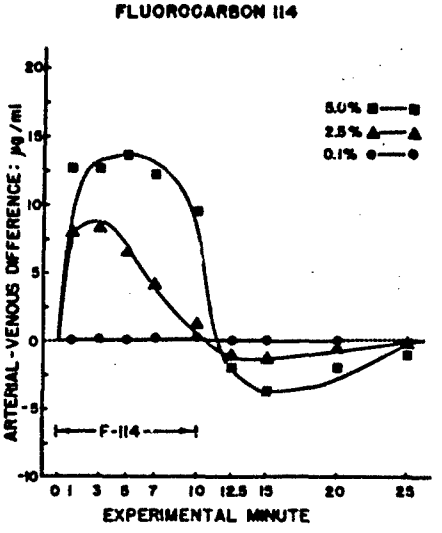

Figure 6. Difference between arterial and venous fluorocarbon 114 concentration in beagle dogs during and after 10-minute exposures to three inspired levels (4 dogs/level).

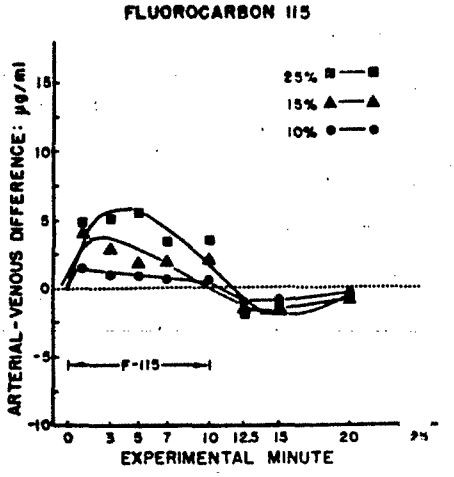

Figure 7. Difference between arterial and venous fluorocarbon 115 concentration in beagle dogs during and after 10-minute exposures to three inspired levels (4 dogs/level at 15.0 and 25.0%, 6 dogs at 10.0%).

In our previous studies (Reinhardt, 1971 and 1972), cardiac sensitization was determined by injecting an intravenous challenge dose of epinephrine (0.008 mg/kg) after the dog had been inhaling the compound under study for five minutes. Table 5 shows arterial and venous fluorocarbon concentrations at the five-minute sampling time for various inspired levels of fluorocarbons 113, 114, and 115; previous cardiac sensitization test results are also given. Although a wide range of inspired concentration - 0.5% fluorocarbon 113, 2.5% fluorocarbon 114, and 15.0% fluorocarbon 115 - is required to produce cardiac sensitization, the blood concentrations associated with these sensitizing levels are similar for fluorocarbons 113 and 114, 12.5 and 13.8 μg/ml, and slightly lower for fluorocarbon 115, 5.8 μg/ml. However, previous test results in Table 6 show higher sensitizing blood levels in the 25 to 35 μg/ml range for fluorocarbon 11 (0.5%) and fluorocarbon 12 (5.0%).

TABLE 5. BLOOD CONCENTRATIONS OF FLUOROCARBONS 113, 114, AND 115 ASSOCIATED WITH CARDIAC SENSITIZATION IN DOGS

Fluorocarbon	Formula	Exposure Concentration (vol. %)	Number of Dogs Sensitized*	Blood Concentration** μg/ml Arterial	Venous
113	CCl_2F-$CClF_2$	0.1%	N.D.	2.6	1.5
		0.25%	0/12	N.D.	N.D.
		0.5%	10/29	12.5	4.9
		1.0%	3/4	18.0	12.1
114	$CClF_2$-$CClF_2$	0.1%	N.D.	0.4	0.2
		2.5%	1/12	13.8	7.2
		5.0%	7/12	23.6	10.0
115	$CClF_2$-CF_3	10.0%	N.D.	2.8	1.9
		15.0%	1/13	5.8	3.9
		25.0%	4/12	11.4	5.9

* For fluorocarbons 114 and 115 data from Reinhardt et al., Arch. Environ. Health 22, Feb. 1971; for fluorocarbon 113 data from Reinhardt et al., presented at the Society of Toxicology Annual Meeting, Williamsburg, Va., March 1972.

** Mean concentration at five minutes during a 10-minute exposure.

N.D. = Not determined.

TABLE 6. BLOOD CONCENTRATION OF FLUOROCARBON 11 AND 12 ASSOCIATED WITH CARDIAC SENSITIZATION IN DOGS*

Fluorocarbon	Formula	Exposure Concentration (vol. %)	Number of Dogs Sensitized	Blood Concentation** µg/ml Arterial	Venous
11	CCl_3F	0.1%	0/12	10.9	6.6
		0.5%	1/12	28.6	19.7
		1.0%	5/12	53.2	37.2
12	CCl_2F_2	0.1%	N.D.	1.0	0.9
		2.5%	0/12	N.D.	N.D.
		5.0%	5/12	35.3	22.8
		10.0%	N.D.	46.3	39.8

* From Azar et al., presented at the A.I.H.A. Conference, San Francisco, Calif., May 1972

** Mean blood concentration at 5 minutes.

N.D. = Not determined.

DISCUSSION

Previous investigators (Reinhardt, 1971 and 1972; Clark, 1971) have shown that a critical inspired concentration of fluorocarbon can sensitize the dog heart to the arrhythmic actions of epinephrine. However, sensitization is only a temporary effect since an epinephrine injection given 10 minutes after exposure to a known sensitizing concentration never resulted in arrhythmias (Clark, 1971). Results from this study and others (Clark, 1971; Reinhardt, 1971 and 1972; Shargel, 1972) have also shown that fluorocarbons are rapidly eliminated from the bloodstream on cessation of exposure. Thus, cardiac sensitization probably requires a threshold level of both fluorocarbon and epinephrine in blood (or tissue) in order for an arrhythmia to occur.

Our blood level data on fluorocarbons 11 and 12 (Azar, 1973), and on fluorocarbon 114 are comparable to those reported by Clark and Tinston (1971). Blood concentrations of fluorocarbons 113 and 115 associated with cardiac sensitization have not previously been reported.

For fluorocarbons 11 and 12, we (Azar, 1973) reported an arterial-venous difference during and after exposure. Similar results were found for fluorocarbons 113, 114, and 115. This suggests that these fluorocarbons are taken up by certain body tissues, primarily by fat and adrenals (Dollery, 1972; Jack, unpublished), and released after exposure. Although relatively small

concentrations were found in whole sections of heart (Dollery, 1972; Jack, unpublished; Van Stee, 1971), the site where a critical fluorocarbon concentration is needed to sensitize the heart may be at the nerve cell level.

For the C_2 fluorocarbons, the inspired concentrations required to sensitize the dog heart to exogenous epinephrine cover a wide range, for example, 0.5%, 2.5%, and 15.0% of fluorocarbons 113, 114, and 115, respectively (Table 5). However, corresponding arterial blood levels after five minutes exposure are similar for fluorocarbon 113 (12.5 μg/ml) and fluorocarbon 114 (13.8 μg/ml), but slightly lower for fluorocarbon 115 (5.8 μg/ml). The overall lower blood levels for fluorocarbon 115, as seen in Table 4, may reflect its relatively high volatility and insolubility, properties that necessitated a headspace analytical technique rather than hexane extraction. In contrast, arterial blood concentrations of fluorocarbons 11 and 12 associated with sensitization were in the 25 to 35 μg/ml range (Table 6). In Table 7, some of the physical and chemical properties of our test fluorocarbons are tabulated. There are no apparent differences in physical properties to account for our finding that C_2 fluorocarbons can sensitize the beagle heart at lower blood concentrations than C_1 fluorocarbons.

TABLE 7. SOME PHYSICAL AND CHEMICAL PROPERTIES OF TEST FLUOROCARBONS*

Fluorocarbon	Structure	Mol. Wt. (g)	Density, Liquid at 25°C (g/cc)	B.P. at 1 atm. (°C)	Vapor Pressure at 25°C (atm.)	Sol. in Water at 1 atm. and 25°C (Wt.%)
11	CCl_3F	137.4	1.476	23.8	1.05	0.11
12	CCl_2F_2	120.9	1.311	-29.8	6.46	0.028
113	CCl_2F-$CClF_2$	187.4	1.565	47.6	0.44	0.017 (Sat'n. Pres.)
114	$CClF_2$-$CClF_2$	170.9	1.456	3.8	2.14	0.013
115	$CClF_2$-CF_3	154.5	1.291	-39.1	8.84	0.006

* Freon® Products Information B-2. "Freon" Fluorocarbons. Properties and Applications. E. I. du Pont de Nemours & Co., Organic Chemicals Department, "Freon" Products Division, Wilmington, Delaware. 1969.

For comparison with sensitizing inspired levels, dogs were exposed to fluorocarbons 113 and 114 at 0.1% (1000 ppm), the threshold limit value (American Conference of Governmental Industrial Hygienists, 1972) for these compounds, and to 10.0% fluorocarbon 115. These inspired concentrations do not produce cardiac sensitization (Table 5). Arterial blood levels are several orders of magnitude lower - 2.6 μg/ml (fluorocarbon 113), 0.4 μg/ml (fluorocarbon 114), and 2.8 μg/ml (fluorocarbon 115) - than concentrations associated with cardiac sensitization. Although fluorocarbon 115 was not tested at 0.1% (1000 ppm), results shown in Figure 3 suggest that arterial levels after five minutes exposure would be well below 1 μg/ml.

This type of information on fluorocarbon blood levels may be compared to levels obtained in man during normal use of products containing these fluorocarbons, or to industrial exposure levels, when such data become available.

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DISCUSSION

DR. AVIADO (University of Pennsylvania): Since you were m suring arterial blood levels of fluorocarbons, I wondered if you had measured venous blood levels which would be more related to the effect on the organ? Could you compare the venous blood concentrations of these various propellants, the sensitizing dose please, rather than the arterial blood?

DR. TROCHIMOWICZ (E. I. duPont de Nemours and Company): Venous blood levels for fluorocarbon 113 at 0.5% concentration were 4.9 μg/ml and above. For fluorocarbon 114 at 2.5% concentration, it was 7.2 μg/ml; and for fluorocarbon 115 at a 15% concentration, it was 3.9 μg/ml. Freon 11 at a 0.5% concentration produced a venous level of 19.7 μg/ml; for fluorocarbon 12 at 5%, the venous level was 22.8 μg/ml.

DR. AVIADO: So there's a closer agreement then?

DR. TROCHIMOWICZ: I can't calculate the ratios in my head, but certainly the fluorocarbon 11 and 12 levels in venous blood were somewhat higher than those found in fluorocarbon 113, 114 and 115 exposures.

DR. TAYLOR (National Institute for Environmental Health Sciences): I have a question for Dr. Aviado. Why is the venous level so much more important than the arterial level in determining the toxicity?

DR. AVIADO: I think this is just a general phenomenon in anesthesia. With halothane, the blood levels in the effluent blood leaving the organ correlate more with effect on the heart or on the brain.

DR. HARRIS (National Institute of Occupational Safety and Health): In view of the observations you made there on fluorocarbon 113, 114 and 115, would you care to speculate on what you expect to find with fluorocarbon 116 which is a totally fluorinated hydrocarbon?

DR. TROCHIMOWICZ: In an unpublished study conducted in our laboratory, I tested a 20% concentration of fluorocarbon 116 in air using a standard cardiac sensitizatic.. test and, of course, no added oxygen. I exposed 12 dogs and gave a challenge dose of epinephrine but found nothing. I found no serious arrythmia occurring, no cardiac sensitization. The blood levels of fluorocarbon were not measured, but I would assume that they would be even lower than those for fluorocarbon 115.

DR. FIELDING (Naval Research Laboratory): Do you have any data on the half-time or 90% time for equilibration with the gas?

DR. TROCHIMOWICZ: I don't have any specific data on that although the next speaker does have some long-term inhalation exposure data, 6-hour exposure to fluorocarbon 115, and possibly this information may be extrapolated from that data.

DR. FIELDING: These would be the 90% time taken to achieve maximal effect, I suppose.

DR. TROCHIMOWICZ: I don't want to give his paper.

DR. GEHRING (Dow Chemical Company): You said that the animals exposed to one of these levels, to 0.5% concentrations, become refractory to sensitization by epinephrine. If you increase that level of exposure from 0.5% to 1% or 2%, would that increase render them sensitive again or would they stay refractory to the higher levels?

DR. TROCHIMOWICZ: At 0.5% fluorocarbon 113, something like 10 of 29 dogs were sensitized. When we increased the fluorocarbon exposure concentrations using a different group, more dogs showed this effect. Now, I have to tell you that most of our dogs do survive our tests and they are given a minimum of about 3 days rest after which they may be used again on a subsequent experiment or with another compound, for that matter. We've never noted any increased sensitivity in a dog used repeatedly. Is that what you were referring to?

DR. GEHRING: That isn't exactly what I meant. You said that within a few minutes after exposure to a concentration of 0.5% the individuals become refractory. I was wondering if you increased the concentration to 1%, would those same dogs again become sensitive or do they stay refractory? What I'm trying to understand is the effect of physical chemical adaptation. Or is this process of sensitization a change of equilibrium?

DR. TROCHIMOWICZ: We never exposed an animal immediately after recovery from one experiment to a higher concentration. In general, we test the same dogs at a higher concentration 3 or 4 days later.

DR. AZAR (Ohio State University): The point, I think, that Dr. Trochimowicz was making was not that the animal is refractory. I think that's a poor choice of words. The point is that there is apparently a critical concentration of fluorocarbon and epinephrine that must be present simultaneously for sensitization to occur. And if you take the animal off the exposure for 10 minutes, you will see a rapid fall in fluorocarbon concentration in the blood. If you then give an injection of epinephrine, you won't get sensitization effects. I think it's more dependent on the blood level. I know that Dave Clark at ICI has done what you suggested. You do have to go back up to a higher blood concentration of fluorocarbon before you get sensitization. It shouldn't be inferred that the animal becomes refractory.

AMRL-TR-73-125

PAPER NO. 12

ARTERIAL/VENOUS BLOOD LEVELS OF CHLOROPENTAFLUOROETHANE: INHALATION VERSUS ORAL EXPOSURES

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INTRODUCTION

Chloropentafluoroethane (fluorocarbon 115) is used as a propellant in pressure packaged food preparations. This fluorocarbon has a low order of acute toxicity; the LC_{50} is >80% (v/v, rats) for a four-hour exposure (Clayton et al., 1966). It also has a low order of cumulative toxicity based on a ninety-exposure (six hours each) inhalation study at the 10% (v/v) level (Clayton et al., 1966). Another study in which anesthetized dogs were exposed to a 10% (v/v) fluorocarbon 115 atmosphere reported that aortic blood pressure and heart rate were within the normal range of the controls (Belej and Aviado, 1972).

When the 90-exposure inhalation study with fluorocarbon 115 was carried out, the anticipated human exposure was by inhalation. The primary fluorocarbon 115 exposure is now ingestion rather than inhalation. If inhalation of fluorocarbon 115 places at least as great a toxicological stress on test animals as ingestion, the results of the 90-day inhalation study should be applicable for evaluating stress by ingestion. For this compound, the important toxicological parameter is the amount of compound reaching target organs which is related to blood levels.

The purpose of this investigation was to relate the previous 10% inhalation exposures with an ingestion exposure using the fluorocarbon 115 blood concentration as the correlation parameter. We can then predict the blood fluorocarbon concentration for ingestion of any given fluorocarbon concentration. Also, we can evaluate the safety of periodically eating a given amount of fluorocarbon by correlation of these results and the 90-inhalation exposure.

Previous studies in our laboratory have shown that measurable blood levels of trichlorofluoromethane and dichlorodifluoromethane can be achieved both by inhalation (Azar et al., 1972) and by ingestion (dichlorodifluoromethane) (Terrill, 1972). In the inhalation exposure, venous and arterial equilibrium is achieved in 10 minutes. Therefore, we decided to limit most exposures to 10 minutes. A single 6-hour exposure was carried out to determine whether the kinetics changed appreciably in 6 hours versus 10 minutes.

The following experiments using beagle dogs were planned on the basis of the prior 90-day inhalation work and the previous trichlorofluoromethane and dichlorodifluoromethane blood level studies:

1. A 10-minute inhalation exposure to 10% fluorocarbon 115.
2. Feeding (by intubation) a 6-ounce aerosol can of 8% fluorocarbon 115 in whipped topping.
3. A 6-hour inhalation exposure to 10% fluorocarbon 115.

In each of these experiments, the arterial and venous fluorocarbon blood levels were measured.

EXPERIMENTAL

Male beagle dogs weighing 7 to 11 kg were used in this study. Catheters were surgically implanted in the right carotid artery and right jugular vein. The interior end of these tubes was located near the major vessels of the heart. The tubes traveled subcutaneously from the vessels and were exteriorized at the back of the neck. Eighteen-gauge wire (1/2" long) was used to plug the external ends. The vessels were kept operable by flushing 3 times a week with normal saline followed by 4% sodium heparin in saline. Some animals were maintained for 2 months in this condition. Temperatures and dietary intake were checked daily. Any animal that had a temperature above 103°F or that did not eat was not exposed until food consumption and temperature had returned to normal.

A valved face mask was used for 10-minute exposures (Table 1). The construction of this mask, the system for delivery of a steady state fluorocarbon concentration, and the analytical system for monitoring the exposure concentration have all been previously reported (Reinhardt et al., 1971).

TABLE 1. ANALYSIS OF FLUOROCARBON 115 IN ATMOSPHERE USED FOR 10-MINUTE EXPOSURE

Dog	Time-Weighted Average (%)	Standard Deviation (%)
A	10.1	± 0.30
B	10.0	± 0.20
C	10.1	± 0.20
D	10.0	± 0.05
E	9.9	± 0.17
F	10.1	± 0.30

A 4' x 7/16" (O. D.) x 5/16 (I. D.) Tygon® tube was used for intragastric intubation of the topping. For dosing, the tube was connected directly to the aerosol can by a Fitzall ®* valve. Following insertion of the intragastric tube in the dog, the valve was opened and left open until no more topping would flow through the intubation tube. It was calculated by weight difference that about 150 grams of the can contents and 12 grams of fluorocarbon 115 were given each dog.

Fluorocarbon 115 was monitored in the arterial and venous blood for 24 hours. The sampling times were weighted to favor the first several hours. Also, fluorocarbon 115 was monitored in the exhaled air using a special two-valve face mask. (A picture is shown as Figure 1.) Samples were taken from the section of ~1" diameter hose via a 5 ml gas tight syringe. Fluorocarbon concentrations were determined by a calibrated Varian 600 D gas chromatograph which was equipped with a 1 mv (full scale) Beckman recorder and a flame ionization detector. The column was 5' x 1/8" (O. D.) S. S. 5% SE-30 on Chromosorb® W 60/80 and was operated at 100 C using nitrogen carrier gas at a flow rate of 60 ml/minute.

*Fitzall® is a registered trademark of Virginia Chemical Company.

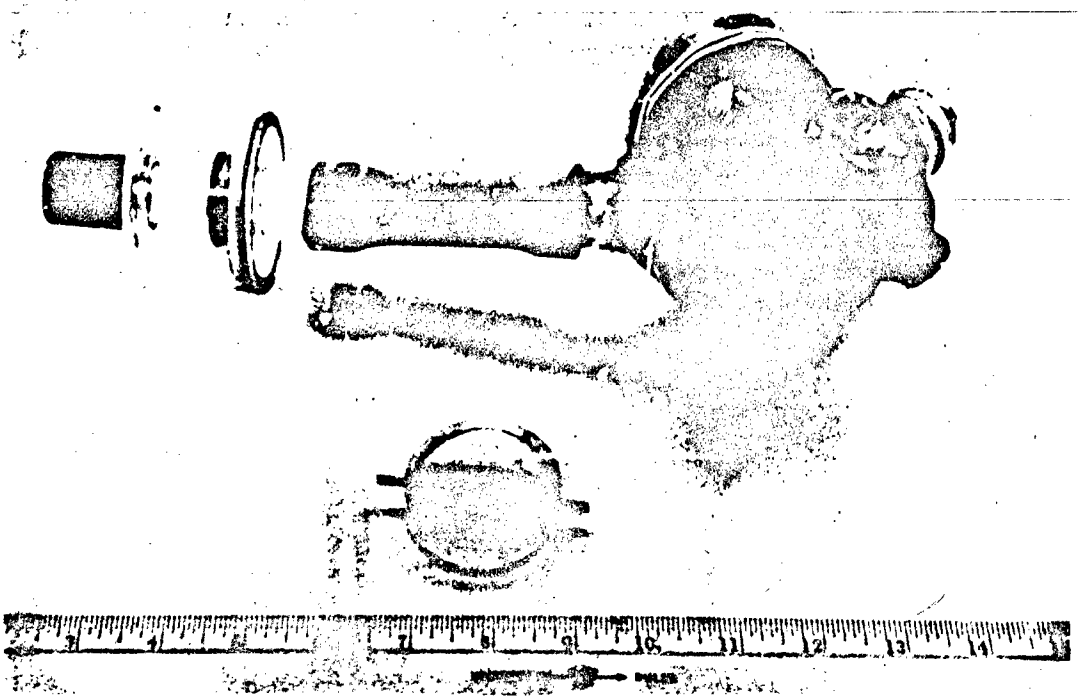


Figure 1. Picture of special two-valve mask used to sample fluorocarbon 115 in exhaled air.

The six-hour inhalation exposure to 10% fluorocarbon 115 was carried out in a $1M^3$ chamber whose exhaust rate was determined to be 15 cfm, using a vane-type anemometer. A mixing chamber (see Figure 2) was used for delivery of a uniform mixture of air, oxygen, and fluorocarbon. The concentration of fluorocarbon 115 in the chamber was monitored by a Varian Model 700 gas chromatograph which was equipped with a Model 7100 B Mosely Stripchart Recorder, a thermal conductivity detector, and a 4' x 1/4" (O.D.) S.S. Porapak® + T 80/100 column. The exposure record for the six-hour study is presented as Figure 3.

+Porapak® T is a chemically bonded stationary phase GC support material available from Waters Associates, Incorporated.

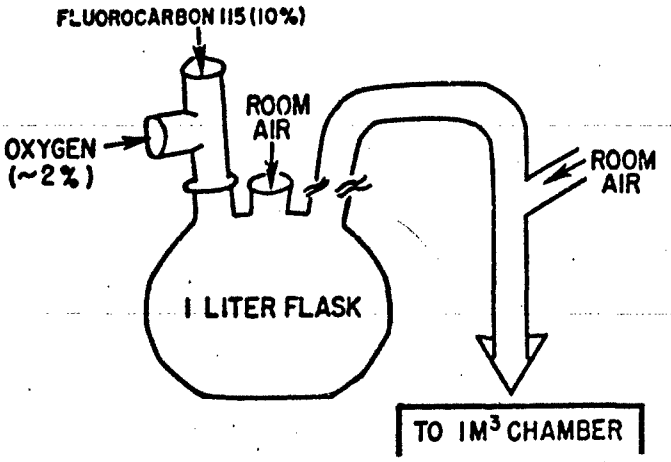


Figure 2. Sketch of mixing chamber used to generate 10% fluorocarbon 115 atmosphere.

NOTE:
1. ALL JOINTS WERE 29/40
2. FLUOROCARBON 115 WAS DELIVERED FROM A CYLINDER USING 3/8" O.D. COPPER TUBING.

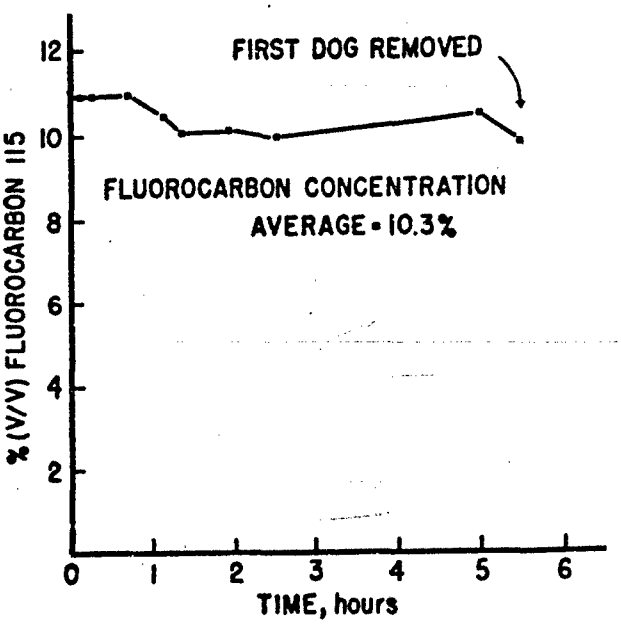
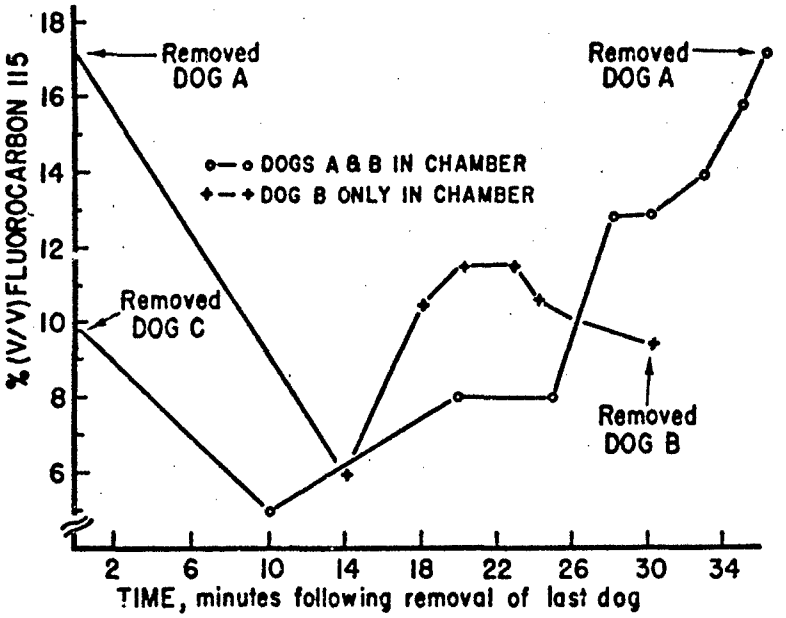


Figure 3. Concentration vs. time for six-hour 1 M^3 chamber exposure.

It was necessary to remove the dogs individually from the chamber in order to take blood samples. As the chamber door was opened, the concentration of fluorocarbon inside the chamber dropped sharply. Efforts were made to restore the concentration to 10% and keep it in the 10% region for 15 minutes before removal of the next dog. A concentration profile against time is shown as Figure 4. Dog C was removed first, dog A was second, and dog B was third.



NOTE:
Line o—o gives concentration of fluorocarbon 115 after DOG C removed, but DOGS A and B remained. At 35 minutes DOG A was removed and time starts at zero for DOG B.

FIGURE 4

Figure 4. Concentration of fluorocarbon 115 in chamber after opening.

Samples of venous and arterial blood (4 ml) were taken periodically with 6 ml syringes via the indwelling catheters. The entire sample was placed in a 10-ml Vacutainer® tube. The concentration of fluorocarbon 115 in the blood sample was determined by headspace analysis procedure which involved an electron capture equipped GC (Terrill, 1972).

RESULTS AND DISCUSSION

The arterial and venous blood levels of fluorocarbon 115 for each type of inhalation exposure are given in Tables 2 and 3.

TABLE 2. ARTERIAL AND VENOUS BLOOD LEVELS OF FLUOROCARBON 115 FOR UNANESTHETIZED BEAGLE DOGS EXPOSED BY FACE MASK METHOD TO AN ATMOSPHERE OF 10% FLUOROCARBON 115 FOR TEN MINUTES[1]

Time (min)[2]	Dog A		Dog B		Dog C	
	Arterial[3,4]	Venous[5]	Arterial	Venous	Arterial	Venous
0	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
1	2.3	1.2	2.2	0.8	2.2	0.83
3	2.5	2.0	2.6	1.8	2.0	1.5
5	3.0	3.0	2.9	2.0	2.3	2.2
7	2.3	2.5	3.0	2.1	2.1	2.2
10	3.7	2.5	2.8	2.1	2.9	1.3
12-1/2	0.12	1.3	0.2	1.2	0.4	0.9
15	<0.06	1.2	-	0.9	<0.06	0.3
20	-	0.6	-	0.4	-	-

Time (min)[2]	Dog D		Dog E		Dog F	
	Arterial	Venous	Arterial	Venous	Arterial	Venous
0	-	<0.06	<0.06	<0.06	-	<0.06
1	2.8	1.1	1.8	0.4	2.9	1.0
3	3.2	1.6	1.8	0.9	3.1	1.8
5	3.0	1.6	-	1.0	3.0	1.7
7	3.0	1.2	1.9	1.3	3.0	2.1
10	2.8	2.6	2.0	1.4	3.4	2.7
12-1/2	<0.06	1.0	0.2	0.8	<0.06	1.5
15	<0.06	0.6	0.08	0.5	<0.06	0.65
20	-	0.3	-	0.3	-	0.3

[1] Concentrations are given in µg per ml of blood
[2] Time from beginning of the ten-minute exposure.
[3] Ten-minute arterial average = 2.9 µg/ml (all dogs).
[4] Blank indicates that a sample was not taken.
[5] Ten-minute venous average = 2.1 µg/ml (all dogs).

TABLE 3. ARTERIAL AND VENOUS BLOOD LEVELS OF FLUOROCARBON 115 FOR BEAGLE DOGS EXPOSED TO AN ATMOSPHERE OF 10% FLUOROCARBON 115 FOR SIX HOURS[1]

Time (min)[2]	Dog					
	A		B		C	
	Arterial[4]	Venous	Arterial	Venous	Arterial	Venous
0[3]	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06
1	0.15	4.1	0.24	2.2	0.48	5.2
3	0.07	3.5	0.12	0.6	0.20	2.3
5	< 0.06	2.0	< 0.06	1.0	0.15	1.9
7	-	1.9	-	1.0	0.3	0.6
10	-	1.2	-	1.6	-	0.7
15	-	1.3	-	0.7	-	0.3
20	-	1.0	-	1.0	-	0.5
24 (hr)	< 0.06	< 0.06	< 0.06	0.3	< 0.06	< 0.06

[1] All blood levels stated in μg of fluorocarbon 115 per ml of blood.
[2] Time began as dog was removed from chamber.
[3] "Zero" time was the pre-exposure level.
[4] Blank indicates that a sample was not taken.

In the 10-minute inhalation exposure (Table 2), the arterial and venous fluorocarbon blood level rose rapidly at the start of the exposure and reached a rather constant value in less than five minutes. Arterial levels were higher (10-minute arterial average 2.9 μg/ml during the exposure but decreased more rapidly than the venous levels following the exposure. The blood levels were compared at 10 minutes and 6 hours. Little difference was observed in the arterial rate of decay, but the venous level decrease appeared to be a bit slower following the 6-hour exposure than the 10-minute exposure (Figure 5). In both cases, the half life for venous fluorocarbon loss following cessation of exposure was less than five minutes. One could predict from these results that individuals exposed to TLV concentrations of fluorocarbon 115 would have arterial ($\sim$0.03 μg/ml) and venous ($\sim$0.02 μg/ml) fluorocarbon blood levels below the sensitivity of our analysis (0.06 μg/ml).

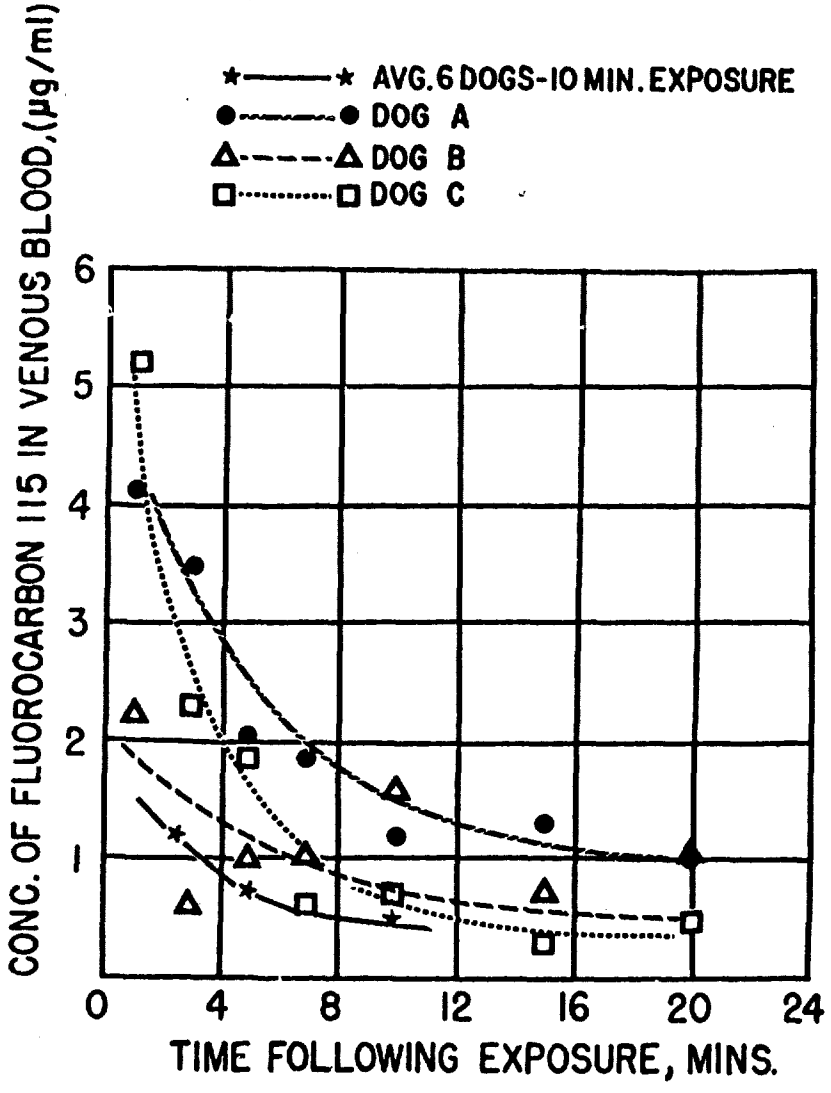


Figure 5. Comparison of venous blood levels of fluorocarbon 115 for 6-hour and 10-minute exposures.

Ingestion of the whipped topping containing 8% fluorocarbon 115 gave an arterial blood level of less than 0.06 µg/ml (minimum method sensitivity). The levels in venous blood during the first 30 minutes occasionally were higher than 0.06 µg/ml (Table 4). These values were probably caused by sampling error or, more likely, inhalation of some fluorocarbon 115 from regurgitated gases (see Figure 6).

TABLE 4. VENOUS BLOOD LEVELS OF FLUOROCARBON 115 DURING THE FIRST 30 MINUTES FOLLOWING INTUBATION WITH 150 G OF WHIPPED TOPPING CONTAINING 8% (W/W) FLUOROCARBON 115

Time (min)	Dog[1,2,3,4]			
	A	B	C	D
0	< 0.06	< 0.06	< 0.06	< 0.06
1	< 0.06	-	< 0.06	0.18
3	< 0.06	-	0.09	< 0.06
5	-	0.4	-	< 0.06
7	< 0.06	-	0.09	< 0.06
12	< 0.06	-	< 0.06	< 0.06
15	-	0.6	-	-
16	< 0.06	-	< 0.06	< 0.06
20	< 0.06	-	< 0.06	0.09
30	< 0.06	< 0.06	< 0.06	0.07

[1]All levels stated in μg per ml of blood.
[2]All arterial levels <0.06 μg/ml of blood.
[3]All venous levels were less than <0.06 μg/ml when measured at 45, 60, 180 minutes and 24 hours.
[4]Blank indicates that a sample was not taken.

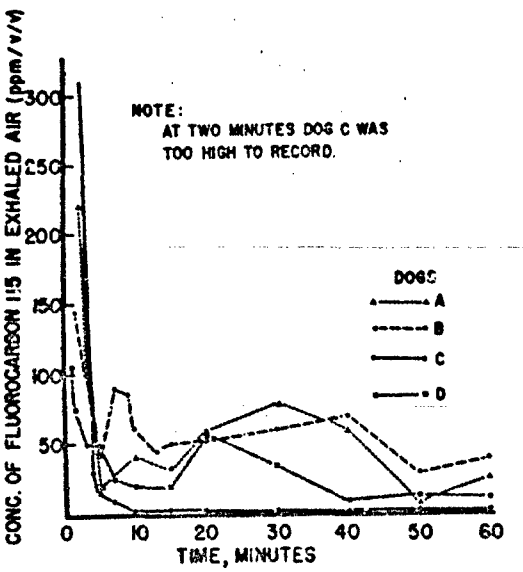


Figure 6. Concentration of fluorocarbon 115 in breath vs. time for dogs intubated with 8% fluorocarbon 115 in whipped topping.

CONCLUSION

The inhalation exposures and feeding exposures can be critically compared on the basis of fluorocarbon blood levels. Most of the venous and arterial blood levels in the ingestion studies were below the sensitivity of the analytical method (<0.06 μg/ml). The differences between these values and those observed in the 10-minute inhalation exposure are 48 fold (arterial) and 35 fold (venous). The topping (150 g) represents a 12-gram dose of fluorocarbon 115 per 10 kg dog or about 1 g/kg of body weight.

Under these conditions of maximal oral exposure, blood levels still were far less than observed in a 10% inhalation exposure. For normal human use, pressure packaged food preparations contain 500 μg fluorocarbon per gram food - at the time of consumption (Daly). The results of this study show a person would have to eat, at one meal, many times his own body weight of an aerosol food preparation to achieve the fluorocarbon 115 blood level reached in a 10-minute (10%) inhalation exposure.

In summary, measurable levels (2 to 3 μg/ml) of fluorocarbon 115 in the blood were found in 10-minute and 6-hour inhalation exposures, a detectable (>0:06 μg/ml) level of fluorocarbon 115 in the blood was not regularly established for an extremely large oral dose of the fluorocarbon. Thus, in the case of fluorocarbon 115, inhalation of a 10% concentration caused a much higher fluorocarbon blood level and may cause a much higher tissue level than could be achieved by ingesting foods saturated with fluorocarbon 115.

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DISCUSSION

DR. AVIADO (University of Pennsylvania): Could you tell me, please, where the venous blood was sampled?

DR. TERRILL (E. I. duPont de Nemours and Company): The venous blood was sampled in the same place as it was in the previous work. The catheter was surgically implanted into the jugular vein and it was pushed down the vein toward the heart. We determined, by x-ray, that the end of this tube was right at the vena cava.

DR. AVIADO: Then this was mixed venous blood above the liver. I think before I accept your statement that there is no fluorocarbon in the venous blood, you'll have to convince me by measuring portal venous blood levels. Any propellant that's absorbed will go through the portal venous blood and then something will happen in the liver. The blood that you sampled had already gone beyond the liver.

DR. TERRILL: The principal thing one would assume to happen in the liver is chemical change of the material. That's one thing you would expect. In this case, the molecule contains only one chlorine atom and it has already been established from the metabolism study that has been done on fluorocarbon 12 that roughly 98% or better of the material remains unchanged when it passes through the body.

DR. AVIADO: You are citing work done of fluorocarbon 12 which is very different from fluorocarbon 115. I think the burden of truth is yours to prove that nothing has happened in the liver.

DR. TERRILL: You're quite right. If you want to get right at the source of the blood coming from the digestive system, you have to get into the portal blood. I won't argue that point at all. However, what I'm saying is that we measured it in mixed venous blood. It is true that the blood coming from the liver is diluted once it gets up to that area of the body and into that circulation and consequently, the blood level might be lower. However, the 35-fold difference suggests that there is extremely little fluorocarbon being absorbed into the body.

MAJOR MC NUTT: How long after you gave the whipped topping did you measure fluorocarbon concentrations in the blood? Was it 10 minutes or was it longer than that?

DR. TERRILL: I believe it was within the first several minutes and it went up to 30 minutes. Because we found that in our work with doing tissue analyses of the liver with fluorocarbon 12, there was a maximum amount of

fluorocarbon 12 in the liver at less than 30 minutes. The time of measurements was 1, 3, 5, 7, 12 and so forth down to 30 minutes. Time zero was when the stomach tube was removed from the dog.

MAJOR MC NUTT: My question really has to do with gastric emptying time of the dog and whether the fluorocarbon really got into the intestinal tract by the time you finished your measurements.

DR. TERRILL: This is really an academic question because we are trying to duplicate a normal human exposure to the material. We inserted the tube into the animal to make sure it got as far as the stomach. Some dogs were observed to burp the material up. This does not guarantee that the topping got into the intestinal tract; however, this is a duplication of human exposure to the material.

MR. WANDS (National Academy of Sciences): I have 2 questions, please. First, have you done any sort of a material balance on the ingested freon? If so, what is its fate? You've got some data showing a little of it comes out in the expired air and you just implied that some of it is eructated. Does any of it come out in flatus? Does any of it stay in any of the fatty tissues? We heard earlier from Dr. Trochimowicz that the adrenal soaks up even more than the heart does. Do you have data of these kinds?

DR. TERRILL: No, I do not have such extensive data. It's very difficult to measure the gas volumes coming up in a sudden burst when the stomach tube is removed. Blood levels to some extent reflect a reversible passage of the material through the tissues. The 6-hour exposures suggest that there is no appreciable fluorocarbon absorbed by the tissue.

MR. WANDS: Did I understand correctly that this product has been approved by the FDA?

DR. TERRILL: That is correct. This product was approved in 1965 in the Federal Register. The reason we reinvestigated this material was because of the development of these techniques for measuring blood fluorocarbon levels. We went back to look at some earlier work that was done to see just what the margin of safety was. We believe from this work that there is a considerable margin of safety.

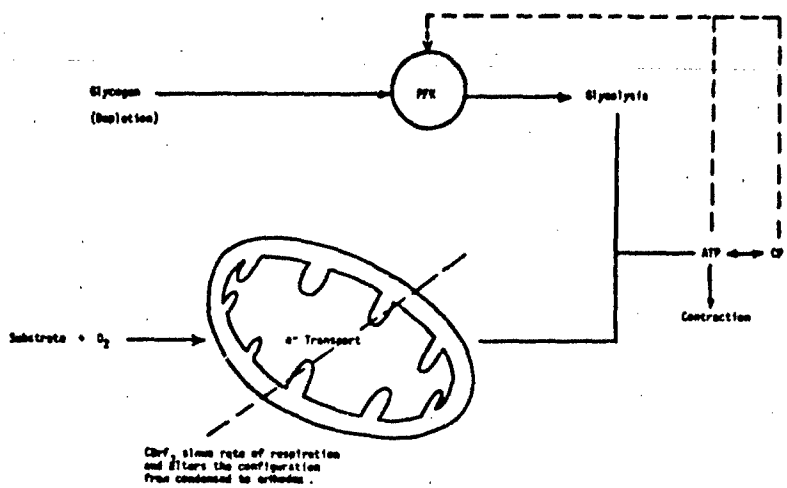
MR. WANDS: And now my second question, please. I understand that fluorocarbon 115 is used in food products in conjunction with other propellants such as nitrous oxide. Have you done any studies with a combination of these on the heart or any other anatomic system?

DR. TERRILL: I have not.

DR. TROCHIMOWICZ (E. I. duPont de Nemours and Company): I mentioned earlier that we had tested, on a limited basis, fluorocarbon 116 at 20% in air and found no effects of cardiac sensitization in 12 dogs. In conjunction with that same study, we exposed dogs to 20% fluorocarbon 116 and 5% nitrous oxide, the remaining balance being air. Again, just one exposure using 12 animals. We again found, at least relative to the cardiac sensitization phenomena with induced epinephrine, no evidence of cardiac sensitization.

OPEN FORUM

MAJOR VAN STEE (Aerospace Medical Research Laboratory): On behalf of my colleagues and myself, I would like to suggest a mechanism that may contribute to the negative inotropic action of $CBrF_3$ that takes into consideration many of the observations that have been made on experimental animals exposed to this compound. The working hypothesis is shown on the screen.



A working hypothesis consistent with some of the observations made on the metabolic effects of exposure to $CBrF_3$. The rate of mitochondrial oxygen consumption is slowed in the presence of $CBrF_3$. Heart mitochondria from guinea pigs exposed to $CBrF_3$ assume the orthodox configuration. Myocardial glycogen is depleted and phosphofructokinase (PFK) is activated. Broken lines represent inhibitory relationships. $CBrF_3$ slows the rate of oxidative phosphorylation that results in a decreased rate of ATP-CP synthesis. ATP-CP inhibition of PFK is released and glycogen depletion follows. Net production of ATP-CP is decreased which results in decreased myocardial contractility.

ATP is synthesized through aerobic oxidation and anaerobic glycolysis. Dr. M. L. Horton of our group has conducted 5 of a projected 20 experiments in which rat liver mitochondria are isolated, exposed in vitro to $CBrF_3$, and their respiration followed by an oxygraph. At this early point in the investigation he has presumptive evidence of a decreased rate of respiration, but no evidence for the uncoupling of oxidation and phosphorylation. In support of this observation, Dr. N. S. McNutt, also of our group,

has demonstrated that mitochondria from hearts of guinea pigs exposed in vivo to $CBrF_3$ assume an orthodox configuration, a cristal modification consistent with a decreased rate of aerobic activity. No evidence of tissue hypoxia such as mitochondrial swelling has been demonstrated.

If, indeed, the rate of mitochondrial respiration is reduced, the rate of ATP synthesis would also be expected to be reduced. Such a reduction would result in the release from inhibition of phosphofructokinase (PFK), accelerating glycogenolysis. Dr. McNutt has observed a significant depletion of myocardial glycogen in the same guinea pig hearts as used in the mitochondrial observations.

Since a normal rate of total ATP synthesis might not be expected from a combination of reduced aerobic activity and increased glycolysis, a diminished availability of ATP to the contractile apparatus could be expected to result in decreased myocardial contractility. The final experimental observation supporting this hypothesis was the highly significant correlation between myocardial oxygen extraction and contractility that was determined in the open-chested dogs discussed earlier today.

DR. AVIADO (University of Pennsylvania): It's rather unfortunate that this meeting is not devoted purely to myocardial infarction because I would like to challenge some of those hypotheses that have been presented. However, since this is a general meeting, I just would like to challenge the source of the material that Major Van Stee described and that is the source of the coronary sinus blood. The figures he gave for oxygen content in the venous blood have never been reported as high before. I've never heard of any reports of venous oxygen concentrations that high and there are only three possibilities. One possibility is that the analysis of blood oxygen content may be wrong, but I think that we can exclude that possibility because the measured arterial blood oxygen content seems to be reasonable. The second possibility is that the dogs in Dayton, Ohio may have tremendously high oxygen content. We teach our medical students that the oxygen content in coronary blood is supposed to be the lowest in the body so that these very, very high values challenge everything that we've learned. Now regarding the dogs in Ohio, I think that we can exclude the uniqueness of Ohio dogs. About 30 years ago, Ogden, at Ohio State University, reported venous oxygen content in dogs using the Van Slyke technique showing that they were the same as others have described. The third possibility is that the so-called coronary sinus blood is not a true sample. I happened to be around when the coronary sinus was first catheterized by Eckenhoff at the University of Pennsylvania about 29 years ago. I was, therefore, warned about the possible dangers in trying to claim that a blood sample is a so-called "true" coronary sinus blood sample. There are tests that have to be done to prove that fact. Dr. Van Stee, how certain are you that the blood that you have drawn from the coronary sinus represents true coronary sinus blood and not a mixture of mixed venous blood? If your sample happens to contain some mixed venous blood, it would explain the very high oxygen content.

MAJOR VAN STEE: We were very certain of the placement of the catheter since this was done from the exterior on the heart in situ. We were able to visually locate the great coronary vein and then we moved from the coronary sinus at that point at which it would empty into the right atrium back a centimeter or so. We then withdrew our blood samples very slowly so that we wouldn't inadvertently be pulling in some blood from the right atrium while we were drawing the sample from the coronary vein. Now, I will have to explain to you how we calculated the oxygen content of the blood. There was a study done by Pickrell and Schluter at the Lovelace Foundation on a colony of beagles. Through a combination of techniques, they developed a very elaborate computer program strictly for beagle dogs that took into account the oxygen dissociation curve for the dog and a number of other factors and corrections were put into this program for body temperature, pH, CO_2 and one thing or another. This whole program was designed strictly for the determination of these various blood gases in dogs specifically. They pointed out that in the past many determinations of blood gases had been erroneous because they were based on factors derived from human blood which was different from dogs. This was one of the purposes then for putting the reference values on our slides. Rushmer, for instance, if we took, I would prefer to do this outside actually, it's a little bit involved but I could explain to you how we arrived at some of these figures. In the case of our coronary sinus oxygen content, it is about 50% higher than that shown in the Rushmer reference and it's also twice as high as shown in the other reference. But we also had rather high hemoglobin values in our dogs.

DR. AVIADO: So what you are saying, then, is that it is probably technique. Incidentally, the values of Rushmer were collected using the Van Slyke technique. I don't think anyone has ever challenged those techniques as far as I know. I know Dr. Van Slyke has died but we still regard the Van Slyke technique with high respect. This modern technique that you used then is an indirect technique, is that right? You derive oxygen tension first and then from that you back-calculate to determine oxygen content?

MAJOR VAN STEE: Right, we measure PO_2, PCO_2, pH, hemoglobin and temperature.

DR. AVIADO: But in the past, instead of measuring all of those, we went directly to the Van Slyke technique and obtained these so-called low values. I don't think anyone would accept those tremendously high oxygen values for coronary sinus blood. At least I won't.

MAJOR VAN STEE: Well, I'd be glad to show you this reference and this program for your criticism. I'd be delighted to find out if there was something radically wrong with it.

MR. ADAMS (USAF School of Aerospace Medicine): Do you have a ballpark figure for the oxygen saturation level?

MAJOR VAN STEE: Yes, this also was provided. I didn't print this out and record it, but the saturations were 97, 98, 98% in almost all cases. The lowest arterial blood at any time was 90% oxygen saturation.

MR. ADAMS: What does that 90% value correspond to in terms of saturation? Is it in the ballpark of 25% saturation? That would be normal, wouldn't it?

DR. AVIADO: The units that he used are out of the ordinary. We usually measure or express oxygen content in the blood in milliliters per 100 ml, rather than milliliters per liter. If you shift the decimal point, that 98 oxygen content in the coronary sinus blood is 9.8 and that is rather high, as I was saying. That's about twice the usual Van Slyke value. You express oxygen content per 100 cc in blood and that saturation will probably turn out to be about 50 or 60 which is not the accepted normal percent oxygen saturation.

DR. TAYLOR (National Institute for Environmental Health Sciences): I can't really get too excited, Dr. Van Stee, about the way you put this material together in your last presentation. If I understand correctly, what you're saying is that perhaps the mechanism of negative inotropism is decreased ATP or decreased high energy phosphate availability. Is that correct?

MAJOR VAN STEE: Not the entire mechanism. That would be one part of it.

DR. TAYLOR: There are a couple of observations that, I think, mitigate against this theory. The first is that the depression in cardiac contractility, regardless of how it's measured, is a beep by beep phenomenon. Recovery from this effect is also a rapid phenomenon. In the rapid freezing studies that were done initially for determining the mechanism of myocardial energy utilization and supply, a number of tests were done in terms of substrate availability and well-oxygenated heart muscles were subjected to perfusates that had no glucose and also no glycogen. The negative inotropism seen in these studies was a much slower phenomenon than was observed with these freon studies. It was something that occurred over 10 to 15 minutes. Over longer periods of time, they did see a loss of glycogen stores but certainly didn't see a loss of glycogen stores quite as fast as could be accounted for if this is indeed a mechanism of action. I think an equally viable possibility is the fact that with the freon, you are running into problems with calcium availability and problems with the EC coupling. I don't think anyone's ruled that out. I think the thing to do if you're really interested in the matter of substrate availability, is to conduct the rapid freezing type studies and find out whether or not you have a depression of high energy phosphate stores with these brief, acute exposures.

MR. ADAMS: One point that both Dr. Taylor and Major Van Stee mentioned in their papers was that these animals were under anesthesia but they failed to regard this in their interpretation of the data. I think it's one thing to describe what you've done but when you start extrapolating from your experimentation, you forget that you don't have a total neural control under these conditions. I'm not so sure that you have a negative inotropic effect from that freon exposure in the conscious state. Do you have any data?

MAJOR VAN STEE: Not yet, but we do have some plans to investigate that point. I think there are some other people here who could comment on that.

DR. AZAR (Ohio State University): I honestly couldn't comment on that, but it brought up the point I want to make. I think it's very important that one include a sham group in these classical pharmacologic studies because of the 90-minute period that the animal is anesthetized. It's fine to use an animal as his own control but then to compare those data that are obtained under this particular methodology with those recorded in the literature can be somewhat misleading. I would like to suggest that a group of animals be anesthetized for 90 minutes as a sham control and the same parameters measured because you do traumatize the heart whenever you put a catheter in the coronary artery.

MAJOR VAN STEE: I wouldn't have the temerity to come in here and offer this without having done that previously. We have already done those experiments but for lack of time did not include these in this particular presentation. We used four dogs treated identically to these experimental animals in which we substituted nitrogen in a gas mixture in the same concentrations as the fluorocarbons were present. And the conclusions that I made here were based upon comparisons to those animals also, to the extent that I said there were no significant changes in these measured variables with the exception of those that had been shown in the past, the cardiovascular dynamic variables that do not change in function in the presence of the fluorocarbon. We use sham treated controls in every type of experiment that we do here. It is not sufficient under the circumstances of stress placed upon these animals to just compare him with himself pre- and postexposure.

DR. TAYLOR: May I also answer that question, since I think it was directed to my study as well as Major Van Stee's. We did similar studies: a number of animals were anesthetized and observed over a long period of time. There were no great changes during the exposure periods that I was using in the parameter I was measuring. Even over more prolonged periods, there was no tailoff in the parameters I was measuring. In terms of the anesthetic itself, both pentobarbital, which is what I was using, and chloralose, which Major Van Stee was using, have been shown to be negative inotropic agents. Because of this factor we did studies in which we anesthetized animals, let them sit for a considerable period of time, and then rechallenged them with pentobarbital. We observed a very rapid depression in dP/dt and cardiac output

which reversed itself within 3 or 4 minutes. It was a very transient type of thing. Studies done in Dr. Braunwall's laboratory in the late 1960's, on awake animals which had been prepared with cardiac muscle strain gauges, showed similar types of things. When awake animals were given a dose of sodium pentobarbital, they did have a depression of these cardiac parameters we were measuring, but this depression was rapidly reversed.

DR. TYLER (University of California, Davis): I'm not a toxicologist but I'm a consumer. And I noticed a complete absence of any information relative to the effects on the organs or systems through which these materials were administered. The route of administration seems to me to have been ignored, both in the oral and in the inhalation exposures to the fluorocarbons. In other words, what are the effects of all this lipid soluble material on the respiratory system and especially on the phospholipids of the respiratory system? I ask this simply because today we're realizing more and more that metabolic functions occur in the lung.

DR. AVIADO: If the consumer would come tomorrow afternoon, I will speak on the effects of the materials on the lung.

MR. FIELDING (Naval Research Laboratory): I am interested in a very specialized type of subject, the intact U. S. sailor, and in his response to Halon 1301 under conditions of stress. The best human data currently available are those obtained at the Haskell Laboratory in 1966. Three men were exposed to 7 and 10% concentrations of Halon 1301 in air. It was found that a 7% concentration, within 4 to 5 minutes they began to get marginal or beginning conditions of narcosis. Now those data are beginning to be taken as standards, that if a person isn't exposed to more than 7% Halon 1301 in air for 5 minutes, he won't be in much trouble. But we're wondering at NRL about the sailor who's in the engine room of a destroyer when a fire breaks out; he's already thermally stressed and presumably he's frightened, he dashes around and turns off a valve or two and then has to climb three decks up a vertical ladder as fast as he can. Are we justified in taking this 7% concentration as safe under these conditions? I presume no one here has done work on stressed men but this panel is as good as any to take a crack at it or would you rather say it's outside of our field?

DR. WILLS (Albany Medical College): Well, I can't answer it directly but there is a study in the literature in which dogs were exposed to freon with and without running on a treadmill. It was found that the cardiac effects of the exposure to freon were identical with these two conditions.

DR. BACK (Aerospace Medical Research Laboratory): Dogs convulse from Freon 1301 so we can't compare dogs with humans.

MAJOR VAN STEE: Excuse me, but Dr. Azar was the author of that paper and he's waiting to tell you about it.

DR. AZAR: There is a great deal more information available. Dr. Hine's group at the University of California, San Francisco, exposed 10 men to Freon 1301, and those results were presented here in the past. The U. S. Navy has also conducted research in this area at the Philadelphia Naval Research Center but I don't know if the results have been published. That group knew everything that we knew about Freon 1301 at Haskell. They had the data from Dr. Hine's exposures and they collected their own data so I think this is a subject that's being looked at quite vigorously. I don't want the group to be misled in thinking that all the conclusions were based on 3 people.

MR. WANDS (National Academy of Sciences): I think, Dr. Fielding, that you would find the proceedings of a seminar held at the National Academy about a year ago does contain a good review of the literature on all of these fire fighting agents, including Freon 1301. There is a fairly extensive literature on both animal and human exposures and some of the animal work was performed under conditions of stress. I might also comment that the recommendation from the Academy to the Navy for an exposure limit was 5 minutes at 6% Freon 1301, not 7%. The 6% limit did include a fair margin of safety because the narcosis that was observed at Haskell Laboratories and also by Dr. Hine, began at 10% concentration of Freon 1301. In the latest review on the Freon 1301 by the Committee on Toxicology at the request of the Navy, the committee refused to go to 7% that your engineers wanted on the basis that there were not sufficient data to justify going to 7%. Now we're suggesting that the Navy do these critical studies under simulated human exposure conditions of heat stress, noise and emotional stress. Until those studies are done, I think this is a moot point at this time.

DR. FIELDING: Perhaps I should have made one point a little clearer. We're not much worried about narcosis. This comes on gradually and almost everyone recovers from anesthesia, but cardiac fibrillation is another thing and we have no data on that. If this is the condition we fear, there's no particular point in citing data regarding narcosis.

DR. BACK: I think that there is something else to be gleaned from those experiments. The central nervous system effects of the compounds take place at a different level than that at which the arrhythmigenic effect occurs. In Dr. Hine's test on 10 individuals, the cardiac effects in only one individual were seen between 10 and 20 minutes of exposure as the concentration was increased. The subject finally said, "I don't feel very good," at somewhere around 13 or 14% Freon 1301 in the air. At that time he was dangerously close to fibrillatory problems. He had AV dissociation. However, he was still lucid and he knew he didn't want any more. Now the point of the matter is that the Committee on Toxicology set the EEL value at 6% Freon 1301 for 5 minutes based on the belief that a man could self rescue, and certainly you wouldn't want a drunken man trying to self rescue. My point is that if you don't go over 6%, no matter how much you stress the individual, the likelihood of

getting a stray cardiovascular effect is relatively nil. Now there's a great deal of difference between 6% and 8 or 10% if you look at the CNS effects as you increase the exposure concentration. At 10% freon concentration, one might not self rescue, but say the heck with it because this compound does give you a "high."

DR. TAYLOR: I think that this group is failing to look at part of the question Dr. Fielding has raised. Certainly to me, it is the most important part of the question. A lot of the people here are Air Force and Navy people who are dealing with healthy 18-year-old sailors. My training has been as an internist, specifically my interests are in clinical cardiology. I generally am much more interested in the little old lady who is found lying on her kitchen floor with pulmonary edema and brought into the emergency room late at night. Epidemiologic studies have not been done and probably won't be done to tell us what exactly happened the five minutes before her congestive heart failure condition worsened and tell us what it was that tipped her over the brink. She had been on digitalis and diuretics for some time but why did she get sick when she did? It could very well be that a depression of her cardiac contractility of 1% would be just enough to throw her into pulmonary edema which is a self perpetuating thing. When you talk about stress for an 18-year-old sailor, you're talking about one thing and when you're talking about people with pre-existing cardiovascular disease, you're talking about another. The fluorocarbon fire fighting agents are chemicals that could very possibly wind up in nursing homes.

DR. BACK: Under those conditions, I would expect that no one would allow 6% for 5 minutes. We're talking about the DOD reason for requesting such an emergency exposure limit, and nobody here is suggesting this level for general population use.

DR. FIELDING: Exactly. But what I'm pointing out is that these are ubiquitous agents. The fluoroalkane gases are found in virtually every household in the country and it's really not known yet at what levels they're found. There are a lot of people walking around in these very households with previous cardiovascular disease and no one really knows whether or not they are being affected by them. As to whether or not you can determine the tensile stress of a vase without breaking it is a moot point. Very little toxicology experimentation has been done in animals with induced disease but it probably will be in the future.

DR. BACK: I would like to ask a question about something along these lines on central nervous system effects. I noticed that Dr. Trochimowicz was using unanesthetized animals with Freons 113, 114, 115 and possibly 116. You were using exposure dose levels of 25% of Freon 115. Did you see central nervous system changes in these dogs in any one of the compounds that you studied?

DR. TROCHIMOWICZ (E. I. duPont de Nemours and Company): At the 25% level of 115, we began to see some central nervous system effects, a slight increase in excitation. Certainly not to the point of struggling out of the sling, but the animal was agitated. Now whether that's due to the compound or the fact that he's tired of standing there for 10 minutes, I'm not sure. But in comparing the controls just on a subjective basis, we began to see some increased activity on the part of the animal and possibly a little salivation; on removing the animal from the exposure, putting him on the ground, there were no effects on gait or equilibrium observed.

DR. BACK: The reason why we don't do too much work on unanesthetized animals with Freon 1301 is because it's a convulsant for dogs and you can't measure cardiovascular changes in a convulsing animal, so Freon 1301 can't be studied for central nervous system effects at the same time cardiovascular effects are being studied in the dog. It's just the wrong animal.

DR. AZAR: I would like to disagree slightly with Dr. Trochimowicz if my recollection of the early CNS signs seen in his studies is correct. These occurred near the level where we usually start to see cardiac sensitization occur. The dogs would start looking edgy and they would begin to fight the mask. Granted they wouldn't jump out of the sling every time, but they were starting to show the early CNS findings. During the study where we put dogs on a treadmill trying to stress them while exposing them to the freons, we would get a certain concentration of a freon and the dog would just sit down. There was no way you were going to get that dog to run. We broke several plastic cages that we used for an exposure chamber by beating on the side trying to get the dog to keep running. This was the same in the mask exposures in that you could run a dog up to a certain concentration and then he'd start fighting it. And the word around the lab was that the dog is smarter than the kid sniffing fluorocarbons to get a "high." The dogs would start holding their breath and fighting the exposure. This made sense to me. You know there's nothing unique about cardiac sensitization. It's been known to happen for years with anesthetic agents. A patient usually gets in cardiac trouble whenever he's light, whenever he's just starting to go under and whenever he's coming out of the anesthetic. So there may well be something there and I think a well needed study would be a look at sensitization and at the same time record EEG levels to see what happens and what's going on in the CNS.

DR. BACK: My point was that you are challenging them with epinephrine, and we were thinking more realistically of spontaneous arrhythmias. These animals convulsed so you would expect them to have a high output of epinephrine from the adrenals and massive CNS discharge through the autonomic nerves, and therefore, if there were any epinephrine effect, you would expect to see at least a good percentage of those dogs spontaneously fibrillate, but they don't. It takes large doses of epinephrine to trigger the ventricular response in the dog. I don't know how much it takes in man or whether stress will produce enough

epinephrine to cause cardiac sensitization in man. I don't know how man acts under these conditions. At least the one Freon 1301 exposed individual that showed AV dissociation was not so excited that he went all the way, although he wasn't convulsing obviously, but he was probably excited.

DR. WILLS: We did a series of experiments on decerebrated dogs to remove any effect of anesthesia and we did find that those animals became arrhythmic under just about exactly the same conditions as animals that had been anesthetized with pentobarbital.

DR. BACK: You're talking about a challenging dose of epinephrine. Well, I'm not surprised at that because that's a relatively rough way to challenge a heart. There's not really a great deal of difference between a microgram per kilo and 10 micrograms per kilo of epinephrine. It's a relatively rough challenge, but that's certainly not anywhere near normal circulating epinephrine levels.

SESSION III

GENERAL TOXICOLOGY

Chairman

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AMRL-TR-73-125

PAPER NO. 13

TOXICITY OF COAL TAR AEROSOL

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The effects of coal byproduct exposures to humans are well documented in literature beginning with the high incidence of scrotal cancer noticed in chimney sweeps as early as 1775. Cancer of the skin in workers of the coal tar and pitch industry was first described by Butlin in 1892. In 1907, the British officially recognized that cancer of the skin could occur in the handling or use of pitch, tar, and tarry compounds by including it in the Workman's Compensation Act of that year.

In 1947 Kennaway and Kennaway reported excess lung cancer mortality for British gas stokers and coke oven chargers. In 1971 Lloyd found that the excess of respiratory cancer reported for coke plant workers is limited to men employed at the ovens, the relative mortality for this disease being 2-1/2 times that predicted. The greatest part of this excess is accounted for by an almost 5-fold risk of lung cancer in men working on the tops of coke ovens. A 10-fold risk of lung cancer is observed for men employed 5 or more years at fulltime topside jobs. Fifteen lung cancer deaths were observed among the 132 men in this group compared to 1.5 deaths expected.

Reid and Buck (1956) conducted a survey of the occupational histories of all men that died after working in British coking plants during the period 1949-54 inclusive. They found that the number of deaths from cancer compared with numbers expected on the basis of mortality experience of other large industries.

Horton et al. (1962) reported an inhalation study of coal tar aerosol in which the solids of the coal tar had been removed. They exposed mice to 300 mg/m^3, 2 hours per day for 3 days per week. All of the mice died by 36 weeks of exposure. They found an increased incidence of squamous cell metaplasia in the exposed mice while the controls remained free of these tumors during and beyond this period of time.

Our initial investigation of coal tar was a pilot study designed to determine the potential carcinogenicity on skin, pulmonary tissues, liver and bladder of the volatile materials from coke oven effluents. The range of concentrations selected were representative of those in actual working conditions found in and around byproduct coke ovens. Our intention was to simulate long periods of interrupted work exposure by use of shorter term continuous exposure and to investigate the possibility of a dose-effect relationship in coal tar carcinogenesis.

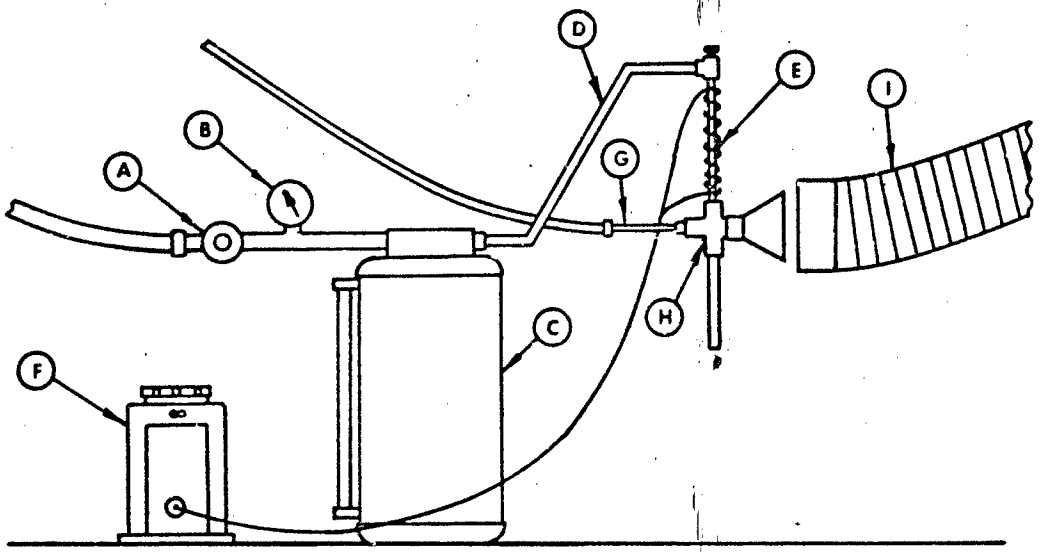
Animals were exposed continuously for 90 days to aerosolized coal tar at concentrations of 0.2, 2.0, 10, and 20 mg/m^3. The animals were observed daily for general appearance, behavior, signs of toxic stress, and lethality. Control groups of each species were maintained for comparison with test animals.

Experimental animals included female Sprague-Dawley yearling rats, male, and female Sprague-Dawley weanling rats, male ICR mice, male CAF-1 mice, male Golden Syrian hamsters and female New Zealand albino rabbits. The hamsters and rabbits were exposed only to the highest concentration (20 mg/m^3) while weanling and yearling rats and both groups of mice were exposed to all contaminant levels.

The most difficult problem in conducting exposures to coal tar aerosol was the generation of the aerosol itself. Coal tar is a complex, viscous mixture of coal pyrolysis products which contains a significant amount of dispersed solids. When commercial oil sprayers or foggers were used in attempts to produce satisfactory aerosols, frequent clogging of the nozzles interrupted the generation. Commercial ultrasonic nebulizers worked satisfactorily for a short time, then failed. This problem occurred because the more volatile materials were being expelled from the nebulizer, leaving a thick residue on the transducer which eventually caused it to stop.

Initial experimentation demonstrated that dilution of the coal tar with an equal volume of benzene decreased the viscosity sufficiently for the solids to be separated by centrifugation. The benzene added could then be removed from the tar by fractional distillation. When the product of these operations was aerosolized through a specially designed generating device, no clogging took place, and a 7-day test at 20 mg/m^3 coal tar concentration was conducted without serious problems. It was, therefore, decided to separate the solids from the coal tar in this manner for conducting the 90-day exposure.

The aerosol generating device (Figure 1) was made from a 1/8" stainless steel "cross" pipe fitting with the exit opening enlarged to 3/4" (H). Stainless steel lines, 1/16" O.D., carried the coal tar and air to the generator where it was aerosolized and blown into the chamber air supply line (I). The generator air line was maintained at a constant pressure of 35 psi. The quantity of coal tar entering the generator was controlled by the air pressure on the reservoir (C). The chamber concentration, therefore, could be regulated by either increasing or decreasing the pressure on the coal tar reservoir. Prior to entering the generator, the delivery line was subjected to mild heat to facilitate the aerosolization process.



(A) Coal Tar Pressure Regulator
(B) Coal Tar Pressure Gauge
(C) Coal Tar Reservoir
(D) Coal Tar Supply Line
(E) Coal Tar Delivery Line Heated with Nichrome Wire
(F) Variable Output Transformer (Variac)
(G) Generator Air Line
(H) Generator Nozzle
(I) Chamber Aerosol Delivery Line

Figure 1. Modified Contaminant Generation System for Aerosolization of Coal Tar Volatiles.

An aerosol particle size determination following the procedure of Vooren and Meyer (1971) was performed on each chamber at the beginning of the study and once monthly thereafter on the Longley Chambers only. Table 1 shows the monthly particle size characterization expressed as a percentage of droplets 5 micrometers or less in diameter. Except for chamber D-2 in the second month, all samples showed more than 95% of the droplets 5 micrometers or less in diameter.

TABLE 1. MONTHLY PARTICLE SIZE CHARACTERIZATION, 90-DAY EXPOSURE TO COAL TAR AEROSOL PERCENTAGE OF DROPLETS 5 MICROMETERS OR BELOW IN DIAMETER

Chamber and Aerosol Concentration	1st Month	2nd Month	3rd Month
A - 20 mg/m^3	100.0	-	-
B - 20 mg/m^3	100.0	-	-
C-1 - 20 mg/m^3	98.7	96.4	97.9
C-2 - 10 mg/m^3	98.8	95.1	95.2
D-1 - 2 mg/m^3	98.7	98.4	99.4
D-2 - 0.2 mg/m^3	97.9	92.1	98.9

Ten percent of the hamsters, weanling rats, and yearling rats from the 20 mg/m^3 and from the control groups were sacrificed at the termination of exposures. The surviving animals are still being observed for various lengths of time up to their natural lifetime. All animals that died were subjected to gross and microscopic pathological examinations. In addition, some of the animals that died from the 20 mg/m^3 exposure group had sections of kidney, liver, and lung submitted to the chemistry section for extraction and analysis of fluorescent compounds. Histologic examination included liver, lung, genito-urinary tract (including bladder), scrotum, skin, spleen, and bone marrow.

The exposures were interrupted approximately 15 minutes each day for routine animal maintenance. All leftover animal food was discarded and replaced with a fresh supply. The rodent cages in the Longley Chambers were rotated daily, the top cage to the bottom and all other cages moved up one level. All cages were changed on a weekly schedule.

Chamber concentrations were monitored using a combination of a hydrocarbon analyzer and a fluorometer. A standard curve was prepared using the results of the fluorometric sampling plotted against the readings of the hydrocarbon analyzer. Fluorometric analysis only was used to monitor the 0.2 mg/m^3 exposure since the hydrocarbon analyzer was insensitive at that concentration. Chamber concentrations were recorded on an hourly schedule.

The daily fluorometric analyses yielded 90-day mean concentrations of 20, 19, and 20 mg/m^3 in the 3 nominal 20 mg/m^3 chambers, and 10, 1.9, and 0.22 in the nominal 10, 2, and 0.2 mg/m^3 chambers as shown in Table 2.

TABLE 2. SUMMARY OF 90-DAY COAL TAR AEROSOL CONCENTRATIONS

	Nominal Concentration (mg/m^3)	90-Day Average Measured Concentration (mg/m^3)
Rochester A	20	20
Rochester B	20	19
Longley C-1	20	20
Longley C-2	10	10
Longley D-1	2.0	1.9
Longley D-2	0.2	0.22

At the conclusion of the exposure period, the animals from the 20, 10, and 2 mg/m^3 concentrations showed considerable accumulation of coal tar on their fur. The accumulation on the fur was dose related in its intensity with the 20 mg/m^3 animals being quite brown. Through preening or grooming, most of the discoloration had disappeared by one month postexposure.

Although mortality from the exposures was considerable, it did not appear to be directly related to coal tar toxicity, but, rather, to the debilitating effects of the exposure which resulted in a greater susceptibility to infections. A high incidence of chronic murine pneumonia was found in animals of all species that died during and after exposure. Cumulative animal mortality showed a general graded response proportional to exposure concentration. Exceptions to this were in the ICR mice exposed to 0.2 mg/m^3 and the control male weanling rats. Both groups showed a surge in mortality during the second and third month of exposure, apparently due to an unidentified infection.

All rabbits housed underneath hamsters died within the first 67 days while only 3 of the 12 rabbits housed beneath rats and mice died during the 90-day exposure. In order to investigate the possibility that this arrangement was deleterious to rabbits, we housed hamsters above 4 control rabbits. Three of the 4 control rabbits died within a month's time. We feel that this particular group of hamsters was carrying a pathogen which was noncompatible to rabbits and, therefore, do not attribute these rabbit deaths to coal tar toxicity.

In every animal species, there was a marked effect of exposure on growth during the exposure period. As can be seen in Figure 2, the significant difference in the CAF-1 mice was dose related and the effect can still be observed 7 months postexposure. Figure 3 shows the same type of body weight effect on the ICR mice. The body weight curves of the yearling rats, hamsters, and rabbits were similar to those found in the 2 mouse groups. The male and female weanling rats, being at the peak of their growth period, were weighed once per month during the study. The body weight effects of the aerosol exposure on these 2 groups are shown in Figures 4 and 5. As can be seen in the graph showing the growth curve of the male weanling rats, all exposed groups gained subnormally during the first 2 months and lost weight during the third month. A definite dose related effect is demonstrated in this group and can still be seen 7 months postexposure. The female weanling rat group, although not as dramatic, shows a similar effect both during exposure and for several months postexposure.

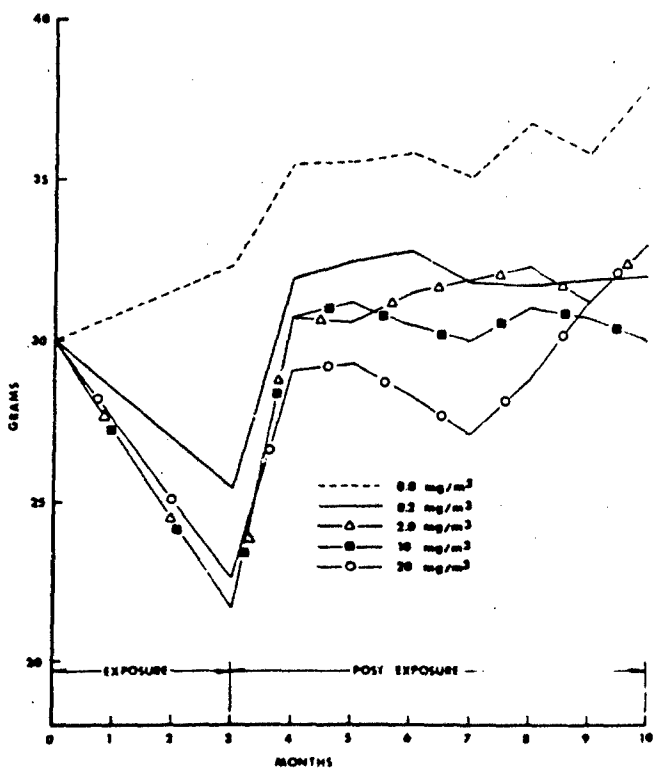


Figure 2. Growth of Male CAF-1 Mice Exposed to Coal Tar Aerosol.

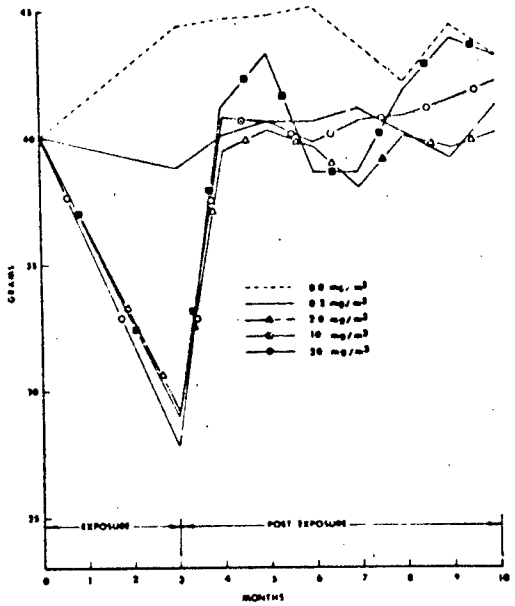


Figure 3. Growth of Male ICR Mice Exposed to Coal Tar Aerosol.

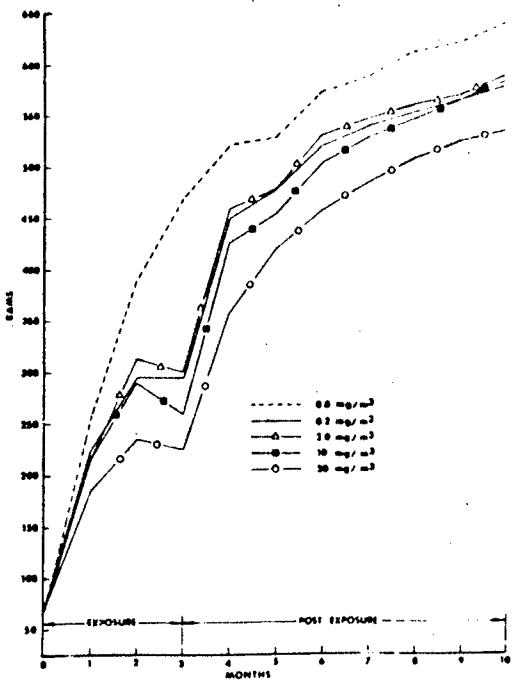


Figure 4. Growth of Male Weanling Rats Exposed to Coal Tar Aerosol.

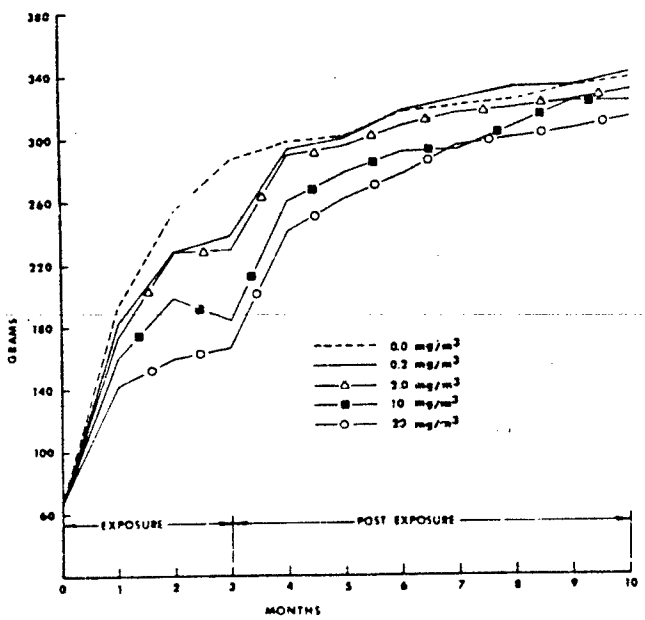


Figure 5. Growth of Female Weanling Rats Exposed to Coal Tar Aerosol.

The measurements of fluorescent materials extracted from animal tissue with toluene after 30 days exposure to 20 mg/m^3 are shown in Table 3. A slight increase in fluorescence can be seen in the kidney and the liver values, while a large increase is seen in the lung values. Also noticeable, when looking at the lung tissue fluorescence values, is what appears to be an increase in the fluorescent content of the lung tissue essentially proportional to animal size.

TABLE 3. TOLUENE SOLUBLE FLUORESCENCE IN ANIMAL TISSUE AFTER 30 DAYS CONTINUOUS EXPOSURE TO 20 mg/m^3 COAL TAR AEROSOL

		μ*/g Wet Tissue		
		Kidney	Liver	Lung
Rat:	Exposed	5.4	174	365
	Control	3.5	100	57
Mouse:	Exposed	9.7	234	253
	Control	3.1	154	4
Hamster:	Exposed	6.5	60	813
	Control	3.1	43	26
Rabbit:	Exposed	5.8	21	1003
	Control	2.7	9	5

*Coal Tar Equivalent.

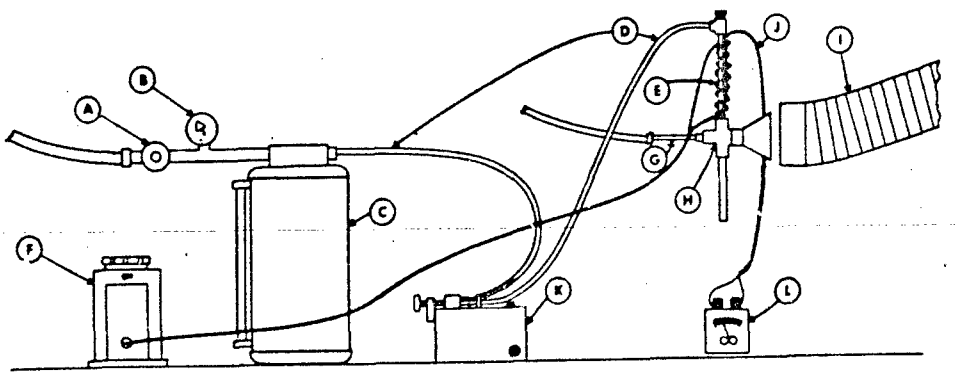
The biggest criticism of this pilot study concerned the fact that the coal tar introduced into the chamber was not exactly representative of the toxic materials to which coke oven workers are exposed since most of the solids had been removed. Moreover, the coal tar did not contain a light oil fraction of the coke oven distillate which is normally removed late in the separation process. This light oil fraction is also known as the BTX fraction and consists primarily of benzene, toluene, and xylene.

As a result of the findings of the initial experiment, three additional experiments were planned using coal tar in which the light oil and solid fractions had been retained; thus making it more comparable to actual coke oven effluent. The three sequential experiments of 90 days each would be to concentrations of 10, 2, and 0.2 mg/m^3.

The aerosol exposure portion of the first of these experiments (the 10 mg/m^3 concentration) has been completed. The experimental animal species remained the same although the numbers were reduced. Again, a control group of each species is being maintained for comparison with the test animals.

One hundred fifty CF-1 mice were added to the experiment for serial sacrifice during the study. Five mice were removed after 1 and 7 days and once per month thereafter. Gross and histopathology were performed on these animals to determine the progressive pulmonary effects of the coal tar aerosol. In an attempt to measure the rate of coal tar buildup on the skin and in the lungs of mice sections of lung and patches of dorsal skin were analyzed for fluorescent compounds. The serial sacrifice of this group of mice is being continued monthly during the postexposure observation period.

The addition of the BTX light oil fraction decreased the viscosity of the coal tar and made possible some improvements in the generation system (shown in Figure 6), although the generating device has remained the same. Between the coal tar reservoir and the generator is a Buchler Polystaltic® pump (K) which is used to regulate the amount of coal tar entering the generating device. Polyethylene tubing carries the coal tar to the pump and from the pump to the generator. Technicon Acidflex® transmission tubing is used to carry the coal tar through the polystaltic pump. A slight pressure is maintained on the reservoir to prevent collapsing of the transmission tubing. The chamber concentration can be regulated by controlling the speed of the polystaltic pump. As in the preliminary study, the delivery line is subjected to mild heat (65 C) to facilitate the aerosolization process.



(A) Coal Tar Pressure Regulator
(B) Coal Tar Pressure Gauge
(C) Coal Tar Reservoir
(D) Coal Tar Supply Line
(E) Coal Tar Delivery Line Heated with Nichrome Wire
(F) Variable Output Transformer
(G) Generator Air Line
(H) Generator Nozzle
(I) Chamber Aerosol Delivery Line
(J) Thermocouple
(K) Buchler Polystaltic Pump
(L) Pyrometer

Figure 6. Contaminant Generation System for Aerosolization of Coal Tar Volatiles.

The chamber concentrations were analyzed using gravimetric sampling to trap the aerosol droplets on a Millipore® filter. The fluorescent materials were then dissolved from the filter with toluene and the fluorescence measured by a Turner Fluorometer. Sampling was done hourly during the first two weeks and every two hours thereafter when it was determined that the concentrations were stable. The chambers were also monitored for total benzene concentration. This was done daily during the first two weeks and twice per week thereafter. The average chamber benzene concentration was 10.7 mg/m^3. At no time did the concentration exceed 19 mg/m^3 in any chamber (below the threshold limit value for benzene).

Aerosol particle size determinations were done on each chamber at the beginning and once per month during the study. The results were similar to the preliminary study, with 95% of the total droplets being 5 micrometers or less in diameter.

Body weight measurements during and after the aerosol exposure showed a weight-gain suppression very similar to that seen in the 10 mg/m^3 animals in the preliminary study. The mean body weights of all animal groups remain statistically lower than their respective controls two months postexposure.

Table 4 shows a summary of the lung and hide fluorescence done on the CF-1 mice serially sacrificed during the study. If the lung control value (64) were subtracted from the test values at one and seven days, the values would be approximately 30 and 200 which would show a 7-fold increase in coal tar deposition. A direct time-dose relationship can be seen in the amount of coal tar deposition as days of exposure increase. The amount of fluorescence found in the lungs of the mice sacrificed after 30 and 60 days postexposure shows that the mice were able to clear a considerable amount of the coal tar after termination of the study. The 90-day results have just been received and the value of the exposed group of mice is approximately one half the value seen at 30 and 60 days. It appears that the coal tar continues to be cleared from the lungs.

The amount of fluorescent compounds found on the hide remained relatively constant throughout the study. When calculated on a $\mu g/cm^2$ basis, the values at each of these periods ranged from 20 to 35 $\mu g/cm^2$. A Millipore filter placed in a chamber for 21 hours as an inert control showed 30.4 $\mu g/cm^2$ of coal tar deposition. Therefore, it appears that only a small amount of coal tar deposited on the skin is removed by grooming during the first 24 hours of exposure. Thereafter, it appears that an equilibrium is reached in the amount of coal tar removed by preening and the amount being deposited.

TABLE 4. SUMMARY OF TISSUE FLUORESCENCE OF MICE EXPOSED TO 10 mg/m³ COAL TAR AEROSOL (EXPRESSED AS μg COAL TAR/g TISSUE)

Tissue	Treatment	Days of Exposure 1	7	30	60	90	Postexposure 30	60
Lung	Control	64	64	84	41	68	54	58
Lung	Exposed	95	268	752	688	2250	444	405
Hide	Control	0	0	0.56	0	0	1.45	0
Hide	Exposed	284	366	178	274	241	1.38	0

0 = Less than 0.5.

Ten percent of the hamsters and 10% of each rat group were sacrificed at the conclusion of the 90-day aerosol inhalation period. These animals were submitted for gross and micropathological examinations and they also had sections of lung removed for analysis of coal tar deposition shown in Table 5. The values for the exposed animals, in every case, indicate good deposition of the contaminant in the lungs. The value for mice at 90 days, Table 4, was 2250 which falls in line with the values of these species.

TABLE 5. SUMMARY OF LUNG FLUORESCENCE OF RATS AND HAMSTERS SACRIFICED FOLLOWING 90 DAYS CONTINUOUS EXPOSURE TO 10 mg/m³ COAL TAR AEROSOL (EXPRESSED AS μg COAL TAR/g LUNG TISSUE)

Species	Sex	Treatment	Number Examined	Mean Fluorescence
Yearling Rats	F	Control	8	5.9
Yearling Rats	F	Exposed	8	2691.0
Weanling Rats	M	Control	4	4.5
Weanling Rats	M	Exposed	4	2147.0
Weanling Rats	F	Control	4	4.1
Weanling Rats	F	Exposed	4	962.0
Hamsters	M	Control	10	24.5
Hamsters	M	Exposed	10	1784.0

The continuous exposure of animals to coal tar aerosol appears to stimulate some changes in lung pathology and cause carcinomas of the skin in mice. The gross and micropathologic results, along with the carcinogenic effect, of both the preliminary study and the 10 mg/m^3 study are the subject of the following paper and will be discussed in depth by Dr. McConnell.

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PAPER NO. 14

LESIONS FOUND IN ANIMALS EXPOSED TO COAL TAR AEROSOLS*

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INTRODUCTION

The materials used and methods of exposure to the coal tar volatiles were described in the previous presentation. We will describe the pathologic lesions found in these animals which were felt to be related to the exposure. Also included are some of our preliminary results of a second study, in which the animals were exposed to 10 mg/m^3 of a similar compound.

In this cooperative study with NIOSH, incidental animals dying during the exposure were necropsied and evaluated by the Pathology Branch (THP) of the Toxic Hazards Division, Wright-Patterson AFB. Periodic animal sacrifices were performed jointly and the tissues evaluated by NIOSH and THP.

The only alteration to this protocol was after skin tumors were first discovered, at which time it was decided that all skin tumor cases would be examined in-house. The remaining cases continued to go to NIOSH for evaluation.

Although the information presented is incomplete because all of the material is not ready for evaluation, there have been some significant results to date.

*This work was partially funded under an Interagency Agreement with National Institutes of Occupational Safety and Health No. PHS 2-3273123.

METHODS

The primary target organ to be investigated was the lung, but evaluation of other major organs was also performed. The skin became of importance during the study when it was shown, in an independent study, that coal tar aerosols stimulated skin tumors when painted on the skin. Therefore, we decided to sacrifice only 10% of the rats and hamsters immediately following termination of the exposure, and to hold the remaining animals for evaluation at a later date.

RESULTS AND DISCUSSION

Rats and Hamsters

Three significant lesions were observed in the 10% sacrifice. First, in the lung there were large numbers of alveolar macrophages which contained both 0.5 micron black ovoid isotropic granules and 0.2 micron amber droplets. This phagocytized material was interpreted as coal tar pigment because it was not found in the control animals and stains for iron and lipofuscin were negative. The pigment was more apparent in hamsters than rats, but was readily seen in both. These pigmented macrophages were also found admixed with other mononuclear cells in the peribronchial lymphoid tissue.

The only other pulmonary disease that was encountered at this time was in rats which had chronic respiratory disease or mycoplasmosis. This is found to a variable degree in most laboratory rat colonies.

Secondly, the liver was grossly more brown than normal, and histopathologically this was represented by pigment-laden macrophages (Kupffer cells). Because this pigment was positive with iron stains, it was assumed to be hemosiderin. It was more apparent in the hamster than in the rat, but was seen in both. The kidneys showed a similar change; here the hemosiderin was restricted to the cytoplasm of the epithelial cells lining the proximal convoluted tubules. Approximately equal amounts were seen in both hamsters and rats. Hemosiderosis is usually the result of hemolysis. Hemolysis must have been very mild or episodic, because hematology parameters did not show evidence of anemia. Also, the hepatic and renal hemosiderosis disappeared by 100 days postexposure.

Thirdly, there was mild central lobular necrosis in the liver. Because it was found only in the exposed animals, it was interpreted as being a result of the coal tar volatile exposure.

Mice

Ninety-nine days postexposure, a significant number of exposed mice had spontaneous skin tumors already referred to. At this point it was decided to sacrifice 50% of the remaining mice that were exposed to 20 mg/m^3 coal tar, and a comparable number of controls. Examination of the lungs from the exposed mice revealed that there was still moderate pigmentation of alveolar macrophages, although less than in the rats and hamsters of the earlier sacrifice. An interesting observation was that alveolar macrophages showed significant pigmentation in 14 of 15 CF-1 mice, but in only 1 of 13 exposed JAX mice. We were unable to explain this but have postulated that there are strain differences such that JAX mice are better able to clear this material from their lungs than CF-1 mice.

Both experimental and control mice had multiple alveolargenic adenomas or carcinomas similar to those commonly found in the lungs of mice. These tumors were all similar in their morphology, consisting of well circumscribed but nonencapsulated clumps of cuboidal cells which line thin alveolar-like stromal septae. Electron microscopic examination of these tumors by Major McNutt (THP) has shown that the tumor cells contain numerous viral particles; again, this is comparable to what has been reported in the literature.

There were no significant differences in the number of pulmonary neoplasms between the control and the experimental JAX mice (3 of 14 and 3 of 13, respectively). In the CF-1 mice there was only one pulmonary tumor found among 14 controls; however, 7 of 15 experimentals showed them. The significance of this data has been somewhat moderated in subsequent findings where the incidence appears to be about equal in the controls and experimentals. The importance of these observations will have to wait until the total data become available. Focal areas of cuboidal metaplasia of the alveolar epithelium were also noted. Again there was a difference in the CF-1 experimentals versus the controls, but not as great as with the tumors. If this difference is real, it may be justifiable to relate this back to the better ability of JAX mice to clear the coal tar pigment from the lung than the CF-1 strain. However, we wish to stress that not all data are available yet. To date pulmonary neoplasms have not been observed in other species of animals used in this study, i.e., rats, hamsters or rabbits.

It should be emphasized that skin tumor bearing mice were purposely selected to fill the 50% quota of experimentals sacrificed 99 days postexposure. Prior to this special sacrifice there were 8 mice with skin tumors, 6 JAX and 2 CF-1 (Figures 1 and 2).

Figure 1. CF-1 mouse showing keratinaceous tumor on left ear.

Figure 2. CF-1 mouse with large horn-like growth which arose from the right ear and which has almost completely eroded away the side of the face.

Seven of these tumors were interpreted as squamous cell carcinomas and one as a squamous papilloma, based on the classic histopathologic criteria. Since that sacrifice several other mice have developed tumors (Table 1). We were interested in studying the behavior of the tumors as to spontaneous regression, invasive qualities, and metastasis. With this in mind, it was decided not to kill the remaining animals with skin tumors, but rather to allow them to live out their normal life spans.

Since then several of the skin tumors have been examined histologically, and we have concluded that a whole spectrum of epithelial tumors, from squamous cell papilloma to keratoacanthoma, to frankly aggressive appearing squamous cell carcinomas are stimulated by the coal tar volatiles, although the majority of these tumors fall in the squamous cell carcinoma category (Figures 3-5). None of these various histologic types have been shown to regress spontaneously but there is also no evidence of metastasis. Histologically and grossly, they resemble very closely those tumors that are produced by skin painting with the various benzanthracene and benzopyrene type compounds (Arcos et al., 1968). The occurrence of new skin tumors continued during the next 322 days making the range 93 to 415 days postexposure altogether. However, almost all the tumors had developed by 199 days postexposure, with only an occasional tumor having been found since (Table 1).

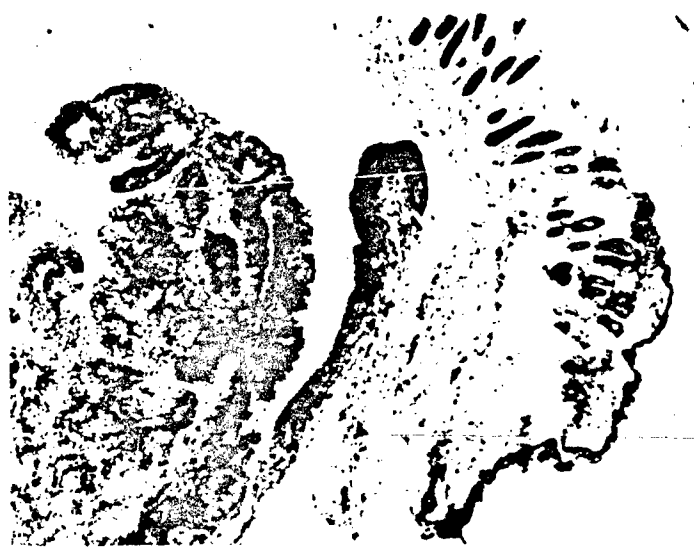

Figure 3. Photomicrograph of the edge of the tumor shown in Figure 1. Relatively normal skin on right with papilliferous growth on left. (HE-X32)

Figure 4. Higher magnification of a different area of the same tumor showing invasion of subjacent muscle by this squamous cell carcinoma. Arrow points to a preexisting muscle cell within the tumor mass. (HE-X240)

Figure 5. Section through base of same tumor showing large anaplastic cell invading adjacent connective tissues. (HE-X480)

TABLE 1. SKIN TUMORS IN MICE EXPOSED TO CTV-I
(Chart showing number of skin tumors in mice found on various examinations. Number in lower left of each square represents the total number of mice in each group at the time of examination, while the number in the upper right corner denotes the total number of animals with tumors to date.)

	ICR 20 mg/m³	JAX 20 mg/m³	ICR 10 mg/m³	JAX 10 mg/m³	ICR 2 mg/m³	JAX 2 mg/m³	ICR 0.2 mg/m³	JAX 0.2 mg/m³	ICR Control	JAX Control
9 Nov 72 (183 days)*	2/36	6/27	0/8	0/12	0/25	0/47	0/2	0/47	0/62	0/74
13 Dec 72 (218 days)	6/18	7/11	1/7	0/12	0/20	0/47	0/2	0/46	0/43	0/57
2 Jan 73 (238 days)	6/18	7/10	1/4	0/11	1/19	0/47	0/2	0/44	0/43	0/57
24 Jan 73 (260 days)	7/15	7/8	2/4	0/10	1/18	0/44	0/2	0/42	0/40	0/57
9 Feb 73 (276 days)	7/14	8/8	2/4	0/10	1/15	0/44	0/2	0/42	0/38	0/57
22 Feb 73 (289 days)	9/14	8/8	3/4	0/10	1/15	0/44	0/2	0/41	0/38	0/57
8 Mar 73 (303 days)	9/14	8/7	3/3	0/10	1/15	0/42	0/2	0/41	0/37	0/56
22 Mar 73 (317 days)	9/13	8/7	3/3	0/10	1/13	0/42	0/2	0/38	0/36	0/55
6 Apr 73 (332 days)	9/12	8/6	3/3	0/10	1/10	0/42	0/2	0/38	0/36	0/55
20 Apr 73 (346 days)	9/10	8/6	3/3	0/8	1/10	0/40	0/2	0/35	0/36	0/52
3 May 73 (369 days)	9/10	8/6	3/3	0/7	1/8	0/40	0/2	0/35	0/36	0/52
15 May 73 (371 days)	9/9	8/6	3/3	0/6	1/8	0/40	0/2	0/30	0/36	0/52
13 June 73 (400 days)	10/6	8/4	3/3	0/4	[illegible]/6	0/38	0/0	0/20	0/30	0/46
11 July 73 (428 days)	10/4	8/4	3/3	0/4	1/6	0/30	0/0	0/17	0/21	0/40
13 Aug 73 (461 days)	10/2	8/3	3/1	0/4	1/2	0/22	0/0	0/16	0/13	0/36
29 Aug 73 (477 days)	10/2	8/3	3/1	0/4	2/2	0/22	0/0	0/13	0/13	0/36
11 Sep 73 (490 days)	10/2	9/3	3/1	0/4	2/2	0/16	0/0	0/13	0/13	0/32
26 Sep 73 (505 days)	10/2	10/3	3/1	0/4	2/2	0/14	0/0	0/11	0/12	0/26

*Number of days postexposure plus 90 days exposure.

A summary of the skin tumor incidence from these mice is shown in Table 2. As of 26 September 1973 (415 days postexposure plus 90 days exposure = 505 days), there was an incidence of 32% in the 20 mg/m^3, 2% in the 2 mg/m^3, and none in either the 0.2 mg/m^3 or controls. This suggests a definite dose response.

TABLE 2. MICE SKIN TUMOR INCIDENCE: CTV-I
(As of 26 September 1973 - 415 Days Postexposure)

20 mg/m^3	-	32%	(20 of 63)
10 mg/m^3	-	15%	(3 of 20)
2 mg/m^3	-	2%	(2 of 72)
0.2 mg/m^3	-	0%	(0 of 49)
Control	-	0%	(0 of 136)

There appears to be a predisposition for the CF-1 strain of mice to develop skin tumors at least at the 10 mg/m^3 and lower concentrations (Table 1). The only JAX mice that showed skin tumor development were those exposed to 20 mg/m^3. This was substantiated in the second study (10 mg/m^3) where only CF-1 mice have shown tumors to date (Table 3).

TABLE 3. LATENT PERIOD OF TUMOR INDUCTION
IN CTV-I EXPOSED MICE

20 mg/m^3	<93
10 mg/m^3	128
2 mg/m^3	142

There also appears to be a time-dose relationship for these tumors. It was not known when the first tumor occurred in the 20 mg/m^3 group, but it was sometime prior to 93 days postexposure since that is when the first tumors were observed. After this all mice were examined at 2-week intervals for skin tumors. The first tumor in the 10 mg/m^3 group did not occur until 128 days postexposure and the first tumor in the 2 mg/m^3 group was not until 148 days (Table 4).

TABLE 4. MICE SKIN TUMOR INCIDENCE: CTV-II (10 mg/m^3)
(Exposure Started 2 April 1973)

	New Tumors	Total	Number Observed*	Days - Postexposure
13 August 1973	1	(1)	164	44
29 August 1973	0	(1)	162	60
11 September 1973	3	(4)	153	73
26 September 1973	3	(7)	149	88

*No tumors in JAX mice to date.

Following the first study, a second study (Coal Tar Volatile II) has been instigated using a 90-day continuous exposure to 10 mg/m^3. The data are just now starting to become available. The first skin tumor that was grossly observable occurred 41 days postexposure. Two weeks later there were no new tumors; however, 3 mice with tumors were found 2 weeks later (69 days postexposure) and 3 more 2 weeks after this (84 days) (Table 4). It was felt that this time ($\pm$ 69 days) represents a more accurate figure of the latent period. These tumors were all in CF-1 strain and this again justifies the speculation that there is a strain predisposition to these skin tumors. One should note that in most carcinogenic studies using mice, there are often strain differences to the carcinogen.

An ulcerated skin tumor was found on the posterior abdomen of one yearling rat exposed to 20 mg/m^3. Microscopically it was compatible with a squamous cell carcinoma, although there was no evidence of metastasis. Since this was the only skin tumor found in any of the rats in this study, it was felt that no definite correlation could be made between CTV exposure and cutaneous neoplasia in this species.

Tumors were found on the inside of the ears of two rabbits in the second study (10 mg/m^3). One was found 8 days and the other 89 days postexposure. They were keratinized "wart-like" growths similar to those observed in the mice. They grew rapidly in size for about 30 days, the largest being a hemispheric shaped growth of 3 cm diameter and 1 cm in height. They remained static in size for 14 days, then decreased rapidly and completely disappeared during the next 40 days. Because the growth behavior was felt to be more important than the microscopic morphology, histopathologic examination was not attempted.

It is beyond the scope of this study to deal in detail with the question of the pathogenesis of the various types of tumors. However, since the coal tar volatiles contain a certain amount of benzopyrene (Fannick et al., 1972; and National Academy of Sciences, 1972), it can be postulated that this is probably the portion of the compound that is carcinogenic. It is also known that skin tumor induction in mice is related to the hair growth cycle, i.e. during the resting phase of hair growth they are more susceptible (Arcos et al., 1968). The reason for this is that the carcinogenic substance is in contact with the target cell, in this case the epithelial cell, for a longer period during the stage. The hair growth cycle is approximately 30 days in mice. Therefore, a continuous exposure of 90 days would expose the mouse during the resting phase at least twice.

It is too early at this point to make any definitive statements about the pathogenesis of the pulmonary tumors.

SUMMARY

In summary, there are four points that can be stated with regard to coal tar volatiles:

- First, they appear to be a significant skin carcinogen for mice and probably less so for rats and rabbits.
- The carcinogenic potential appears to be both time- and dose-related.
- The skin tumors in mice once started do not regress spontaneously and show no evidence of metastasis. In contrast, the tumors in rabbits appear to spontaneously regress.
- Finally, the carcinogenic effect is probably due to the benzanthracene-type compounds within this material.

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PAPER NO. 15

IN VITRO METHODS FOR ASSESSMENT OF TOXICITY AND CARCINOGENICITY

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The state of knowledge of the application of isolated mammalian organs or cells to questions in cell biology, genetics, mutagenesis, teratogenesis and carcinogenesis is advanced enough to recommend that such systems be developed for use in evaluating toxic and carcinogenic effects of environmental chemicals. These systems are most likely to be useful in short-term studies and need to be employed in parallel with studies in intact animals where possible in order that we may build a framework of comparative data with which to judge the relative sensitivity, cost, and utility of in vitro systems. The test systems I will identify may be only a fraction of the number and variety that can be employed. The value at this time of these systems does not depend on their replacing animal and human exposure models unless they prove suitable as substitutes. Their present value lies in serving as adjunctive probes of the biologic action of environmental chemicals with the view to assessing their status as substitutes for animal studies.

CELL CULTURE

Cell culture methods have been applied to a large range of critical biological questions. It is the basic technology for studies of neoplastic transformation by viruses and a variety of chemical carcinogens. Conditions for isolating, maintaining, and growing cells have become highly developed and some characteristics of cells as individuals and as populations are being elucidated. A major gap in this development is in the isolation and the maintenance of cultures of epithelial cells as compared to connective tissue or fibroblastic cells. Epithelial cells are the first cells to be hit upon exposure to environmental chemicals and are the major cell types which metabolize, detoxify, and excrete environmental chemicals. The liver epithelial cell, or hepatocyte, is a major cell type responsible for metabolism and detoxification. Epithelial cells in other organs, including those directly affected in environmental exposures such as lung and skin, are also capable of detoxifying and metabolizing such chemical carcinogens as benzo(a)pyrene to levels of

biological effectiveness whether or not metabolism in these epithelia proceeds to detoxification. Most cell cultures used in carcinogenesis have been fibroblastic cells. These are situated below the surface epithelia and would probably not make the first contact with environmental agents. Studies in epithelial cells have been deficient for a number of reasons, including the difficulty of culturing such cells free from fibroblasts. Development of culture methods for epithelial cells is a high priority objective in research in cancer, lung disease, and environmental toxicology.

Problems arising from the use of epithelial cells as compared with fibroblastic cells are illustrated by a series of experiments in which we attempted to isolate epithelial cells separately from connective tissue cells of the trachea of the mouse. This animal was chosen because a species specific sarcoma virus, Harvey Mouse Sarcoma Virus (HMSV), was available. In the absence of an epithelial transforming virus of mice, this virus was chosen on the hypothesis that an oncogenic virus might transform any cell type without changing the identity of that cell type. These studies were to determine whether HMSV would act in a transforming fashion on cultured mouse tracheal epithelial cells. Parallel experiments were done with fibroblasts from mouse trachea. Both cell types were treated with benzo(a)pyrene as a test of the chemical transformability of the two systems. HMSV was added alone or in combination with benzo(a)pyrene to seek synergism.

Two questions were under examination: can epithelial cells be isolated and can they be transformed by either or both oncogenic agents. Benzo(a)pyrene was toxic to both cell types but transformed only fibroblasts. Harvey Mouse Sarcoma Virus applied on cells that appeared to be epithelial produced apparent transformation by inducing a cell population of irregular morphology. The cells did not appear to be fibroblasts but upon inoculation in animals the presumptive epithelial cells treated with HMSV produced fibrosarcomas.

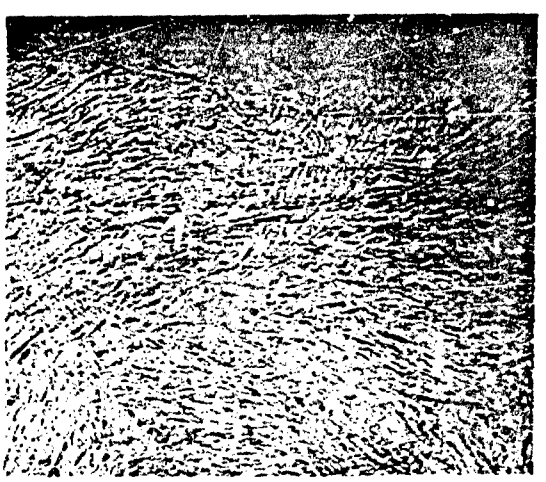

Figure 1. A sheet of fibroblasts which were cultured in parallel with epithelial cells. Phase contrast photomicrographs, low power. Cells are in the fairly orderly arrangement of parallel spindle-shaped fibroblasts.

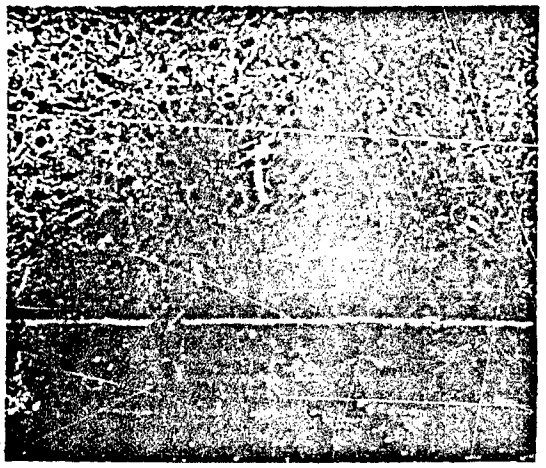

Figure 2. The effect of the treatment of fibroblasts in cell culture with Harvey Mouse Sarcoma Virus. Viral transformation produces foci of abnormal cells within an apparently normal sheet of cells. Cells in foci exhibit irregularity of cell-to-cell relationships, cells lack a spindle shape, and lack formation of typical fibrillar sturctures, presumably collagen.

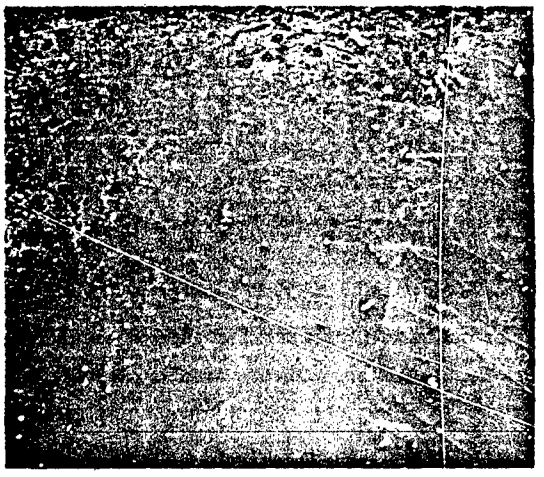

Figure 3. The rather orderly pattern of presumptive epithelial cells which are arranged in a mosaic of roughly hexagonal cells. These cells had been observed from the first days of culture of cells that had been isolated by trypsination from the interior of tracheas. Islands of epithelial-like cells were formed initially. Those islands grew, coalesced and formed a confluent sheet of cells having the rounded or hexagonal appearance of individual cells lying in a close side-by-side pattern without evidence of fibrillar structure. Upon treatment with HMSV, small groups or foci of rounded cells appeared within the sheet. These foci were different from the foci that appeared in sheets of fibroblasts upon treatment with the transforming virus.

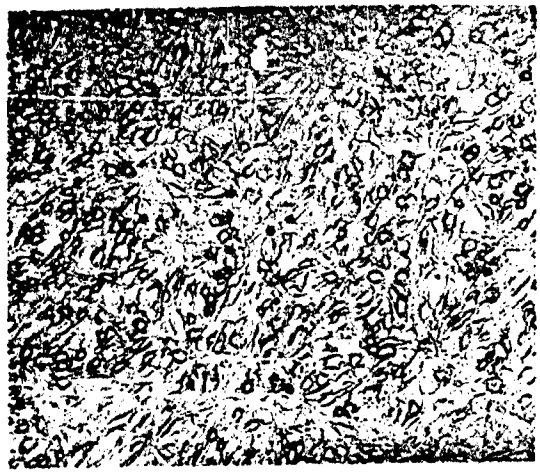

Figure 4. A monolayer of cells of irregular shape and without orderly arrangement emerged from the presumptive epithelial sheet treated with the transforming virus. Some cells are still in a hexagonal or rounded form, but some are elongated and have apparent extensions, although they are not spindle-shaped cells. This was believed to be an acceptable morphological alteration produced in an epithelial sheet by an oncogenic agent although there is little precedent for this conclusion because there has not been prior experience with the cultivation of transformed (neoplastic) tracheal epithelial cells. Transformation of epithelial cells by the chemical carcinogen alone did not occur and there seemed not to be any significant change in the extent of transformation by the virus when the chemical was added to it.

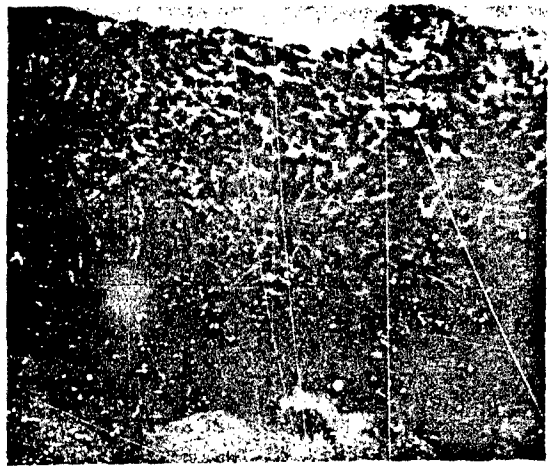

Figure 5. When the presumptive epithelial cells transformed as shown in Figure 4 had been grown to a large enough number to be sub-inoculated subcutaneously in isologous mice, tumors were formed. These were sarcomata as indicated by the pattern of arrays of elongated cells having parallel orientation. The whorled arrangements are characteristic of fibroblastic cells.

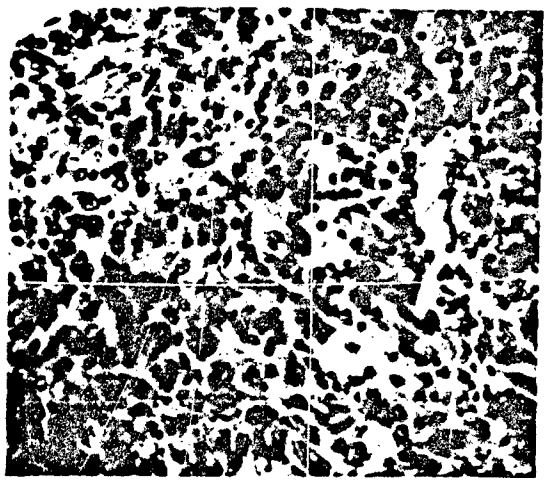

Figure 6. Some of the cells in the sarcomas are large and were regarded as a possible epithelial component. The possibility was considered that a mixture of two cell types had been present in the culture leading, upon inoculation of cultured cells, to mixed epithelial and fibrosarcomatous tumors. Consultations with histologists and pathologists experienced in mouse pathology led to the diagnosis of anaplastic fibrosarcomas containing epithelial-like cell elements which were regarded as histiocytes.

The findings presented thus far can be summarized as follows:

1. Epithelial cells, suitable for studies of environmental toxicity or carcinogenicity in cell culture, may be difficult or impossible to isolate and maintain as pure cultures of epithelial cells.

2. The presumptive epithelial cells obtained in these cultures were susceptible to toxic destruction by benzo(a)pyrene, hence they were able to metabolize this compound to an active form.

3. Morphologic and presumptive neoplastic transformation of presumptive epithelial cells did not occur in cell culture after exposure to benzo(a)-pyrene.

[The word "presumptive" is used to indicate uncertainty of the purity of the epithelial cell culture (i.e., its freedom from contamination by fibroblasts) and to indicate uncertainty as to the appearance of transformed epithelial cells if a pure culture did, in fact, undergo morphologic or neoplastic transformation by benzo(a)pyrene.]

4. Morphologically atypical cells did replace the cells of regular morphology in cultures of presumptive tracheal epithelial cells infected with mouse sarcoma virus and the atypical cultured cells produced fibrosarcomas upon animal inoculation. This result indicates either that epithelial cells were altered by a sarcoma virus to become fibrosarcoma cells or that fibroblasts were present in unsuspectedly large numbers in an otherwise presumptively pure epithelial cell culture and the fibroblasts, transformed by the sarcoma virus, were the origin of fibrosarcomata.

The first interpretation raises new problems because it suggests that epithelial cells do not "breed true" if acted upon by a sarcoma virus. Moreover, the sarcoma virus would appear to be able to convert epithelial cells to a neoplastic state but only by "changing" epithelial cells to fibrosarcoma cells. These are issues for basic cell physiology and pathology rather than for toxicology.

The second interpretation above, which states that fibrosarcomas were produced by the transformation of fibroblasts contaminating an epithelial culture, does raise a problem for toxicology if cell cultures are to be used for toxicologic studies. In vitro systems for use in questions in toxicology as well as in cell biology should be specifiable as to the cell type present in culture. Identity of cell types in cell culture is important not only for the branch of toxicology that deals with environmental carcinogenesis but also for conclusions about the toxic potential of a test substance on epithelial vs. connective tissues.

Some conclusions regarding cell cultures as applied in toxicology would be as follows:

1. Cells and organ pieces do not represent whole animals, but target cells and pieces of target organs may represent suitable test objects for analysis of environmental, chemical and biologic agents.

2. If cells are to be used, they should be as representative of the test animal as possible. Hence, target cells of any type, including epithelial cells, should be obtainable in quantity and in a pure state, or at least in an identifiable state.

ORGAN CULTURE

Identity of cell type in cell culture is a problem that is not encountered in organ culture. This in vitro method has been used for studies of toxic and carcinogenic materials of environmental origin.

We have done organ culture of rodent, simian, and human tracheobronchial structures in order to compare responses of epithelia of these organs to comparable concentrations of polycyclic hydrocarbons under comparable conditions of organ culture. The technique of organ culture differs from cell culture in that the organ is retained with all of the cell types in their normal histologic relationship.

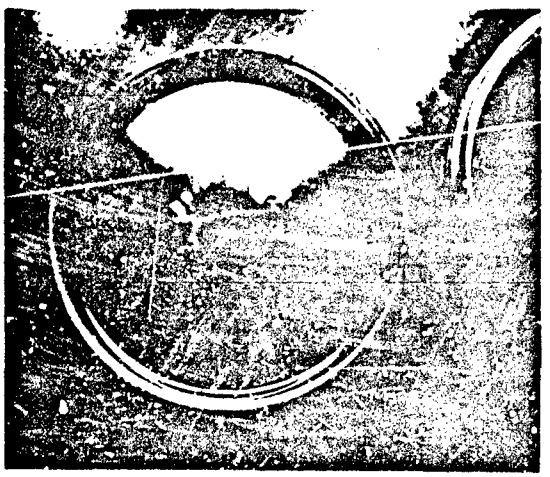

Figure 7. This is a complete tracheobronchial tree from a primate dissected as completely as possible from larynx to some branchings of the bronchi. The animal was a late fetal Bush Baby (Galago crassicaudatus, Family Pro-simii). The fetus was old enough to be free-living. It was taken at this stage in order to assure completely sterile tissue.

Figure 8. This small organ was subdivided into a number of pieces, some of which are lengthwise bits of several small bronchial branches as they may lie beside one another. Whole tracheal pieces were opened lengthwise for use as plaques.

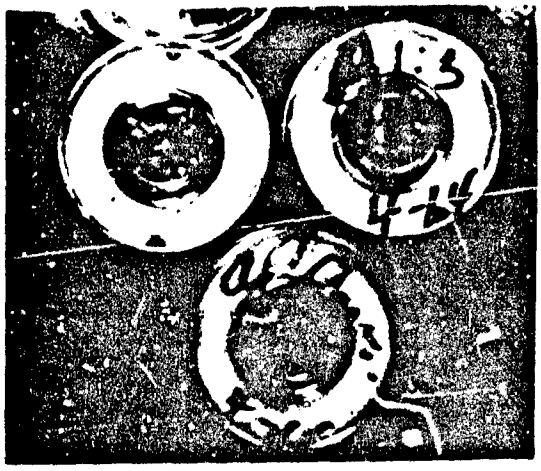

Figure 9. Plaques or tubular structures are shown in a culture vessel. Medium is in the center well and a pad of moist filter paper is in the outer well to give a moist chamber. The bits of the organ are placed on a carrier of rayon mesh. The group of organ pieces can be moved to fresh medium by grasping the mesh. A box gased with 50% oxygen and 3% CO_2 is maintained at 37 C in an incubator.

Figure 10. A low power transmission electron micrograph of hamster trachea shows cilia and microvilli on the surface of the cells with a normal vertical columnar orientation. Basement membrane lying between epithelium and connective tissue is well defined. The orderly arrangement of cell types is maintained over a period of up to three weeks in organ culture.

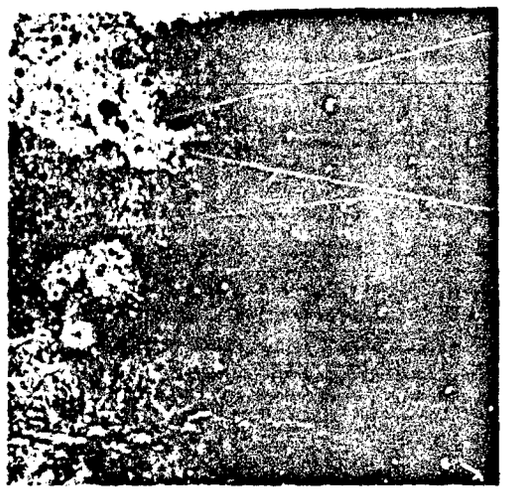

Figure 11. Hamster trachea, cultured in the presence of benzo(a)pyrene added to medium at a concentration of 10 micrograms per cubic millimeter (10^{-5} molar), destroys most connective tissues. Nuclei are rare and fragmented below the basement membrane. The epithelium is disorderly but living, and columnar cells are absent. Pleomorphic cells are present in a layer three to five cells deep. Such cells have lost most surface structures such as cilia and microvilli and secretory goblets of mucous are absent.

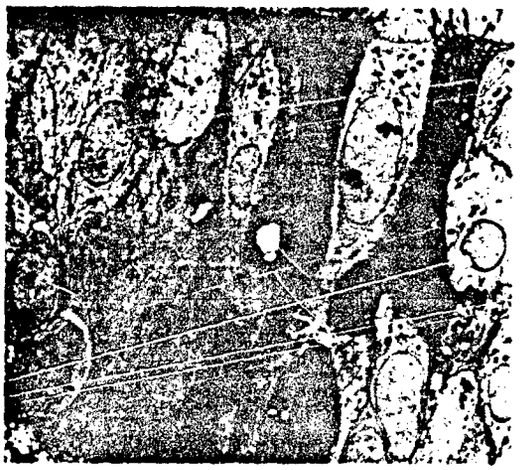

Figure 12. Human fetal tracheas were obtained at the fifth month of gestation by hysteromyotomy from patients undergoing abortion at a late stage in pregnancy for reasons of maternal health. In advanced human fetuses, the respiratory tract reaches a state of complete differentiation. This low-power electron micrograph shows a well-differentiated columnar epithelium.

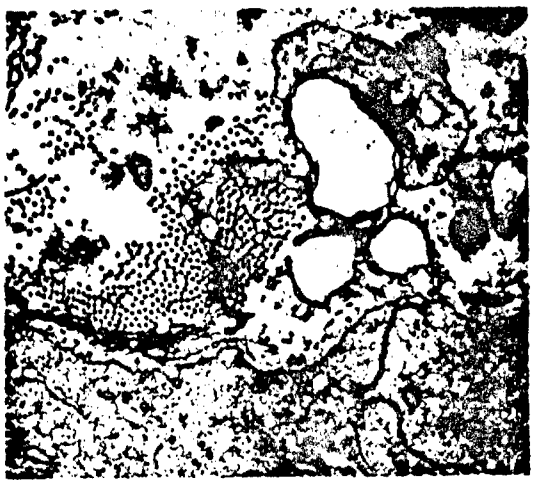

Figure 13. The basement membrane in this low-power electron micrograph is seen as a granular track at a uniform distance from the lowest portion of the cell wall of epithelial cells. The basement membrane was retained in this organ culture for three weeks.

Figure 14. Tracheal explant from an advanced human fetus exposed to the same concentration of benzopyrene which produced lesions in Figure 11 in the hamster trachea. The columnar state of the epithelium is disoriented, surface organelles are lost, and cell walls have deep intercellular interdigitations, analogous to those seen in squamous epithelia. This state can be interpreted as squamous metaplasia consistent with an early phase of response to irritants, carcinogens, and other influences that damage epithelia.

Figure 15. Lack of continuity of basement membrane is shown in human fetal trachea cultured in the presence of benzo(a)pyrene, 10^{-6} M. Numerous sections were examined to determine whether or not discontinuity was a mechanical artifact although such artifacts are rare in well-imbedded plastic material. This defect of the basement membrane was found to be general in human trachea exposed to the carcinogen for three weeks. This same lesion is present in hamster trachea exposed under the same conditions of organ culture.

Metaplasia of epithelia and interruption of basement membrane occur in human and hamster tracheas maintained in organ culture and exposed to benzo(a)pyrene. The same lesions have been found in living hamsters exposed to benzo(a)pyrene by intratracheal installation by a regimen that produces bronchiogenic carcinoma in the hamster lung, bronchi and trachea. The occurrence of similar lesions in hamster trachea in vivo, in hamster trachea in vitro, and in human trachea in vitro indicates that there is a parallel sensitivity of human and hamster epithelium to the effect of this environmental carcinogen. Parallelism between the lesions in the hamster system in vivo and in vitro supports the notion that data from organ culture could be used to predict an effect in vivo in hamsters. The further correlation between the short-term lesion in the animal and the long-term emergence of cancer following exposure to benzo(a)pyrene further supports the concept that the short-term lesion is relevant to carcinogenesis in the animal. The short-term lesion produced in the course of carcinogenesis in the living animal and produced after a three-week period of organ culture is therefore proposed as the standard against which to match the human epithelial lesion in the short term culture of human trachea. By this criterion the hypothesis is proposed that the human respiratory tract, exposed to benzo(a)pyrene in vivo would develop cancer. It is further proposed that human fetal trachea would be as susceptible to carcinogenesis as adult hamster trachea.

The one missing element is that data cannot be had to extend the in vitro data to living man. The remaining unknown is the effect of long-term human exposure to benzo(a)pyrene. The organ culture represents, therefore, a system having potential for the application of in vitro methods in short-term studies to the determination of risk of carcinogenicity in man. Application of organ culture on a wide enough scale to fulfill the demand for testing of potential carcinogens would require an organized approach to the procurement of fetal or other normal human tissue that would clearly strain our resources.

This presentation has illustrated a direct approach to the use of in vitro systems in one area of concern to the field of toxicology. The conclusions are that technologic and procurement limitations upon general use of direct tissue-chemical interaction work are severe and that extensive development would be needed to overcome them. The most useful finding is that these data, even if obtained from a limited number of animal in vivo-in vitro and human in vitro studies, support the concept that hamster observations on carcinogenesis in the respiratory epithelium are relevant to human respiratory carcinogenesis by benzo(a)pyrene. This is by no means a negligible contribution to the translation of animal data to applicability for man. Thus, while in vitro methods cannot at this time replace animal studies, in vitro methods do permit comparisons between animal and human tissues. The most relevant system for this purpose is the organ culture method.

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PAPER NO. 16

THE INFLUENCE OF ALCOHOL ON CARBON MONOXIDE TOXICITY IN HUMANS

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INTRODUCTION

The experiments described in this report were conducted at the Ohio State University from January to August, 1973. The study was supported jointly by the Environmental Protection Agency and the Coordinating Research Council. The experiments described are part of a continuing study designed to assess the effects of carbon monoxide on men performing tasks related to safe and efficient driving. In the present state of the investigation, we were to evaluate the effects of carbon monoxide on persons who were dosed with ethyl alcohol.

METHODS

One of the goals of the research was to relate any observed effects to carboxyhemoglobin (COHb) levels rather than to atmospheric carbon monoxide (CO) exposure levels. In the original series of experiments, we had studied subjects with COHb levels of 7-8%, 12-14%, and 20-22%. As the program developed, all effort was directed to lower level exposures, since it was agreed by both sponsors and researchers that motor vehicle drivers would seldom experience COHb levels in excess of 10-12% (Weir and Rockwell, 1973). Therefore, in the present series of experiments, target levels of COHb were:

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1. Control level of 1-2%
2. A low exposure level of 6-7%
3. A high exposure level of 10-12%.

For the experiments involving ethyl alcohol (ETOH), consideration was given to several important areas in the choice of dose. On one hand, we were interested in assessing the contribution of blood alcohol levels (BAL's) that would not completely mask any CO effect that might be present. On the other hand, we wanted to investigate BAL's that would be sufficient to produce at least a threshold effect on performance. A third and important consideration was to balance these experimental requirements against the ethical considerations of the Medical Review Committee at the Ohio State University and the insurance carrier. As a result of these considerations, the target BAL in this series was 0.05% (50 mg/100 ml blood).

Subjects used in this series were paid volunteers, male and female, age 21-28. All subjects were professed nonsmokers with essentially uneventful driving histories. All had to pass standard physical examinations conducted in our laboratory.

From the 90 applicants responding to our advertisements, we selected 20 males and 3 females for use in various portions of the study.

Three separate experiments were conducted in this series. In each of these experiments, we attempted to maintain a double-blind situation; e.g., neither the subject nor the researcher dealing with the collection and interpretation of the data was aware of the dose levels of CO or ETOH being administered. Maintenance of the double-blind arrangement was not always possible with the alcohol experiments, since it was difficult to mask the presence of this material in any compatible diluent.

The three series of experiments consisted of (1) a driving study, (2) a parallel but independent laboratory study, and (3) a laboratory dose-response of alcohol experiment.

Experimental design in the driving investigation was a random order 2 x 3 block design using 8 subjects, consisting of a control, a low dose of CO, and a high dose of CO with and without the addition of ETOH as follows:

Control - COHb No Alcohol	7% COHb No Alcohol	12% COHb No Alcohol
Control - COHb 0.05% BAL	7% COHb 0.05% BAL	12% COHb 0.05% BAL

In this series, there were 48 experiments using 8 subjects for each of 2 levels of alcohol and 3 levels of CO.

For the second series of experiments conducted in the laboratory, we essentially used the same 3 block design but used only 6 subjects; e.g., six subjects for each of 2 levels of alcohol and 3 levels of CO exposure (36 experiments). For the dose response to alcohol experiments, we used a random order 4 x 4 block design using 4 subjects in a series of 16 exposures. The levels of ETOH chosen for this third exposure were control, 0.05% BAL, 0.05% BAL, and 0.08% BAL.

We exposed subjects to CO in a 2.43 cubic meter exposure chamber of the Edgewood type operated in a dynamic flow mode using 0.8 air changes per minute. The door of the exposure chamber was modified for collection of blood samples during the exposure. Nondispersing infrared spectrophotometry (NDIR*) was used for continuous analysis of the chamber atmosphere for CO. A second method was utilized periodically to assure the accuracy of the NDIR method. This method consisted essentially of a gas chromatographic separation followed by conversion of the CO to methane using a nickel catalyst and analysis of the methane with the flame ionization detector.

Blood from all subjects was routinely taken for chemical analysis of COHb. For most samples, capillary blood from a lanced digit was collected in heparinized, microhematocrit tubes. Approximately 0.04 ml of blood was adequate for each analysis. Blood from the cephalic vein was withdrawn from selected subjects for comparison of venous and capillary samples. The spectrophotometric method of Commins and Lawther (1965), modified as suggested by Buckwald, was used for all determinations.

For the CO exposures, subjects were exposed for 120 minutes to either air (for the control experiments), or 135 ppm CO (for the 7% COHb experiments), or to 240 ppm CO (for the 12% COHb experiments).

For any experiment in which ETOH was administered, one-half hour before the end of the CO exposure, the subject was requested to drink orange juice containing 10% ETOH. The volume of the cocktail was calculated on the basis of the subject's body weight. For control experiments, the subject was given orange juice without the alcohol added.

Blood alcohol measurements were conducted periodically on all subjects using the gas chromatographic method of Curry et al. (1966).

For the driving experiments, it was necessary to refresh the subjects each 30 minutes during the 2.5-hour road experiment. To accomplish this, subjects were administered either air or a mixture of air and CO (1000 ppm)

*Beckman model 215B infrared analyzer.

by mask from a pressure cylinder through a demand regulator. The quantity of gas administered was measured by passing all expired air through a volumetric gas analyzer. Calculations for quantity of gas to be administered were based on the Coburn formula (Coburn et al., 1965). Blood samples were collected routinely and stored for subsequent COHb and BAL determinations in the laboratory.

For the laboratory experiments, a series of simple psychomotor tasks were utilized. One task was a simple rail walking test described by Heath (1949). This test has been administered to innumerable subjects and has been described as being useful for measurement of all senses of coordination. A second test utilized in this series was the Minnesota Spatial Relations Test which has been described as being a measure of mechanical ability independent of verbal intelligence. A third test used in this series was the reversed image star tracing test reported to be valuable for assessment of fine coordination and tremor. All these tests are considered to be good indicators of central nervous system depression.

For the road test, a 1971 Chrysler was modified to allow use of the "eye movement camera" equipment developed by the Systems Research Group and described by Rockwell (1972). This equipment enables the accurate determination of driver eye movement to yield information regarding the driver's visual sampling behavior.

Experimentation conducted on the road consisted of a series of driving tasks conducted under two modes: the normal vision mode and the occluded vision mode. The normal vision mode is self-explanatory. In the occluded vision mode, the subject was requested to keep his eyes closed as much of the time that he felt was possible while maintaining headway and lateral position. The tasks employed were as follows:

a. Open road driving - 30 mph - normal vision
b. Open road driving - 30 mph - occluded vision
c. Open road driving - 50 mph - normal vision
d. Open road driving - 50 mph - occluded vision
e. Car following - 50 mph - normal vision
f. Car following - 50 mph - occluded vision.

All tasks were performed in low density traffic on limited access highways.

The voluntary visual occlusion tasks were of particular interest because they involved secondary loading of the driver, thereby consuming his "spare visual capacity." Generally, a driver is not very busy in an automobile and does not spend much of his time in a driving task. Under control conditions, some of our subjects could spend up to 80% of their time with their eyes shut. However, some of them could not spend more than 20% of the time with their eyes shut. These times were very consistent within a

particular subject but very inconsistent among the various subjects. We assumed that some subjects just accept a greater degree of risk than others under the same circumstances.

RESULTS

Statistical analyses of variance of results from the driving experiments have not been completed. However, preliminary evaluation of the data reveals little or no difference in driving performance between the CO series and the CO plus ETOH series at either of the COHb levels tested.

One interesting, but not statistically significant, difference was observed between control and the 7% COHb levels in the open road driving occluded vision tasks. The presence of alcohol appeared to shorten the "mean open time" per observation in both control and 7% COHb situations. Results of 4 subjects are summarized in Figure 1.

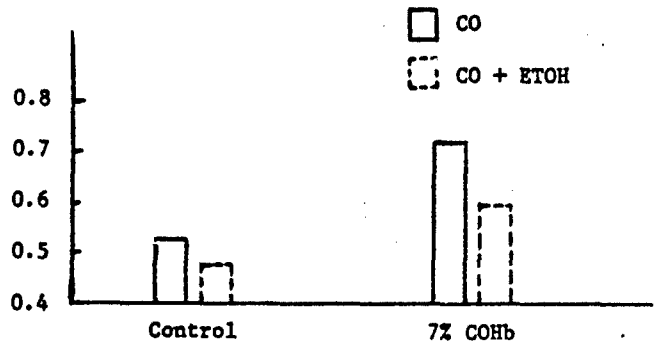

Figure 1. Effect of CO and CO + ETOH (4 subjects) on visual occlusion task.

The presence of alcohol appeared to depress the response to CO in this test. Perhaps alcohol allowed the subject to accept a greater degree of risk than normal. These results parallel many of the data collected in the driving research study.

In the laboratory experiments involving CO and ETOH, the 0.05% BAL did not remarkably influence the test performance of subjects at any

COHb level tested. The mean response under the influence of alcohol was usually decreased, but the variance was increased so that no statistically significant differences were noted.

Results of the dose response to alcohol experiments likewise demonstrated little or no effect attributable to the alcohol at BAL's up to 0.05%. However, there were significant ($p = 0.05$) reductions in performance when the BAL increased to 0.08%. The results of the spatial relations test and the rail walk test are summarized in Tables 1 and 2.

TABLE 1. INFLUENCE OF ALCOHOL ON RESPONSE TIME TO PERFORM MINNESOTA SPATIAL RELATIONS TEST

BAL %	Response Time (sec)	
	Mean of 4 Subjects	Standard Deviation
Control	130	19.2
0.02	137.4	26.4
0.05	136.5	25.4
0.08	157.9*	35.9

* ($p = 0.05$ from control)

TABLE 2. INFLUENCE OF ALCOHOL ON RAIL WALKING PERFORMANCE

BAL %	Distance Walked (ft)	
	Mean of 4 Subjects	Standard Deviation
Control	7.1	3.3
0.02	7.6	2.9
0.05	8.0	1.9
0.08	5.3*	2.7

* ($p = 0.05$ from 0.05% BAL)

DISCUSSION

The results of these experiments demonstrate little or no interaction between the effects of low doses of alcohol (BAL = 0.05%) and commonly incurred levels of COHb (7% and 12%). If the central nervous system depressive activity of these materials were additive or synergistic, an incremental change should have been noted in the responses of the alcohol-dosed subjects when the COHb levels increased. This did not occur. Carbon monoxide did not act as a classical central nervous system depressant in these experiments and did not add significantly to the alcohol effect at the concentrations tested.

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DISCUSSION

COL. KAPLAN (School of Aerospace Medicine): I'd like to ask Dr. Weir whether he ran an 80 mg % alcohol control in those tests. You made the statement that when you went from 50 mg % to 80 mg % blood alcohol you did get significant differences. The question is, was the difference due to the alcohol or the alcohol plus the CO?

DR. WEIR (General Motors Technical Center): This last series was a dose response relationship test only for alcohol, so we ran 0.02, 0.05 and 0.08% alcohol alone. The only studies conducted in combination with carbon monoxide were at the 0.05 grams % alcohol level.

DR. NETTESHEIM (Oak Ridge National Laboratory): I would like to make a few comments on Dr. Crocker's paper and also on the inhalation study. Dr. Crocker, I'd like to point out that I'm absolutely convinced that the approach using the in vitro methods is going to be the only way that we can go to survive experimentally with the large numbers of chemical compounds and air contaminants that we are confronted with. If we want to test them all, I think this is the only realistic way to go. If we gather the kind of data that you are gathering and biochemical data, I think we will eventually have the scientific justification for using this kind of system. I'd like to point out two things. Number one, the first slide that you showed was not a thick section, it was an EM picture, just for your own credit. The other is that the loss of the basement membrane is a very nonspecific phenomenon. We have shown with formaldehyde, and Kay Kilbourne has recently shown with SO_2, that you find not only loss of basement membrane but also what looks like microinvasion. If you would show these slides to the classical pathologist, he would say it was the beginning of invasion, which of course, it is not. I think you have to be very careful with that particular parameter because you can get it with all kinds of toxic injuries in respiratory tract epithelium. I would like to point out a few things about inhalation experiments. There are not only macrophages in the alveoli but there are also cells that are called type 2 alveolar cells. Some of the cells that were shown were that kind of epithelial cell. The mouse tumor that was presented was not of bronchial origin. This has been shown over and over again. That is an alveolargenic tumor and is a type 2 cell alveolar tumor. Whether one calls it a carcinoma or adenoma is also a matter of controversy. Some of these tumors do, indeed, become invasive at a later stage, but they are not of bronchiolar origin; they are of alveolar origin.

DR. CROCKER (University of California, Irvine): I'm grateful for your comments, Dr. Nettesheim. The matter of the basement membrane lesion was one of interest to us chiefly because the same degree of metaplastic change induced by Vitamin A deficiency is not associated with basement membrane lesions. The basement membrane lesion seen with

metaplasia has been suggested as a possible index of the short-term effects that might have significance. Certainly additional findings of the kind that you described with other irritant materials, unless they are also shown to be carcinogenic, would tend to reduce the value of those particular indices of the lesion. I would still like to emphasize your major point which is that the in vitro test is necessary for handling a large number of chemicals that need to be tested. Indeed, it can be used with a very highly sensitive source of cells. In so doing, it will give us a number of false positives which will have to be dealt with by more complex and sophisticated levels of screening. It may be a very useful first try and is not likely to give very many false negatives. It will still need a fair amount of validation by testing the positives to determine whether they are false. In the other in vitro system application, mainly the organ culture, one has at least seen the lesion, which the histopathologist can recognize. The pitfall of looking only for potential microinvasion and loss of basement membranes is that not only does it extend to irritant chemicals, but also to influenza infection as shown in influenza virus in mouse trachea cultures. Thus even there, there may be false positives. However, the false positive tends to have more relevance in this case to a real lesion which may, in fact, correlate with other susceptibilities to superimposed infection. Which then would lead me, if I may go this far, to ask questions of Dr. McConnell. He observed a significant increase in spontaneous infection among the animals exposed to the inhaled coal tar. This impresses me as an indication of some alteration in capacity to resist infection or of activation of latent microorganisms. While his objective was inhalation carcinogenesis, he may very well have realized a different one which was alteration of competence of the respiratory system to resist infections of a latent nature. The question I would like to ask him is whether he looked at laryngeal tissue.

MAJOR MC CONNELL (Aerospace Medical Research Laboratory): Dr. Crocker, in response to your second question first, after talking with you earlier this year, I definitely did watch for laryngeal lesions much more closely and I have not seen a single one to date. I have wondered if this could be related to the fact that we don't have a particulate cocarcinogen. Wasn't that a factor in previous studies?

DR. CROCKER: In the Dontenwill experiment, the aerosol was tobacco smoke. Certainly an aerosol is an important element of the effect of tobacco smoke. The lack of laryngeal lesions suggests to me that you applied the most critical test of whether or not the inhalant was reaching concentrations adequate for lung carcinogenesis. I think it may not have been in spite of the high load of fluorescent material.

MAJOR MC CONNELL: Regarding your first question about effects on resistance to infection, the same thing impressed me and 2 weeks ago, I asked the statisticians to review this data and some from other studies to

see if we had a higher rate of chronic respiratory disease, at least based on histopathologic recognition of the disease. The analysis has not yet been completed.

DR. DREW (National Institute for Environmental Health Sciences): Dr. McConnell, did you see any lung tumors in hamsters? I ask the question because many people are using the hamster as a model animal for inhalation carcinogenesis, and to my knowledge, at least thus far, no one has ever produced a tumor in a hamster by inhalation. I am just curious.

DR. MC CONNELL: We did not see a single tumor in hamster lungs, but I think this has been done by putting material in the lung.

DR. DREW: But not by inhalation. There's a big difference. I think that this fact emphasizes the point that the hamster is not the species of choice when looking for carcinogenesis. The second question is to Mr. Kinkead. You mentioned that you separated the soluble fraction from the solid particulates. Did you then reexpose the animals to the particulate, or was that part of the coal tar product completely eliminated from the exposure?

MR. KINKEAD (University of California, Irvine): In the first study, we took the solids out, and the animals weren't exposed to any solids. In the second study, the solids were left in. We didn't remove any solids in the second study.

DR. MAC EWEN (University of California, Irvine): The solids that were removed from the coal tar are commonly called the C_1 fraction which is almost a pure carbon particulate. However, it was a particulate aerosol that the animals were inhaling. Once it got to the lung, it was pretty much like hard tar. It wasn't fluid and you could see particles or aggregates of particles inside the macrophages under the microscope. The animals were exposed to an aerosol, but it was not an aerosol with a mineral dust in it, as in the second series of experiments.

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PAPER NO. 17

ROLE OF THE ALVEOLAR MACROPHAGE IN PULMONARY DEFENSE AGAINST BACTERIAL INFECTION

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INTRODUCTION

The initial host-parasite encounter is a critical event in the determination of pulmonary bacterial defense. If the entering microorganisms are killed by host defense mechanisms, or are removed mechanically, the potential infection is averted. If neither of these events occurs, the bacteria may multiply and invade the pulmonary parenchyma.

Previous investigations have established the importance of non-specific intrapulmonary phagocytic systems in the initial defense of the lung against inhaled bacteria (Green and Kass, 1964). Because the proportion of intrapulmonary bacteria that are killed by macrophages has not been determined, the relative significance of this defense system in relationship to other non-phagocytic defenses and to the overall defense of the lung against bacterial invasion is unknown. An additional query in this regard concerns the pathogenesis of disease induced impairments in bacterial killing. To what extent are these abnormalities due to defects in the component processes of phagocytosis, bacterial ingestion and intracellular killing, and to what extent, if any, are the abnormalities due to disturbances of non-phagocytic defense, such as enzymatic digestion (Green, 1970).

Because the rate of bacterial ingestion governs the extent of phagocytosis, comparisons of this rate with that of bacterial inactivation would delineate quantitatively the maximum proportion of inhaled bacteria that are susceptible to destruction by phagocytosis. This report details a method for measuring in vivo the rate of bacterial ingestion by alveolar macrophages. The technique consists of infecting rats with concentrated and finely dispersed aerosols of staphylococci. Sufficient numbers of bacteria are inhaled to allow statistical analysis of the proportion of intracellularly located bacteria at various time intervals after infection. These rates of phagocytic ingestion are then compared in the same animals with simultaneously measured rates of bacterial inactivation.

In a second series of experiments, intrapulmonary bacterial inactivation was impaired by exposure to ozone. This noxious gas was chosen because it inhibits pulmonary bacterial killing, and allows bacterial proliferation without altering mucociliary transport rates (Goldstein et al., 1971). The effect of the pollutant on murine rates of bacterial inactivation and ingestion was measured as a means of delineating the mechanisms responsible for the impairment in bacterial defense. According to the data that were obtained, exposure to ozone causes severe defects in the intracellular killing of bacteria and lesser defects in the rate of bacterial ingestion.

Material and Methods

Animals

Sprague-Dawley CRD-free rats weighing 120-150 grams were used in these experiments. The animals were housed two to three per cage and fed food and water ad libitum.

Infection Schedules

Two liters of trypticase soy broth were inoculated with Staphylococcus aureus and cultured in a shaker water bath at 37 C for 16 hours. The bacteria was sedimented by centrifugation and resuspended in 8.0 ml of saline. Approximately 5.0 ml of this suspension was aerosolized into an exposure chamber which permitted quantitative infection of the lungs of rodents (Laurenzi et al., 1964). The nebulizer was specifically designed to provide concentrated and finely dispersed particles, the majority of which were 1.0 - 3.0 μ in size (DeOme, 1944). At 0, 1/2, 1, 2, 2-1/2, 4, and 5 hours after infection groups of 5 infected rats were sacrificed with ether. The lungs of each rat were exposed, aseptically. A ligature was placed securely about the left main stem bronchus and this lung was excised for determination of the numbers of viable staphylococci. The right lung was perfused with 2.4 percent glutaraldehyde in cacodylate buffer at 10 cm of pressure via an intratracheal cannula. After fixation, this lung was used to determine the anatomic location of individual staphylococci.

Bacterial Clearance Rates

The numbers of viable staphylococci that were present in the left lung were determined by standard pour plate techniques. A mean bacterial count for each group of rats at each time period was calculated, and bacterial clearance was expressed as the number of bacteria present immediately after exposure (N_O) minus the number present at time T (N_T) divided by the initial number of bacteria (N_O)

$$\text{Bacterial Clearance} = \frac{N_O - N_T}{N_O}$$

These data were analyzed for significance of difference by the theorem of Wilks (Green and Kass, 1964; Wilks, 1962).

Bacterial Localization

The right lung was embedded in formalin. Sections of 4 to 5 μ in size were cut from the medial aspect of the right median lobe. These sections were stained with the Brown and Brenn tissue stain for bacteria (Preece, 1972), and scanned for staphylococci at 1,000 x magnification with a Leitz Orthoplan microscope. The intra- or extracellular location of 100 consecutive bacteria was determined for each lung. On occasion clumps of 10 or more bacteria were found in which it was impossible to determine the exact number of bacterial cells. These groupings were tabulated separately and their number of individual bacteria were excluded from the 100 bacteria that were counted. Wherever possible the bronchial or alveolar location was noted. The proportion of bacteria that were intracellular was compared for each of the time periods studied. These data were also analyzed by the theorem of Wilks.

Ozone Exposure

Groups of 15 rats were infected with bacterial aerosols as in previous experiments. Five of these animals were sacrificed immediately to determine the anatomic location and viability of the inhaled bacteria. Half of the remaining animals were exposed to 2.5 ppm of ozone for four hours in an air pollution chamber (Goldstein et al., 1971). The ozone was generated from oxygen by silent electrical discharge. The concentration of ozone was determined by microcoulomb ozone sensors attached to a multiple point recorder. Control animals were exposed to identical air flows containing 21% oxygen.

RESULTS

In each of the experiments more than 10^{10} bacterial cells/ml were cultured from the aerosol nebulizer. Table 1 contains the data for the particle size distribution of the aerosols (Andersen, 1958). Ninety-nine percent of the bacterial particles were less than 3.5 μ in size, with the greatest percentage of these particles being in the respirable range of 1.0 to 2.0 μ. Since the instrument sampled one cubic foot of air per minute (Andersen, 1958), and the infection period was 20 minutes, $>10^8$ staphylococci of respirable size were present in each cubic foot of infected air. The pulmonary deposition of staphylococci within the left lung varied from 743,000 to 2,072,000 bacterial cells in the six experiments.

TABLE 1. PARTICLE SIZE DISTRIBUTION OF STAPHYLOCOCCUS AUREUS IN THE INFECTION CHAMBER [a]

Andersen Sampler Stage	Aerodynamic Size[a]	Number of Particles	% of Total
1	>8.3	$10.7 \cdot 10^5$	.04
2	5.0 - 10.5	$4.3 \cdot 10^5$	.02
3	3.0 - 6.0	$64.3 \cdot 10^5$	.22
4	2.0 - 3.5	$21.3 \cdot 10^7$	7.79
5	1.0 - 2.0	$22.8 \cdot 10^6$	83.42
6	<1.0	$23.2 \cdot 10^7$	8.49

[a] From Andersen, 1958.

Each of the microscopic sections usually contained 8 or more bacteria. The black staining staphylococci were easily identified and their number as well as their location was apparent (Figure 1, 2). Microorganisms that had been ingested were intracytoplasmic. Since the nucleus stained pink, confusion due to this structure was minimal. The occasional problem of determining if a bacteria lay on or adjacent to a phagocyte, rather than within its cytoplasm, was frequently resolved by viewing the spatial relationship of bacteria and cell at different focal depths. Unless the bacteria could be clearly delineated, as inside or outside of a macrophage, its location was recorded as indeterminant. Bacteria that were tabulated in this manner were excluded from the numerical count.

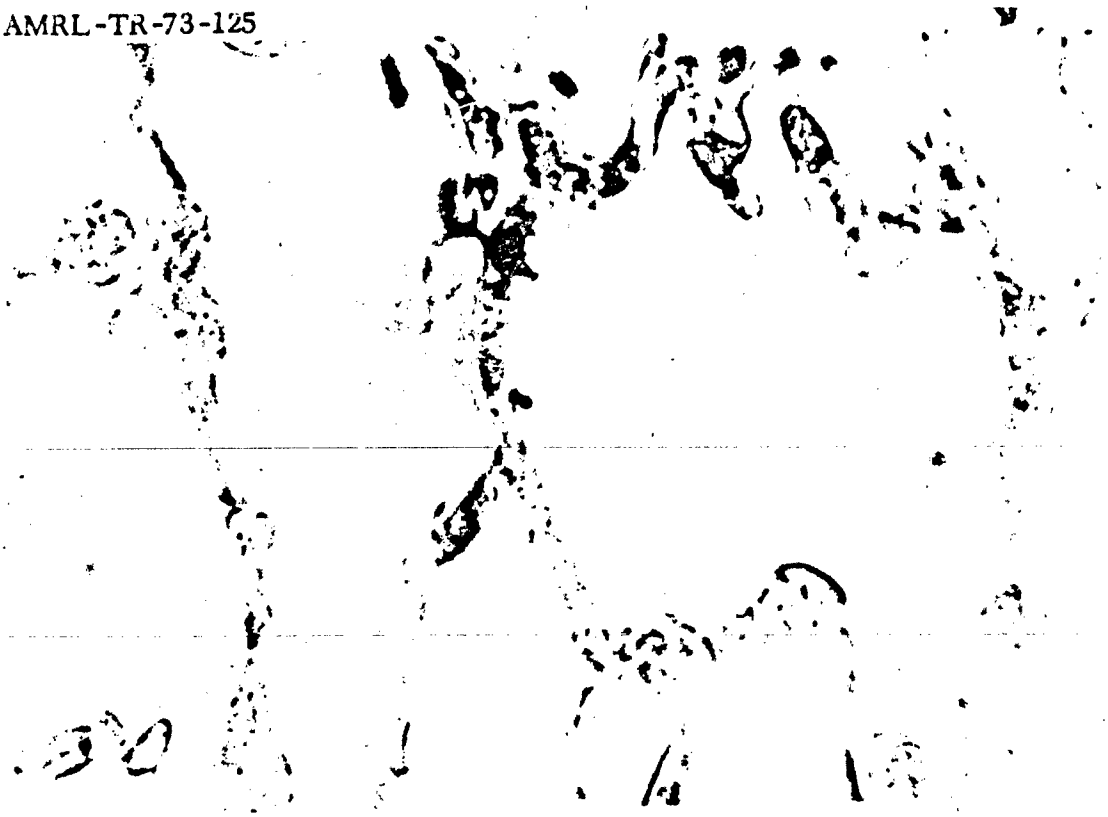

Figure 1. An alveolar macrophage from a normal rat with four intracytoplasmic staphylococci. One of the staphylococci is at a slightly different depth. Brown and Brenn stain x 1,000.

Figure 2. Two alveolar macrophages from a normal rat with ingested staphylococci. The staphylococci are visible within the cellular cytoplasm. Brown and Brenn stain x 1,000.

Table 2 contains the data from an illustrative example of the six control experiments in which the rate of bacterial ingestion by pulmonary phagocytes was determined. The percentage of ingested staphylococci increased from 53.4% in rats that were sacrificed at 0 hours to 93.6% for animals that were sacrificed at 5 hours after the aerosol infection. In this experiment fewer than 3% of the observed bacteria could not be classified as to their intra- or extracellular location.

TABLE 2. LOCATION OF INHALED STAPHYLOCOCCI AT VARIOUS TIMES AFTER EXPOSURE TO AEROSOLS OF BACTERIA

Time after Exposure (hrs)	Animal Number	Bacterial Site (Right Lung)			Phagocytic Ingestion %
		Extracell	Intracell	Indeter	
0	1	27	73	0	73
	2	41	59	1	59
	3	66	34	0	34
	4	65	35	1	35
	5	34	66	2	66
				Mean ± S.E.	53.4 ± 8.0
2-1/2	6	5	95	0	95
	7	13	87	1	87
	8	14	86	3	86
	9	16	84	0	84
	10	12	88	2	88
				Mean ± S.E.	88.0 ± 1.9
5	11	7	93	1	93
	12	5	95	1	95
	13	1	99	0	99
	14	1	99	2	99
	15	18	82	1	82
				Mean ± S.E.	93.6 ± 3.1

Table 3 is a comparison of murine bacterial clearance and ingestion rates for all of the experiments in which control animals were studied. According to these data, the rate of bacterial ingestion increased from 54.9% to more than 90% at four or five hours. The rate of bacterial clearance increased from 23.2 to 80.9% during the same time period. Inspection of these data indicates that the rate of bacterial ingestion exceeded the rate of bacterial clearance for each of the time periods.

TABLE 3. COMPARISON OF MURINE RATES FOR STAPHYLOCOCCAL INGESTION (RIGHT LUNG), AND STAPHYLOCOCCAL CLEARANCE (LEFT LUNG), AT VARIOUS TIMES AFTER EXPOSURE TO BACTERIAL AEROSOLS

Time after Exposure (hrs)	Number of Rats	Bacterial Ingestion %	Bacterial Clearance %
0	30	54.9 ± 2.6	----
1/2	5	64.4 ± 5.2	23.2 ± 12.0
1	10	77.7 ± 3.0	28.0 ± 14.0
2-1/2	10	89.8 ± 1.8	50.3 ± 9.5
4	10	94.4 ± 0.9	63.6 ± 6.9
5	10	91.6 ± 3.6	80.9 ± 3.6

The data for the experiments in which rats were infected with staphylococci and then exposed for four hours to 2.5 ppm of ozone are shown in Table 4. Exposure to ozone reduced the pulmonary bacterial clearance rate from a control value of 63.6% to a treatment value of -15.3% ($P<0.01$). A significant but much smaller decrease in the percentage of intracellularly located bacteria was found in the rats that were exposed to ozone (78.7% vs 94.4%; $P<0.05$). Figure 3 depicts the occurrence of clumps of bacteria within the phagocytes of the ozone treated animals. Twenty-five of these intracellular foci were found in the treated animals as compared to four foci in the controls ($P<0.05$). Figure 4 shows the clumps of extracellularly located staphylococci were also occasionally present in the treated animals. Vascular hyperemia was the only histological abnormality that was found in these pulmonary specimens. The bronchial and alveolar architecture appeared normal and neither inflammation nor edema was present.

TABLE 4. EFFECT OF A 4 HOUR EXPOSURE TO 2.5 PPM OF OZONE ON THE MURINE RATES OF PULMONARY CLEARANCE AND PHAGOCYTIC INGESTION OF STAPHYLOCOCCUS AUREUS

Experimental Group	Bacterial Clearance %(a)	Bacterial Ingestion %(a, b)
Control	63.6 ± 6.9	94.4 ± 0.9
Ozone	-15.3 ± 21.1(c)	78.7 ± 5.2(d)

(a)Mean ± S. E.
(b)Clumps of uncountable bacteria: controls - 4; ozone - 25; $P<0.01$
(c)$P<0.01$
(d)$P<0.05$

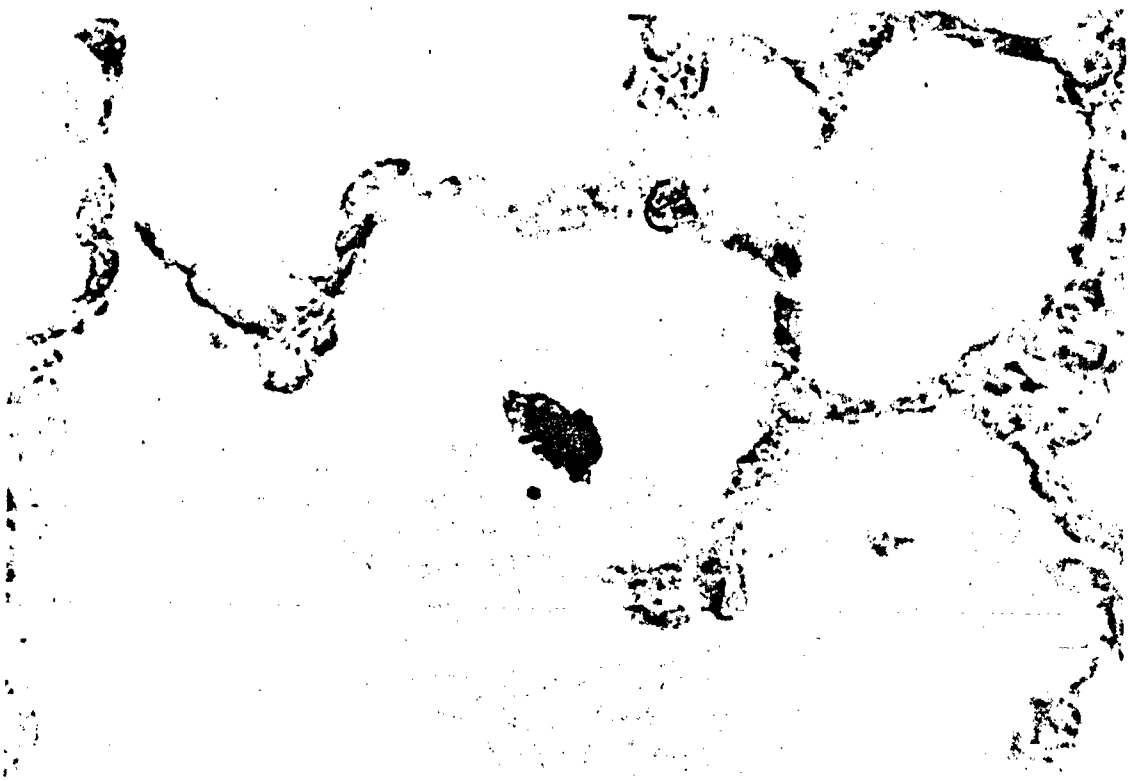

Figure 3. A clump containing numerous staphylococci within the cytoplasm of a macrophage from a rat that had been exposed to 2.5 ppm of ozone for four hours. A single extracellularly located staphylococcus is also present. Brown and Brenn stain x 1,000.

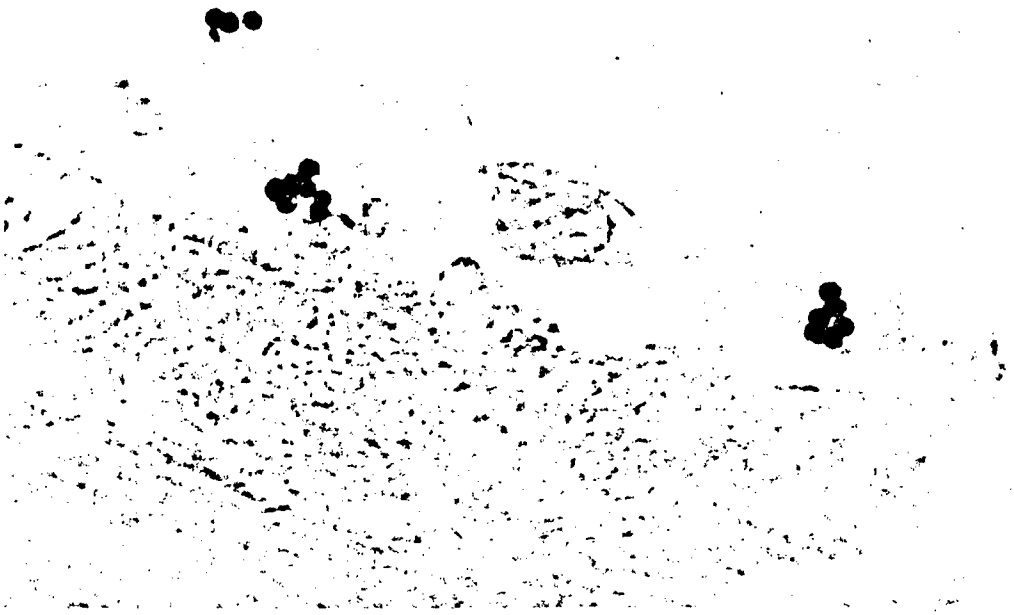

Figure 4. Clumps of intra- and extracellular staphylococci adjacent to bronchial epithelium in a rat that had been exposed to 2.5 ppm of ozone for four hours. A small group of three extracellulary located staphylococci is also present.

DISCUSSION

These experiments demonstrate that phagocytosis by the alveolar macrophage is the principal mechanism by which the lung protects itself against inhaled staphylococci. Approximately 50% of bacteria that are inspired are ingested within minutes after they reach the lungs, 80% are ingested within the first hour of residence, and 90% are ingested at 2-1/2 hours. Since this rate of bacterial ingestion exceeds the rate of intrapulmonary bacterial inactivation, phagocytosis can account in entirety for bacterial death.

These studies also indicate that non-phagocytic defense mechanisms are much less important in the initial defense of the lung against bacterial invasion. Because 50% of inhaled bacteria are present within phagocytes at 0 hours, extracellular killing can be of significance only if: 1) dissolution of large numbers of extracellular bacteria had occurred prior to the microscopic examination at 0 hours; or 2) the ingested bacteria were rendered non-viable prior to phagocytosis. Previous studies with radiophosphorus labelled staphylococci make these possibilities very unlikely (Green and Kass, 1964; Green and Goldstein, 1966; Goldstein et al., 1970; Jakab and Green, 1972). When radiophosphorus bacteria are aerosolized, the ratio of viable bacteria to radiophosphorus counts of the infected lung at 0 hours is only slightly less than the ratios of the nebulizer suspension (Green and Goldstein, 1966; Goldstein et al., 1970) or the aerosol (Jakab and Green, 1972), a finding which proves that intrapulmonary killing and dissolution of large numbers of extracellular bacteria does not occur during the 20-minute period of bacterial infection. Similarly, the possibility that the ingested bacteria were non-viable is contrary to the observation that the numbers of culturable bacteria at 0 hours is only slightly less than the actual numbers of inhaled bacteria (Green and Goldstein, 1966; Goldstein et al., 1970). The necessity to aerosolize maximum numbers of bacteria prevented the use of special media to allow testing with radio-labelled bacteria. However, since the experimental model is the same as the one in which the radiotracer experiments were conducted, the evidence from the radiotracer experiments should be applicable to the present studies.

The conclusion that phagocytosis is the principal mechanism of pulmonary bacterial defense is in accordance with our present knowledge of the macrophage system. These phagocytes exhibit positive chemotaxis towards bacteria (Jacoby, 1965; Rabinovitch, 1967). They are able to ingest numerous bacteria in periods as brief as one hour (Gill and Cole, 1965; Green and Carolyn, 1967), and they are sufficiently numerous to insure the likelihood of proximity to inhaled bacteria. Quantitative studies from lung lavages have shown that there are more than 5×10^6 free phagocytes per gram of lung tissue (Brain, 1970; Brain and Frank, 1968). The actual number of pulmonary macrophages is probably much higher since only a fraction of these cells are removed by the lung wash. These considerations suggest that even though 10^6 bacteria were deposited within the lung, the bacterial burden is unlikely to overload the phagocytic system.

The majority of inhaled staphylococci were found within the alveoli. Although the exact distribution of bacteria between bronchial and alveolar regions cannot be definitively assessed because no attempt was made to sample different pulmonary regions, the following arguments suggest that the high proportion of staphylococci which were observed within alveoli was probably an accurate reflection of bacterial distribution. Particles that have a diameter of 1.0 to 2.0 μ such as single or paired staphylococci settle out largely at the bronchiolar to alveolar levels of the lung (Green, 1968). These particles also

tend to deposit uniformly throughout the lung (Jakab and Green, 1973). The observation that these bacteria traversed the region of the mucociliary stream is consistent with previous findings that bactericidal mechanisms rather than physical transport accounts for pulmonary sterility (Green and Kass, 1964; Green and Goldstein, 1966; Goldstein et al., 1970).

The experiments in which the effect of ozone on pulmonary defenses was studied demonstrated that the pollutant inhibited the intracellular killing of already ingested staphylococci. According to these data, bacterial clearance did not occur in the ozone treated rats despite the ingestion of 70% of staphylococci. Since clumps of 10 or more staphylococci were found within macrophages (Figure 4), and less frequently extracellularly (Figure 4), these groupings probably represented bacterial proliferation in ozone treated rats. It should be noted that clumps of staphylococci were infrequent occurrences in control animals. This finding that ozone allows the intracellular multiplication of staphylococci accords with previous reports of in vitro studies in which similar exposures to ozone resulted in staphylococcal proliferation (Coffin et al., 1968). Although of lesser importance, the ozone induced decrease in the rate of bacterial ingestion suggests that the pollutant may also have inhibited phagocytic chemotaxis and ingestion. The biochemical mechanisms by which ozone damaged cellular function may involve the inactivation of enzymes (Hurst and Coffin, 1971), peroxidation of lipids (Balchum et al., 1971), or denaturation of cellular protein (Menzel, 1970).

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AMRL-TR-73-125

PAPER NO. 18

GUIDES FOR SHORT-TERM EXPOSURE LIMITS TO HYDRAZINES

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INTRODUCTION

At the request of the Environmental Protection Agency and following the suggestions of several other Federal Agencies, the Committee on Toxicology of the National Research Council has undertaken the preparation of a series of Guides for Short-Term Exposure of the Public to Air Pollutants. Three of these are the subject of this paper. The compounds are hydrazine, monomethylhydrazine, and 1,1-dimethylhydrazine and a preview will be given of the limits which will probably be recommended. The physical and chemical properties of these compounds are shown in Table 1.

TABLE 1. PHYSICAL CHEMICAL PROPERTIES

	Hydrazine	Monomethyl-hydrazine	1,1-Dimethyl-hydrazine
Molecular Formula	H_2NNH_2	CH_3HNNH_2	$(CH_3)_2NNH_2$
Molecular Weight	32.05	46.07	60.10
Boiling Point °C	113.5	87.5	63.3
Melting Point °C	2.0	-20.9	-58.0
Odor Threshold ppm	3-4	1-3	0.3-1
Density (25/25) g/cc	1.00	0.87	0.78
Vapor Density (air = 1)	1.1	1.6	2.0
Vapor Pressure, mm Hg (25°C)	14.38	49.63	157.0
Flash Point, °F (TCC)	100	80	5
Flammability Range in Air % Vol., 1 atm.	4.7-100	2.5-98	2.5-95
1 ppm Vapor, 25°C/760 mm	0.001309 mg/l	0.001881 mg/l	0.002554 mg/l
1 mg/l vapor, 25°C/760 mm	764 ppm	532 ppm	408 ppm

In preparing the Guides for the hydrazines, the Committee on Toxicology was assisted by an ad hoc sub-committee consisting of the following members: C. Boyd Shaffer, Chairman, Kenneth C. Back, Frank N. Dost, Richard Henderson, and James D. MacEwen, with Keith Jacobson and Dale Clark acting as independent reviewers.

In spite of the excellent work conducted at the Aerospace Medical Research Laboratories, the Committee has found that the animal toxicity data are not really adequate. They are inconsistent and their application to human exposures is further complicated by the fact that there are marked differences in species response to these agents. As a result it has not been possible to survey these compounds as a homogenous group because their differing physical characteristics and physiologic actions give rise to wide variations in potential toxic effects. Accordingly, the toxicity of each compound will be reviewed briefly. Then a basic problem will be discussed and preliminary recommendations will be given.

Hydrazine Toxicology

Acute exposures of animals to hydrazine may lead to death within a few hours from convulsions, respiratory arrest, or cardiovascular collapse. Others may die two to four days later of kidney or liver injury. The single-dose LD_{50} does not vary much with different routes of administration or among several species. It is generally well below 100 mg/kg (Weir et al., 1964; Witkin, 1956; Krop, 1954). The four-hour LC_{50} of hydrazine vapor is 322 mg/m^3 for mice and 759 mg/m^3 for rats (Jacobson et al., 1955).

The lethality of hydrazine is approximately the same whether it is given in a large single dose or in an equivalent amount in small repeated doses. The accumulated lethal dose appears to be similar for all routes of absorption, including inhalation (Patrick and Back, 1965; House, 1964).

The liver is the primary target organ in most animals under most conditions of exposure (Amenta and Johnston, 1962). Hydrazine also produces pathologic changes which are apparently not wholly attributable to irritation from inhalation. Thienes and co-workers (1948) observed that toxic doses by any route of administration affected the endothelium of blood vessels, especially those in the lung. Monkeys have been shown to be more sensitive to liver injury by hydrazine than other animals (Patrick and Back, 1964).

Hydrazine is slowly excreted unchanged in the urine of experimental animals at the rate of about 25% of the dose per day. It interferes with metabolism of carbohydrates and fat and also inhibits the production of insulin (Taylor, 1966; Aleyassine and Lee, 1971). There are also reports of effects on amine metabolism but these do not appear related to any of the overt toxic effects of hydrazine.

Human exposures to low concentrations of hydrazine produce local irritation. Heavy exposure to the vapors causes prompt severe irritation of the nose and throat. Later the eyes itch, burn, and swell. Temporary blindness may develop lasting for about a day (Comstock et al., 1954). Ingestion of hydrazine solutions has caused severe effects and death. Dermal sensitization with extensive cross-reactions have been reported.

Monomethylhydrazine Toxicology

Acute exposure of animals to MMH results primarily in effects on the central nervous system. The LD_{50} values for various species and routes of administration are between 12 and 96 mg/kg (Witkin, 1956; Rothberg and Cope, 1956). The 1-hour LC_{50} values are as follows: mice 229 mg/m^3, rats 459 mg/m^3, dogs 181 mg/m^3, squirrel monkey 154 mg/m^3, and rhesus monkey 305 mg/m^3. The 4-hour LC_{50} data are: mice 105 mg/m^3, rats 139 mg/m^3, hamsters 737 mg/m^3 (Jacobson et al., 1955; Haun et al., 1970). These inhalation data demonstrate a wide species variation.

The signs of MMH toxicity in animals include irritation of the nose and eyes, salivation, emesis, diarrhea, hyperactivity, tremors, and severe tonic-clonic convulsions leading to death. At concentrations below those producing any of these overt signs subtle changes in behavior can be observed (Sterman et al., 1969). In addition, MMH produces hemolytic, renal, and hepatic effects.

Chronic dosing of animals with MMH shows that it also accumulates as does hydrazine. A "no observed effect" dose in monkeys appears to be 2.5 to 5 mg/kg for up to 20 consecutive intraperitoneal injections. Repeated daily inhalation exposures to MMH produces a dose-related hemolytic anemia with Heinz body formation which has no threshold and appears to be reversible upon termination of exposure, at least at levels as high as 9.4 mg/m^3 (MacEwen and Haun, 1971).

The respiratory and urinary elimination of MMH varies both between species and with the dose. The clearance mechanisms in mice, rats, and monkeys appear to be different from that of dogs (Pinkerton et al., 1967). The data indicate a limit to the rate at which MMH can be excreted.

As with hydrazine, MMH affects carbohydrate and amine metabolism. Intensive studies have so far failed to elucidate the mechanism by which MMH affects carbohydrate metabolism (Dost et al., 1973). The inhibition of monoamine oxidase by MMH appears to cause a depressor effect on the cardiovascular system (Weir et al., 1964).

Carefully controlled human exposures to MMH were conducted at the Aerospace Medical Research Laboratory. Seven volunteers were exposed for 10 minutes at 169 mg/m^3 (90 ppm). There was only slight irritation of

the eyes, nose, and throat with no overflow of tears, no coughing. Fourteen clinical chemistry parameters were followed for 60 days postexposure with no changes being found. Heinz bodies to the extent of 3-5% were found by the seventh day and declined after two weeks. Spirometry data were normal on six volunteers while the seventh had decreased pulmonary function accompanied by symptoms of a respiratory infection.

1,1-Dimethylhydrazine Toxicology

The acute toxic effects of UDMH are primarily convulsions and respiratory irritation. In the mouse, rat, dog, and monkey lethal doses produce tonic-clonic convulsions terminating in respiratory arrest (Back and Thomas, 1963). The LD_{50} data in several species by various routes (i.v., i.p., p.o.) all lie between 60 and 290 mg/kg with most of the values between 100 and 125 mg/kg. The ready evaporation of UDMH minimizes the systemic toxicity from skin applications.

The 4-hour LC_{50} values for UDMH vapor are: for mice, 439 mg/m^3; for rats, 644 mg/m^3; and for hamsters, 1001 mg/m^3 (Jacobson et al., 1955). LC_{50} data for shorter exposures have been determined for rats and dogs. For rats the values are: 60 minutes, 3,520 mg/m^3; 30 minutes, 10,000 mg/m^3; 15 minutes, 20,660 mg/m^3; 5 minutes, 61,300 mg/m^3. For dogs the values are: 60 minutes, 2,450 mg/m^3; 15 minutes; 8,950 mg/m^3; 5 minutes, 55,700 mg/m^3. When these data are plotted on a logarithmic scale they reveal a linear relationship within the span of the data between the dose of UDMH and its lethality. Minimal toxic signs in dogs were observed at C x T-doses equivalent to 290,000 mg minutes/m^3 (Weeks et al., 1963).

Repeated exposures to UDMH suggest that it is accumulated when the dose absorbed exceeds some maximum amount related to the organism's capacity to metabolize and excrete the material (House, 1964; Cornish and Hartung, 1969; Rinehart et al., 1960; Weeks et al., 1963). These chronic exposures at tolerated doses produced only minor pathologic changes in the liver and kidney.

Although the toxicity data indicate that UDMH accumulates, the information on its absorption, metabolism, and excretion do not give strong support for accumulation. UDMH can be detected in the bloodstream and urine very shortly after administration with up to 50% being excreted in 5 hours (Back and Thomas, 1963; Smith and Clark, 1971; Dost et al., 1966).

As with the other hydrazine compounds UDMH affects carbohydrate metabolism as well as suppressing amine oxidases and transaminases. Although these appear related to the convulsive effects the mechanism is far from clear. It is a strong diuretic but has little or no effect on blood pressure.

The few human exposures to UDMH that have been observed have produced respiratory irritation, nausea, and vomiting. Heavy exposure produced paleness, sweating, wheezing, twitching of the extremities and clonic movements (Azar et al., 1970).

Tumorigenesis

Several workers have shown that isonicotinic acid hydrazide can enhance tumor production in animals. A postulated mechanism is its metabolic hydrolysis to hydrazine. This has led to several studies on the tumorigenic properties of hydrazine and its derivatives including MMH and UDMH. At the present time the significance of these findings to short-term exposures of the public is far from clear. The experimental doses were high and repeated for long periods. These findings are offset by the work of Ochoa at Sloan-Kettering on the experimental use of hydrazine sulfate for the treatment of cancer patients. At present it appears that these compounds may be at most promoters rather than de novo carcinogens. Accordingly, the Committee's recommendations for Short-Term Public Exposure Limits (STPL's) and Public Exposure Limits (PEL's) are subject to possible extensive revision downward pending review of further work at AMRL and elsewhere (Toth, 1972).

RECOMMENDATIONS

The final report from the Committee on Toxicology on these compounds, hydrazine, MMH, UDMH, has not yet been released. Accordingly, we can only share with the limited group of scientists in this audience some of the present thinking of the subcommittee and ask for your comments. Table 2 summarizes their views on the subject. Your reactions and comments are solicited.

TABLE 2. RECOMMENDED LIMITS FOR EXPOSURE (STPL's)

	STPL's (25 C/760 mm Hg)		
	Time	Limit	
Compound	min.	ppm	mg/m³
Hydrazine	10	15	20.
	30	10	13.
	60	5	7.
	*	2.5	3.
Monomethylhydrazine	10	9	16.9
	30	3	5.6
	60	1.5	2 8
	*	.3	.56
1, 1-Dimethylhydrazine	10	50	127
	30	25	63.5
	60	15	38.2
	*	1	2.55

*5 hours/day, 3-4 days/month.

TABLE 2a. RECOMMENDED LIMITS FOR EXPOSURE (PEL's)

Compound	PEL's (25 C/760 mm Hg) Time min.	Limit ppm	mg/m³
Hydrazine	10	30	39
	30	20	26
	60	10	13
	-	-	-
Monomethylhydrazine	10	90	169
	30	30	56
	60	15	28
	-	-	-
1, 1-Dimethylhydrazine	10	100	255
	30	50	127
	60	30	76.5
	-	-	-

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PAPER NO. 19

EXPERIMENTAL APPROACHES TO THE IDENTIFICATION OF CARCINOGENIC AIR CONTAMINANTS*

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It is estimated that 95% of all lung cancers are caused by pollutants of the breathing air. While epidemiological data clearly indicate that inhalation of tobacco smoke constitutes the major risk factor, a number of occupational pollutants (radon daughters, mustard gas, arsenic, nickel, beryllium, asbestos) have also been demonstrated, either epidemiologically or experimentally, to constitute lung cancer risks.

With an increasing number of potential inhalation hazards appearing on the horizon, it becomes urgent to decide which of the various laboratory approaches currently used for respiratory-tract carcinogenesis studies are best suited (practical and reliable) for detection of carcinogenic air contaminants.

I have attempted to summarize what I consider to be major considerations in selecting the appropriate bioassay for the detection of carcinogenic air pollutants (for review see Shabad, 1962; Kuschner, 1968; Saffiotti, 1969; Kuschner and Laskin, 1971).

Of foremost importance is the clear and precise definition of the research objectives to be accomplished:

1. identification of (co-)carcinogenic air contaminants,
2. determination of (co-)carcinogenic potency,
3. singling out the principal factor(s) responsible for the increased lung cancer incidence in a particular population group (occupational group),

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4. identification of the tumorigenic principle responsible for tumor induction distant from the point of entry,

5. pathogenetic mechanisms (conditions influencing carcinogenic efficacy) - exogenous factors (e.g., particle size, cofactors); endogenous factors (e.g., genetic or age-related susceptibility, concurrent diseases, impaired host defenses).

Depending on the research goal(s), the selection of the test system has to be made from a rather limited arsenal of currently available bioassays unless new ones are developed. These are the options:

1. in vitro assays - (a) mutagenesis (e.g., Ames et al., 1973); (b) malignant transformation (e.g., Sivak and Van Duuren, 1970; DiPaolo et al., 1973); (c) cytopathology of respiratory-tract epithelium (e. g., Palekar et al., 1968; Dirksen and Crocker, 1968);

2. in vivo assays - (a) test-site nonrespiratory-tract tissue (e.g., mouse-skin carcinogenesis assay), (b) test-site respiratory tract (intratracheal injection, bronchial and lung-pellet technique, inhalation exposure).

The next important decision concerns the selection of the test animal, provided we have decided the task before us requires an in vivo test. In making this choice we need to consider:

1. "spontaneous" diseases, neoplasms in particular,

2. average survival time of the species,

3. susceptibility to chemical carcinogens (carcinogen metabolism),

4. histologic type and site of origin of induced tumors,

5. genetic homogeneity,

6. logistics of animal care (number of animals that can be maintained at a "reasonable" cost).

Another critical and usually very controversial decision that has to be made is that concerning the specific modalities of exposure. The question here is: Can we and should we attempt to reproduce "realistic" human exposures in terms of dose levels and concentration, and of single, multiple, or

continuous chronic-exposure episodes? It is important to remember that, no matter how much effort is made to simulate human exposure conditions, the experimental situation will always be vastly different from the "real world." It is simply unreasonable to try to induce a cancer death rate of 40 per 100,000 in the laboratory, or to reproduce tumor induction times of 20-30 years. In carcinogenesis studies we almost always have to resort to "unrealistically" high and intense exposures.

Other factors which will influence the selection of experimental methods are the physicochemical properties of the test material and the problems related to obtaining or generating such material, for example, physical state (gas, liquid, solid, or mixtures) and stability (chemical and physical).

The most formidable challenge of all is: How do we relate the experimental findings to man? This question will persist for some time, even if much care has been taken to pattern the experiment after real life. The magic conversion factor that will compensate for the differences in the experimental and human situation in individual and cumulative dose; in life span, exposure time, and tumor induction time; and in anatomical, physiological, and biochemical species and target-tissue peculiarities has not yet been invented.

After this brief outline of some of the important considerations that guide us in the selection of the appropriate bioassay system, I will summarize the most important facts currently available on the three most popular approaches used in respiratory tract carcinogenesis studies.

Intratracheal administration of carcinogenic test substances has been successfully performed in rats, hamsters, mice, rabbits, and primates (attempts in guinea pigs have so far not been successful). The most important substances (or mixtures) that have been tested in this fashion are aromatic polycyclic hydrocarbons, N-nitroso compounds, metals, asbestos, radionuclides, tars, soots, etc. The tumor type induced with this method of application depends to some extent on the administered material, but perhaps more so on the animal species used: rats develop mostly bronchioloalveolar tumors, usually squamous cell carcinomas, and occasionally adenocarcinomas and adenomas (Schreiber et al., 1972). Good dose-response curves are not available; however, as a point of reference it may be useful to know that 10 mg of benzo[a]pyrene (BaP) given as a single dose (or in two applications of 5 mg each) induces multiple squamous cell carcinomas in nearly 100% of Fisher-344 rats (induction time 12-18 months). Hamsters have been more widely used in intratracheal injection studies and a number of extensive dose-response experiments have been published (Saffiotti et al., 1968; Saffiotti, 1970; Feron et al., 1973). Tumors develop in all parts of the respiratory tract distal to (and inclusive of) the larynx. The tumor types most commonly seen are squamous cell and adenocarcinomas,

undifferentiated carcinomas, adenomas, and papillomas. It was originally thought that for effective induction of respiratory tract tumors with carcinogenic polycyclic hydrocarbons (in rats and hamsters) some carrier particle (e.g., ferric oxide) had to be added to the carcinogen suspension; however, recent experiments suggest that, given a large enough particle size (reduction of elimination rate?), BaP and 3-methylcholanthrene (MCA) are effective carcinogens without addition of extraneous material (Feron et al., 1973). It may be mentioned as a point of comparison that a cumulative dose of 15 mg BaP (given in 15 weekly applications) induces only a 30% tumor incidence. The major differences between rats and hamsters in terms of the tumor response are the greater sensitivity of the rat to carcinogenic polycyclic hydrocarbons (possibly also to other carcinogens), and a predominantly bronchiolo-alveolar tumor response in rats as opposed to a predominantly tracheo-bronchial response in hamsters. In mice, intratracheal injection of MCA has been shown to induce a high tumor incidence with a short induction time (Nettesheim and Hammons, 1971). Six x 0.5 mg MCA induced a near 100% incidence of peripheral adeno-squamous carcinomas; however, lower doses of MCA induced only typical alveologenic adenomas and adenocarcinomas. In rabbits and primates, peripheral adenocarcinomas and squamous cell carcinomas have also been induced with intratracheal injection of polycyclic hydrocarbons (Hirano et al., 1968; Crocker et al., 1970).

While the method of intratracheal administration has some very obvious advantages, notably simplicity of procedure, it also has a number of limitations: toxicity can be very severe (intratracheal tumor induction with $CaCrO_4$ has failed for this reason) and the distribution of the injected material in the respiratory tract is artificial, uncontrolled, and mostly peripheral - the tumor response except in hamsters (reasons are not clear) is mostly bronchiolo-alveolar. The procedure also causes traumatic tissue effects in the respiratory tract and requires narcosis with each instillation.

Bronchial and pulmonary pellet technique involves insertion of a carcinogen-containing pellet into the bronchus or the pulmonary parenchyma (surgical procedure required in both cases). It has been used with aromatic polycyclic hydrocarbons, chromates, tars, and radionuclides in rats and hamsters. Depending on the site of application, bronchial or pulmonary squamous cell carcinomas develop (Kuschner et al., 1957; Kuschner, 1968; Laskin et al., 1970; Stanton et al., 1972). The advantages are: a well-defined target area and a well-defined target dose; slow release of the carcinogen, which results in a high carcinogenic efficacy (with the pulmonary pellet technique, squamous cell carcinomas have been induced with as little as 5 μg of MCA); and only single application is required. The limitations of the method are: traumatization of tissue, only stable compounds are testable (they must be available in pellet form or incorporated into a carrier material), and only single application is feasible (dose is limited).

Last but not least, we have to discuss the most obvious, but also the most complicated and most expensive exposure method - inhalation exposure. The species that have been used most commonly with this approach are mice, rats, hamsters, and dogs (also primates). Materials that have been studied include polycyclic hydrocarbons, N-nitroso compounds, halogenated ethers, metals, asbestos, urethanes, radionuclides, tobacco smoke, and different pollutant mixtures (e.g., auto exhaust, smog). The most common tumors induced by this method are: in mice, alveologenic tumors (e.g., Nettesheim et al., 1971); in rats, nasal and bronchiolo-alveolar cancers (Laskin et al., 1970, 1972); in hamsters, laryngeal tumors (Dontenwill, 1970); and in dogs, peripheral adenocarcinomas and mixed adeno-squamous tumors (Howard, 1970). The major attraction of inhalation exposure lies in the fact that it is a physiological approach and, in fact, the only physiological approach to expose respiratory-tract tissues to air contaminants. However, the problems connected with this method are considerable. Because of the complexity of the technology required (e.g., generation, monitoring, and containment of the particulate and gaseous air contaminants and the long-term nature and large-scale effort usually required) the cost involved is vastly greater than that using any of the other options. Another problem is that of achieving adequate dosing of epithelial tissues below the larynx - a high tumor incidence has so far been induced only with very few and very potent carcinogens, such as halogenated ethers. The seriousness of this problem is illustrated by the fact that, in spite of the overwhelming epidemiological evidence for the carcinogenicity of tobacco smoke, the numerous tobacco-smoke inhalation studies conducted to date with a variety of experimental animal species have failed so far to demonstrate the carcinogenicity of tobacco smoke for tracheobronchial epithelium. This experience may serve as a warning for those who believe that inhalation exposure is the only legitimate or the best way to demonstrate the carcinogenicity of air contaminants. One complication related to dose is the filtration effect of the nasal turbinates in rodents, which are obligatory nose breathers. Another problem is related to definition of major target sites in the respiratory tract, and to measuring the dose for such sites. It is virtually impossible to calculate or estimate the absorbed dose per unit area of epithelium, or the amount of chemical released to a specific area. While many questions relating to detection of carcinogenic air contaminants can be adequately answered with simpler methodologies, some of the more complex inquiries into the pathogenetic mechanisms of lung cancer will have to be conducted with inhalation exposures.

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PAPER NO. 20

TOXICOLOGY OF METHYLCYCLOPENTADIENYL MANGANESE TRICARBONYL (MMT) AND RELATED MANGANESE COMPOUNDS EMITTED FROM MOBILE AND STATIONARY SOURCES

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INTRODUCTION

Regulations requiring that an unleaded grade of gasoline be marketed for the 1975 automobile models prompted testing of other potential antiknock agents. As a result, methylcyclopentadienyl manganese tricarbonyl (MMT), an organic manganese (Mn) compound, has received renewed attention because of its antiknock effectiveness.

DEVELOPMENT AND USAGE OF MMT
(Cope, 1970; Faggin et al., 1974)

The Ethyl Corporation carried out extensive studies on the development of MMT in the 1950's. MMT is an organo-metallic compound; its structural formula differs from cyclopentadienyl manganese tricarbonyl (MCT), which is used in USSR, by the presence of the methyl group (CH_3).

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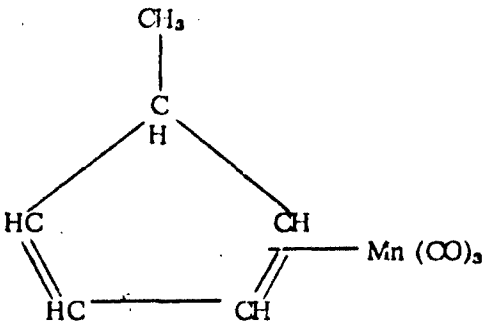

Structure of MMT or C.I. -2

MMT has been marketed as a supplement to tetraethyl lead in the antiknock compound (TEL Motor 33 Mix) which contained about 0.05 g Mn/ml Pb. Later, in the '60's, MMT was introduced as a fuel oil combustion improver under the trade name "Ethyl Corporation Combustion Improver-2 (C.I. -2)," and as a smoke suppressant for gas turbines using liquid fuels. Typical concentrations are 0.025 g Mn/gal of fuel oil, and 0.08-0.5 g Mn/gal for turbine fuels, depending on the type of fuel and turbine. At the present time, most of the MMT marketed is used as a smoke suppressant in gas turbine generators. Within the last few years, the Ethyl Corporation has conducted studies using this compound to replace lead completely as an antiknock additive. The MMT was registered with the EPA's Office of Fuel and Fuel Additives Registration as "Ethyl Antiknock Compound - Manganese." In these studies it was determined that the optimal additive rate is a concentration of 1/8 g Mn/gal of gasoline. Since MMT additive contains approximately 25% of Mn by weight, the increased utilization would be rather significant since it would reflect in higher levels of manganese emitted into the atmosphere. As a theoretical exercise, if one uses the gasoline consumption rate in the U.S. in 1971 and assumes that MMT is added at this rate to both regular (52.5×10^9 gals) and premium gasoline (36.7×10^9 gals), then about 24.5×10^6 lbs of Mn would be used yearly in the gasoline due to the additive. Assuming further - on the basis of data with tetraethyl lead - that about 1/3 of the added Mn is eventually emitted as fine particulates and another 1/3 as coarse particulates in the auto exhaust, then the potential increase of the total manganese injected into the environment may be up to 100 times as compared to the present rate of use if the emissions from automotive and turbine engines are similar (1 million lbs of the MMT additive used presently represents about 250,000 lbs of manganese). The Ethyl Corporation air monitoring data which were taken in the vicinity of power stations that use relatively large quantities of MMT as a smoke suppressant indicated levels from .01-.05 $\mu g\ Mn/m^3$.

In another estimate, the Ethyl Corporation staff suggested that if MMT was used at a rate of 0.25 g Mn/gal in 50% of all gasoline consumed in the U.S., then the motor vehicle exhaust would add to urban air .05-.2 μg Mn/m³. At this concentration, Mn would represent 1-4% of the total emitted particulates. Rural concentration is estimated to be about 5×10^{-3} μg Mn/m³. The authors further estimated that for an urban dweller, it would result in an exposure dose by inhalation of 1-2 μg Mn daily, and for the rural dweller about 0.1 μg (Ter Harr). The authors contrasted this value with the estimated 500-7000 μg Mn ingested daily in the diet (Table 1)(Schroeder et al., 1966).

TABLE 1. ESTIMATED DAILY INTAKE OF MANGANESE IN MAN*

Source	Mean, μg/day	Range, μg/day
Food	2400	500 - 7000
Water	5	0 - 1000
Air	2	0 - 7
Total	2407	500 - 8007

*Taken from Schroeder et al. (1966).

PUBLIC HEALTH ASPECTS OF MANGANESE

Manganese is an essential trace element for all living things and it is extremely ubiquitous in its distribution in nature (Schroeder et al., 1966; Cotzias, 1962). Its primary function in the body appears to be as a coenzyme in various metabolic processes involving such diverse reactions as: ribonuclease activity (Alger, 1970), mucopolysaccharide synthesis (Leach, 1971), and oxidative phosphorylation (Hurley et al., 1970; Cotzias). In trace amounts manganese is beneficial; however, industrial overexposures of workers to various manganese compounds have been associated with two different clinical disease syndromes - chronic manganese poisoning affecting the central nervous system, and a manganic pneumonia. High levels of manganese may become a hazard in the mining and processing of manganese ores and in the use of manganese alloys in the steel and chemical industries. The principal source is dry high speed drilling where the dust contains a large percentage of manganese dioxide (MnO_2). This, coupled with poor ventilation, provided the setting for most of the reported cases of chronic manganese poisoning in the literature, although additional sources are known (Rodier, 1955). Chronic manganese poisoning can result after exposure to high concentrations of manganese dust of only a few months duration, although the average exposure required was usually reported to be 2-3 years. Manganese may be absorbed by inhalation, ingestion, or through the skin, but the majority of reported effects were the result of prolonged inhalation. Nevertheless, other studies

have shown that a greater amount of manganese metal enters the body by the intestinal absorption route. The produced effects are reversible if subjects are removed from exposure; however, evidently a sensitivity can develop since persons who have recovered seem to be prone to contract the illness again (Couper, 1837; Von Oettinger, 1935; Cotzias, 1958; Whitlock et al., 1966; Tanaka and Lieben, 1969; Dogan and Benitic, 1953; Mena, 1967; Stara et al., 1973).

MMT AS AN AIR POLLUTANT

In considering the potential public health hazard of MMT used as a fuel additive in automobiles, it should be stated that 99+% of Mn in MMT was emitted from the automobile exhaust in the form of inorganic oxides, primarily as Mn_3O_4. Further, the remaining trace of MMT present in the exhaust decomposed rapidly in sunlight; experimentally it was found that the half-life of MMT was less than 2 minutes under average climatic conditions; under ideal conditions of bright sunlight, the half-life was found to be less than 30 seconds. Photolytic decomposition converted the remaining MMT into a mixture of manganese oxides and carbonates. There was no evidence of the presence of manganese carbonyl compounds in the monitored samples. The organic portion of the solids appeared to be a mixture of acid esters and polymers (Faggin et al., 1974). In comparing the particle size data for manganese and lead emitted from a car operated on the 7-Mode Federal Cycle, the particle size distributions were found to be nearly identical (Table 2).

TABLE 2. AIR-SUSPENDED LEAD AND MANGANESE FROM CAR EXHAUST

	Aerodynamic Mass Median Diameter, Micron	
	Cold Cycle	Hot Cycle
Manganese (0.125 g/gal)	0.38	0.30
Lead (0.5 g/gal)	0.40	0.32

ACCIDENTAL EXPOSURE IN WORKERS

In industrial experience routine handling in an open system involved the pouring of MMT from storage tanks into fuel containers. Normal transfer and distribution of the product have not resulted in known cases of illness or reactions in workers. Several accidental cases of other types of human exposure have been reported. Two individuals, while wearing airline masks, were accidentally exposed during a maintenance operation by having their clothes thoroughly soaked by a spray of MMT, creating a potential skin

contact of approximately 1.5 hours. The immediate symptom was a slight burning sensation of the skin. Urinary manganese levels rose to 46 and 137 μg Mn/l following this exposure. Urine samples taken a few weeks later showed the Mn levels to be within the normal 2-3 μg/l range. The men did not exhibit additional significant symptomatology (Ethyl Corporation, 1968). In another accident, a worker spilled 5-15 ml of MMT on his hands. Within 5 minutes the symptoms included: "thick tongue," giddiness, nausea, and headache. A fellow worker, handling a rag soaked with MMT noted a metallic taste in his mouth (Flight Surgeon News, 1969).

Exposure to MMT by inhalation and topically through the skin of the hands and forearms have been reported in six individuals; before the skin was cleaned, exposure period was estimated at 5-30 minutes. The symptoms, summarized in Table 3, began 5-60 minutes after exposure and in most cases completely subsided within two hours, with the exception of two workers in which a vague abdominal distress persisted for 2 days. No persistent changes on physical or neurological examination were noted in any of the men (Ethyl Corporation, 1968).

TABLE 3.

Symptom	Number of Workers Reporting Symptom
Metallic Taste	6
Headache	4
Nausea	4
G.I. Upset	3
Dyspnea	3
Tightness in Chest	1
Paresthesias	1

EXPERIMENTAL TOXICITY OF INORGANIC MANGANESE COMPOUNDS

In order to study the pathology of manganese poisoning, several investigators have induced toxic manganese effects in experimental animals. Baxter et al. (1965) studied the effects of acute manganese excess in rats to which manganese chloride in doses of 5 to 150 mg/100 g body weight was administered subcutaneously. Analysis of blood samples at 4 hours showed increases in hemoglobin, hematocrit and mean corpuscular volume, as well as serum chloride, phosphorus and magnesium in a group dosed with 15 mg/100 g body weight. Serum calcium and iron were markedly decreased. The maximum response in hematocrit and hemoglobin was increased by doses of 17 to 30 mg/100 g, and a measurable response occurred at 5 mg/100 g. From peripheral blood smears anisocytosis, basophilic stippling, and hypochromia of red cells were noted. Necrotic changes in hepatic tissues

were seen at 18 hours following injection of 17 mg/100 g or more. There was also an apparent increase in iron content of spleen and liver at 48 hours after injection of 30 mg/100 g.

Jonderko and Szczurek (1967) in a study using male rabbits, gave intravenous manganese chloride in doses of approximately 1.1 to 1.45 mg/kg for up to 32 days. Symptoms began to appear after the first week during which the animals became excited and aggressive followed by apathy and refusal to eat. At the end of the intoxication period, tremors of the head and legs were observed. Pathomorphologic changes were most severe in the liver showing necrosis and inflammation, with an appearance similar to acute hepatic cirrhosis. The kidney and G.I. tract were affected the least. In further experiments (Jonderko and Szczurek, 1969) after a dose of 0.5 to 2 mg Mn/kg subcutaneously as manganese chloride on alternate days for 16 weeks, changes were noted in brains of animals with elevated Mn content in cerebral tissue. The authors suggest that manganese affected directly the brain parenchyma.

Chandra and Srivastava (1970) injected rats with 8 mg/kg of $MnCl_2$ I.P. daily for 180 days; this experiment demonstrated that the maximum number of degenerated neurons was present when the manganese concentration in brain was at a maximum, indicating again that the extent of damage to brain cells is directly related to the amount of manganese in the cerebral tissues. The authors could not determine whether manganese has a direct toxic effect on the brain tissue or whether excess manganese is complexed with other protein molecules to form a toxic compound which may then either damage brain cells directly or carry the Mn ions across the blood-brain barrier in greater than normal concentrations.

Neff et al. (1969) administered a MnO_2 suspension subcutaneously to monkeys and showed that the caudate nucleus of the corpus striatum had significantly reduced levels of dopamine and serotonin, whereas the level of norepinephrine in cerebral brain stem was not affected. In addition, the dopamine depletion appeared to be related to the degree of toxicity; animals on the highest level of MnO_2 showed the greatest caudate nucleus dopamine decrease and the most marked disturbance of motor function. There were no histological changes reported in other areas of the brain. Vacuolar changes were noted in the livers of the test animals. Additional work implicating the liver in the pathogenesis of manganese encephalopathy was reported by Witzleben (1969). Doses of 5.3 to 6 mg/100 g body weight in rats showed that an acute manganese overload caused a rapid, marked decrease in the ability of the liver to clear bilirubin into the bile. Also noted was a rapid development of ultrastructural changes characteristically found in many instances of cholestasis. While there are several mechanisms that could be responsible for the reduced bilirubin clearance, it was noted that reduction in the bile flow rate was present 4 hours after acute manganese overload.

The role this process plays in relation to the development of the lesions of the basal ganglia is as yet undetermined. However, the inability to clear bilirubin in the newborn led to a condition known as kernicterus in which high levels of bilirubin were shown to be toxic to the CNS, as manifested by accumulation in the basal ganglia and by subsequent motor disturbances.

Borisenkova (1967) compared in rats the effects of inhalation to dusts containing ferromanganese, silicomanganese, manganese dioxide, and iron oxide for four months at a level of 0.07-0.15 mg/l. The motor reflex, as indicated by differences in chronaxie and rheobase of flexor and extensor muscles of the hind limb, was shown to be affected at 7-12 weeks after exposure to ferromanganese. With silicomanganese, changes occurred by the 15th to 16th week, while manganese dioxide effects were noted at intermediate times between the former and the latter. Iron oxide did not show a difference from control animals. Changes in morphology of the brain were consistent with changes seen in the motor reflex, suggesting again that the primary effect was on the central nervous system. In addition after four months of inhalation, all compounds were reported to produce chronic interstitial pneumonia. In this case, ferromanganese was the most toxic; the author suggested that permissible concentrations of manganese containing alloys should be based upon manganese content of a compound rather than on its oxidation state.

Davies (1946) studied in more detail the effects of manganese in producing manganese pneumonia. In this experiment, mice were exposed to manganese dioxide dust twice daily for 70 minutes, 5 days a week, for a total of 34 exposures. The animals were subsequently exposed to sprays of pneumococci cultures; the susceptibility to pneumonia was no different in controls from the manganese-treated groups. However, manganese dust was toxic by itself and did affect all parts of the respiratory system, producing a condition which the author prefers to call "manganese pneumonitis".

TOXICITY OF MANGANESE CARBONYL COMPOUNDS

Rather meager information is available in the literature on the toxicity of organic manganese compounds which are being used primarily as fuel additives. In the USSR the additive used in gasoline is manganese cyclopentadienyl-tricarbonyl - C_5H_5-$Mn(CO)_3$ (MCT). Several reports on toxicity of MCT are available in the Russian literature. In the U.S., the compound manufactured is the methylated chemical form methylcyclopentadienyl manganese tricarbonyl $CH_3C_5H_5$-$Mn(CO)_3$, (MMT). Some aspects on the toxicity of this compound were studied by the Kettering Laboratory, University of Cincinnati, in the late 50's. Summary of the experimental data was reported to the U. S. Environmental Protection Agency and the National Academy of Sciences - Panel on Manganese (Pfitzer et al., 1972). More recently, various toxicological aspects of this compound have been studied in our laboratory (Hysell et al., 1973; Moore et al., 1973; Moore et al., 1973).

TOXICITY OF MCT

Arkhipova et al. (1963) conducted inhalation exposures in rats and found that a concentration of 0.12 mg/l was lethal in 80% of the animals after a one-time 2-hour exposure, while 0.02 to 0.04 mg/l represented a sublethal dose of MCT (Table 4).

TABLE 4. LETHAL DOSES OF MANGANESE COMPOUNDS FOLLOWING ADMINISTRATION BY VARIOUS ROUTES IN DIFFERENT SPECIES. EACH TREATMENT WAS A SINGLE ADMINISTRATION EXCEPT WHERE INDICATED. ALL VALUES ARE EXPRESSED AS mg/kg EXCEPT WHERE INDICATED.

Animal	Compound	Treatment	LD_{100}	LD_{50}	$LD_{min.}$	Reference
Mouse	$MnCl_2$	ip	50	37.5	<25	Rogier et al., 1954
	$MnCl_2$	sc	210			Sullivan, 1969
	$MnSO_4$	sc	210			Sullivan, 1969
	MCT	oral	200	≈150	100	Arkhipova et al., 1965
	MCT	daily-18 days oral		25		Arkhipova et al., 1965
	MMT	oral		352		Pfitzer et al., 1972
Rat	MCT	oral	120	≈80	40	Arkhipova et al., 1963
	MCT	inhaled-2 hr.	0.12 mg/l	80% deaths		Arkhipova et al., 1965
			.04 mg/l	0% deaths		
	MMT (male)	oral		175 ± 33		Pfitzer et al., 1972
	MMT (female)	oral		89 ± 14		Pfitzer et al., 1972
	MMT	oral		58		Hysell et al., 1973
	MMT	inhaled-1 hr.-LC_{50}		.22 mg/l		Pfitzer et al., 1972
Guinea Pig	MMT	oral		905		Pfitzer et al., 1972
Rabbit	MnO_2	iv	45			Sullivan, 1969
	MMT	iv		6.6		Pfitzer et al., 1972
	MMT	oral		95		Pfitzer et al., 1972

In another experiment, animals were also exposed over an 11-month period for 4 hours/day; the only changes noted were increases in the threshold for neuromuscular excitability and a decrease in resistance to infection. Acute poisoning was accompanied by vascular changes and hemorrhages, hypoxia and atrophic changes in the cells of the central nervous system. Chronic poisoning was characterized by renal and nervous damage and histologic changes in the respiratory tract. When tetrahydrofuran was used as the solvent the toxicity of MCT was increased, probably due to absorption of the substance through the skin. Another study by Archipova et al. (1963) confirmed these findings and the author discussed the possibility that hypoxia was produced by interference of MCT with oxidative phosphorylation. In a third study, Archipova (1965) established the oral LD_{100} of MCT administered in sunflower seed oil to white mice at a dose of 200 mg/kg; the LD_{50} dose was approximated at 150 mg/kg. White rats showed a greater sensitivity, the LD_{100} being 120 mg/kg and LD_{50} 80 mg/kg. The minimum lethal

dose was 40 mg/kg and the maximum tolerable dose was 20-30 mg/kg. Necropsies performed on animals that died disclosed raspberry-colored blood and a brownish tinge of the kidney and liver tissues. Because of the change in color of these organs, the osmotic erythrocyte resistance was tested and determined to be significantly lowered. The unusual color may have been due to hemosiderin accumulation within the tissues. Repeated oral administration of 25 mg/kg to white mice or 10 mg/kg to rats proved to be toxic, indicating that MCT or its metabolic product effects accumulated in the organism after repeated dosing. Chronic administration of 5 mg/kg MCT over a period of 2 months decreased significantly the osmotic erythrocyte resistance. Repeated subcutaneous administration of 30 mg/kg for 2 months produced serious disturbances of the central nervous system and gastrointestinal tract, with death occurring within 10-12 days.

TOXICITY OF MMT

Lethality Studies

Lethal effects after i.v., oral, and inhalation administration of MMT are compared with other manganese compounds in Table 4. There was a high degree of variability, particularly the results for rats, depending on sex, concentration of ingested MMT, and vehicle of delivery. Other species tested were mice, guinea pigs, and rabbits. The lethality values were also compared whenever feasible with reported results on inorganic Mn compounds experimentally administered by various routes (Moore et al., 1973).

Dermal LD_{50}

Only one study dealing with the absorption of MMT through the skin was reported. The number of experimental animals was limited, however. Table 5 lists the only available LD_{50} data for percutaneous absorption (Pfitzer et al., 1972).

TABLE 5

Species	Length of Skin Contact in Hrs.	Vehicle	LD_{50} (in mg/kg)
Rat	6	Peanut Oil (10%)	665 ± 60
Rabbit	24	Kerosene (10%)	1350 ± 680

Inhalation Toxicity

The effect of MMT inhalation in the rat has been studied in several experiments. The one-hour LC_{90} for the rat was .22 mg/l or 25 ppm (Table 4). A list of mortalities at various dosages following a single inhalation dose is presented for a number of species in Table 6 (Pfitzer et al., 1972).

Repetitive inhalation exposures for 7 hrs/day for 150 days at a level of .015 mg/l resulted in mortality for rats and mice, but not for guinea pigs, rabbits, cats, or dogs (Pfitzer et al., 1972). Histopathological examination of tissues was limited; changes were observed in the liver and kidneys. Repetitive exposure for the same duration at .006 mg/l did not produce mortality in any of the species. No significant pathological abnormalities were found in the tissues studied from the experimental animals at the latter exposure level (Pfitzer et al., 1972).

TABLE 6. MORTALITY IN VARIOUS SPECIES FOLLOWING SINGLE INHALATION EXPOSURES TO METHYLCYCLOPENTADIENYL MANGANESE TRICARBONYL*

Expt. No.	Concentration Time Product (mg/l x hour)	Concentration (mg/l)	Time (hour)	Mortality (No. Dead/No. Exposed)					
				Cat	Dog	Guinea Pig	Mouse	Rabbit	Rat
17D	.0318	.106	0.3	-	-	-	1/10	-	0/10
19	.0456	.228	0.2	-	-	-	0/10	-	0/10
15A	.0684	.228	0.3	-	-	-	0/10	-	0/10
17C	.112	.112	1.0	-	-	-	2/10	-	3/10
9	.113	.378	0.3	0/1	-	-	1/10	-	2/5
20G	.120	.0177	7	-	-	-	2/10	-	-
18F	.122	.035	3.5	-	-	-	1/20	-	-
16	.216	.216	1.0	0/1	-	0/6	-	0/4	-
7	.218	.218	1.0	-	-	-	1/10	-	2/5
18E	.266	.038	7	-	-	-	1/20	-	-
17B	.290	.083	3.5	-	-	-	3/10	-	2/10
18A	.294	.042	7	-	-	-	3/10	-	-
11	.338	1.127	0.3	1/1	-	0/6	3/10	0/4	7/10
4	.470	.470	1.0	1/1	-	0/6	7/10	0/4	7/10
13	.524	.308	1.7	-	0/	-	-	-	-
17A	.679	.097	7	-	-	-	6/10	-	10/10
6	.798	.228	3.5	-	-	-	10/10	-	9/10
12	.831	.489	1.7	-	1/1	-	-	-	-
15	.858	.245	3.5	0/1	-	0/6	-	0/4	-
10	1.237	1.237	1.0	1/1	-	6/6	10/10	4/4	10/10
3	1.302	.372	3.5	1/1	-	0/6	10/10	0/4	10/10
8	1.561	.223	7	1/1	-	0/6	10/10	0/4	10/10
1,2	2.877	.411	7	2/2	-	1/6	10/10	5/7	10/10

*Taken from Pfitzer et al. (1972), "Toxicity of Methylcyclopentadienyl Manganese Tricarbonyl".

In our laboratory, rats were exposed to MMT at a concentration of .002 μg/l for 4 hours. Although no gross abnormalities were noted, there appeared a cloudy swelling of the hepatic parenchyma at 1 and 2 days postexposure. Prominent cytoplasmic vacuolar changes, similar in appearance to lipidic degeneration of the liver, were present in several animals by 21 days postexposure (Dogan and Benitic, 1953). Additional data on chronic inhalation toxicity at low levels are needed to establish more precise parameters of pathogenicity.

Skin and Eye Irritation

Neither rats nor rabbits demonstrated significant dermal irritation in response to an acute contact with MMT. Repetitive skin contact with leaded and unleaded gasoline containing MMT revealed no adverse effects which could be attributed to the MMT additive alone.

Following application of a 10% MMT solution in kerosene into the eyes of rabbits, a very milky cornea and transient hyperemia was occasionally observed (Pfitzer et al., 1972).

Uptake, Distribution and Excretion of ^{54}Mn Following Oral ^{54}Mn-MMT

Rapid G.I. uptake of MMT was evidenced in our laboratory by the increase in urinary manganese following ingestion by rats (Hysell et al., 1973). Similar increases were also seen following inhalation exposures in both rabbits and dogs (Schroeder, 1970).

The whole body kinetics of the labeled ^{54}Mn-MMT was studied in our laboratory following the ingestion of the compound in rats (Moore et al., 1973). The percent retention of ^{54}Mn following a single oral dose of radioactive ^{54}Mn labeled MMT is plotted as a function of time in Figure 1. ^{54}Mn was rapidly eliminated from the body with approximately 27% remaining after 24 hours. It was evident that the retention pattern contained at least two major components, a rapid elimination component followed by a long-term retention component. The long component resulted in a half-life of about 7 weeks ($T_{1/2}$ = 48 days). The half-life of the second component was based on measurements made from the 26th day to the 107th day.

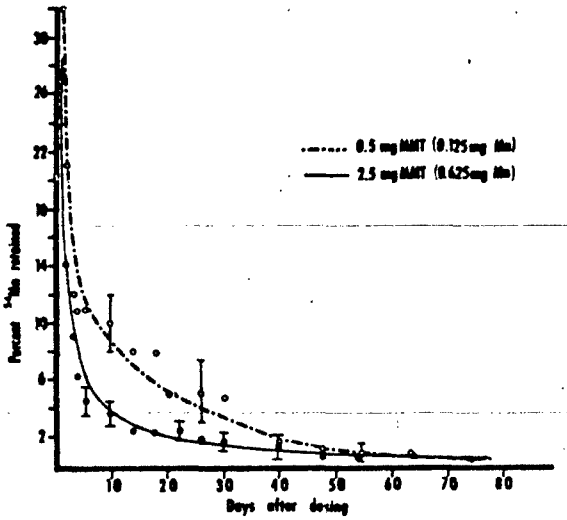

Figure 1. Whole body retention of ^{54}Mn following intragastric administration of ^{54}Mn tricarbonyl.

^{54}Mn Excretion Pattern After ^{54}Mn-MMT

The excretion data shown in Figure 2 reveals that the G. I. tract is the more important pathway for MMT excretion. However, the difference is not large since the observed urine:feces (U/F) ratio ranges only from .68 to .25 (Moore et al., 1973). This is in contrast to the excretion pattern of tested inorganic manganese compounds; in the latter case, the kidneys play an extremely minor role, since practically all of the metal is excreted by the fecal route. Comparison data are presented in Table 7.

It is not known what percentage of the ingested ^{54}Mn-MMT was absorbed by the body; thus, the larger excretion of ^{54}Mn-MMT by the G. I. tract may possibly be attributed to purely passive rather than active excretory mechanisms.

The analysis of individual organs for manganese content revealed that the highest concentrations were found in liver, lung, and/or kidney, pancreas, and heart, in order of decreasing concentration. This pattern of distribution is similar for both oral and inhalation exposures.

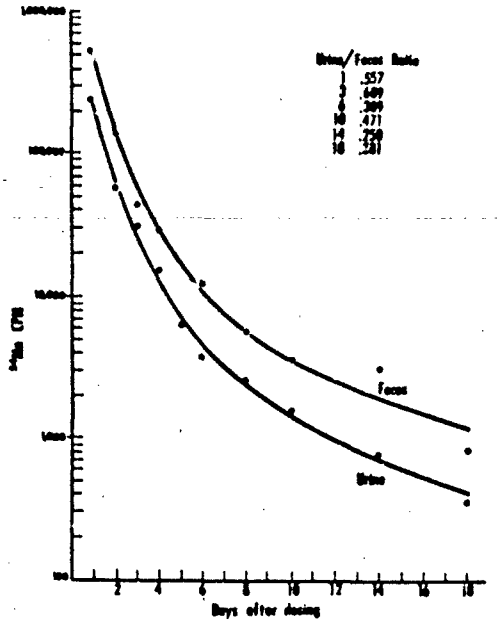

Figure 2. Excretion of ^{54}Mn following intragastric administration of ^{54}Mn tricarbonyl.

TABLE 7. EXCRETION PATHWAYS OF Mn IN RATS

Compound	Adm. Route	% of Total Excreted Urine	Feces	References
MMT	Oral	~30	~70	31
$MnCl_2$	Oral	.1	99.1	39
$MnCl_2$	Oral	N.D.	100	40
MMT	I.V.	~22	~78	31
$MnCl_2$	I.V.	trace	99+	31
$MnCl_2$	I.P.	N.M.	90	40
$MnSO_4$	S.C.	N.D.	100	41

N.D. - Not Detectible

N.M. - Not Measured

Distribution of ^{54}Mn in Pregnant Rats Following Oral Administration of ^{54}Mn-MMT

In another experiment performed in our laboratory, pregnant rats were given a single oral dose of 2.5 mg of ^{54}Mn-MMT during the period of fetal organogenesis (7-10 days postconception). The females were sacrificed on the 20th day of gestation, i.e., just prior to parturition, and the fetuses, placentae, and tissues from the dams were then assayed to determine the radiomanganese concentration. The mean concentrations of ^{54}Mn in various tissues are presented in Table 8.

TABLE 8. ^{54}Mn ACTIVITY IN SELECTED TISSUES AND FETUSES

	^{54}Mn, mean counts/min/gram							
	Maternal Tissues							
	Placenta	Bone	Liver	Lung	Kidney	Pancreas	Blood	Fetus
Activity	173	91	916	455	470	658	470	88

General Toxicity and Pathology

Toxic symptoms resulting from the administration of lethal doses of MMT were similar in all species studied regardless of the route of administration. They consisted of initial mild excitement and hyperactivity, tremors, severe toxic spasms, weakness, slow-labored respiration, occasional clonic convulsions, and terminal coma. Animals given sublethal doses exhibited similar but less severe manifestations, and recovered in 2 to 6 weeks. No residual neurologic effects were apparent (Pfitzer et al., 1972; Hysell et al., 1973).

The kidneys and livers were the primary sites of pathologic alterations following exposure to MMT. The kidneys and livers were swollen, all viscera were hyperemic, and petechiae were noted in the lungs. Histologic examination revealed hepatic centrilobular congestion, necrosis and later vacuolar (probably lipidic) changes. Necrosis of the renal tubules was evident. Lung injury was characterized by perivascular edema and swelling and pyknosis of the cells of the intima and media (Hysell et al., 1973).

DISCUSSION

Under normal circumstances the major route of entry of manganese into the body is through the G.I. tract. The Mn content of the diet varies greatly and daily intake has been estimated to have a range from 0.5-7.0 mg

(Schroeder, 1970; Cotzias, 1958). Water contributes a small fraction to the daily intake with a range from 0-10 μg (Schroeder, 1970). The amount of manganese in ambient air is extremely low and, with the rare exception of a volcanic eruption, the higher concentrations of Mn in the atmosphere are the result of industrial pollution (Department of Health, Education, and Welfare, 1968). The average quarterly Mn values for urban atmosphere reported in 1965 ranged from below minimum detectible to 0.31 $\mu g/m^3$. The maximum values for individual samples collected from some cities were considerably higher, i.e., the highest value was 9.98 $\mu g/m^3$ for Charleston, West Virginia in 1964. The U.S. industrial threshold limit for an 8-hour day has been set at 5,000 $\mu g/m^3$ while in USSR the recommended 24-hour limit is 10 $\mu g/m^3$. The U.S. industrial limit could theoretically result in pulmonary absorption of 5 mg Mn/day, if we assume 100% absorption; this would represent nearly 1/2 the body pool. Even if the pulmonary absorption was, for instance, only 50%, the inhalation contribution to the body pool would still be rather significant (Schroeder, 1970). In addition, the lungs serve as an abnormal route for Mn. Surveys of manganese poisoning reports in humans show that most of the cases resulted from the inhalation of manganese dust or fumes, even though recent information suggests that the bulk of inhaled Mn becomes transferred to the gastrointestinal tract from where a major part of it is excreted (Bertinchamps et al., 1966; Mena et al., 1969). Experimental evidence suggests that the development of chronic manganism is usually associated with (a) inhalation of Mn dust, (b) possible accumulation of Mn in the lungs, and (c) differences in turnover rates (Cotzias et al., 1969).

Therefore, the most important factor that should be considered in determining the environmental impact of MMT is whether its usage will cause a significant increase in the ambient levels of Mn in the atmosphere in highly populated areas, and the effects of Mn on other atmospheric pollutants. It appears that there are advantages of using Mn over lead as a fuel additive; however, it should be noted that industrial sources stated that the use of manganese as an antiknock agent "would not in any sense be a substitute for lead, not would it even noticeably decrease the problems and penalties caused by lead removal; however, such usage potentially represents a useful additional degree of flexibility in the refiners' activities associated with producing an unleaded grade of gasoline."

The site and mechanism action of MMT or its metabolites have not been satisfactorily identified as yet. However, evidence from the studies at this laboratory suggests that the liver and kidneys are the most important organs. This statement is supported by the fact that the manganese is normally secreted in the bile and that urine was an important route of Mn excretion in experiments with MMT. However, when the Mn is given in the inorganic form, it is almost completely excreted in the feces. These findings are supported by data from accidental exposures to MMT. Increased

levels of Mn have been found in the urine of workers exposed to MMT, whereas normally only minute quantities of Mn are excreted in the urine.

It would appear biologically reasonable to use a low concentration of MMT as partial or complete replacement for lead in gasoline, assuming lead remains in gasoline in some level. In one study at this laboratory, animals (rats and hamsters) were exposed for 8 hours a day for 2 months to automotive emissions where gasoline containing 1/8 g MMT was used as a fuel. No significant pathological lesions were found that could be attributed to the use of MMT. A range of Mn concentrations was found in the tissues taken from the control and exposed groups of animals with considerable overlap between tissue values of the two groups. Additional chronic studies using proposed concentrations of MMT in gasoline (1/8 g, Ethyl Corporation) are needed to determine lung burden and the overall biological effect. Although Mn appears to have a very low toxicity through normal routes of exposure, any Mn compounds that through widespread use would increase ambient air levels excessively should be carefully evaluated.

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PAPER NO. 21

THEORETICAL EXAMINATION OF CARBON MONOXIDE KINETICS WITH A COMMENT ON TIME-WEIGHTED AVERAGE EXPOSURES

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Because carbon monoxide toxicity appears to be a function of the carboxyhemoglobin level, it would be useful to have an equation which could accurately predict blood carboxyhemoglobin concentrations under different exposure conditions without actually measuring any parameter in the subject. Factors affecting the carboxyhemoglobin level include the duration of exposure, the concentration of carbon monoxide in the air, the degree of physical activity, the initial carboxyhemoglobin level, the postexposure time, the body size, the endogenous generation of carbon monoxide, and the oxygen content of the air. There have been many attempts to mathematically describe the uptake and elimination of carbon monoxide (Coburn et al., 1965; Pace et al., 1946; Forbes et al., 1945; Peterson and Stewart, 1970; Forster et al., 1954). Previously, the only equation which contained all eight of these variables was that of Coburn et al. (1965). Using Coburn's equation, Peterson found he could accurately predict the levels of carboxyhemoglobin in inactive men exposed to low concentrations of carbon monoxide. One limitation of Coburn's equation is the fact that the arterial oxyhemoglobin, which varies with changes in the carboxyhemoglobin level, must be known or approximated. At low carboxyhemoglobin levels the arterial oxyhemoglobin concentration can be assumed to be constant and equal to 20 ml O_2/100 ml blood (hemoglobin saturated with oxygen). At higher carboxyhemoglobin levels, this assumption is not valid. Using Coburn's equation one would predict the equilibrium level of an exposure to 500 ppm carbon monoxide to be about 83% carboxyhemoglobin. Previous studies have found the blood carboxyhemoglobin level resulting from a continuous exposure to 500 ppm carbon monoxide would be approximately 44% (Hanne, 1935, 46%; Haldane, 1895, 40%; Forbes et al., 1945, 44.8%). The error involved in using this equation is appreciable at high carboxyhemoglobin levels because the arterial hemoglobin can no longer be assumed to be saturated with oxygen. Because the arterial oxyhemoglobin concentration is a function of the carboxyhemoglobin level, it is impractical to use this equation under all conditions to predict carboxyhemoglobin levels.

*Presented by H. C. Hodge, University of California, San Francisco Medical Center.

The equation below, based on the work of Haggard (Henderson and Haggard, 1943) on the uptake and elimination of inert gases, was adapted to predict blood carboxyhemoglobin levels under a wide range of exposure conditions. The derivation of the equation, which can be found elsewhere (MacGregor, 1973), is based on the following assumptions:

1. The gas is absorbed and eliminated solely via the lungs.
2. The substance is inert in the sense that it is not metabolically altered in the body.
3. Lung washout time is not significant.
4. There is instantaneous mixing throughout the body compartment.
5. Diffusion across the alveoli is rapid; the blood and alveolar air equilibrate with each breath.
6. Diffusion within the alveolus is rapid, and therefore the concentration of gas at all sites in the lung is the same.
7. The inspiratory and expiratory volumes are assumed to be equal.
8. Ventilation is pictured as a continuous, not a cyclic, process.
9. The pulmonary blood flow is equal to the cardiac output.
10. The air concentration of the gas is constant.

Equation: $(P_b) = k\ in/k\ out + [P_b(O) - (k\ in/k\ out)]\, e^{-k\ out\ t}$ ..... (1)

$[P_b(O)]$ = Initial concentration of carbon monoxide in the blood, μg/ml
(P_b) = Concentration of carbon monoxide in the blood, μg/ml
(P_a) = Concentration of carbon monoxide in the air, μg/ml
V = Alveolar ventilation rate, L/min
CO = Cardiac output, L/min
VD = Volume of distribution of carbon monoxide, blood volume, L
t = Time, min
k in = Uptake rate constant, (μg/ml)/min
k out = Elimination rate constant, min^{-1}
λ = Partition coefficient of carbon monoxide in the blood. It can be obtained from either:
1. The in vitro ratio of carbon monoxide concentration in blood/carbon monoxide concentration in air.
2. The in vivo equilibrium ratio of carbon monoxide concentration in blood/carbon monoxide concentration in air.

$$k\ in = \frac{V(P_a)}{[V + (CO \cdot \lambda)]} \cdot \frac{CO}{VD} + \text{Endogenous rate of carbon monoxide formation} \quad \text{..... (2)}$$

$$k\ out = \frac{V}{[V + (CO \cdot \lambda)]} \cdot \frac{CO}{VD} \quad \text{..... (3)}$$

For truly inert gases and vapors, the concentration dissolved is always proportional to the partial pressure of the substance in air (hence the ratio of the concentration dissolved to the concentration in air is a constant). The "apparent solubility" of carbon monoxide in blood, however, is limited by the number of binding sites available on the hemoglobin molecules. For carbon monoxide the ratio of the concentration in blood to that in air is not a constant but decreases with increasing carbon monoxide concentrations in air. The partition coefficient was calculated from data on either the in vitro or in vivo equilibrium ratio of carbon monoxide in blood to that in air. Values for exposures to carbon monoxide concentrations of 50, 200, 500, 1000, and 2000 ppm were equal to 365, 271, 205, 141, and 82, respectively. When using the equation, the appropriate value for the partition coefficient, λ, should be used.

There are only two studies in which carboxyhemoglobin levels were measured in humans under controlled experimental conditions where enough information was reported to enable one to utilize the equation above to calculate the predicted carboxyhemoglobin level. Pace et al. (1946) measured carboxyhemoglobin levels after exposures to high levels of carbon monoxide for relatively short times. During every exposure the blood volume, minute volume, initial carboxyhemoglobin level, concentration of carbon monoxide in the air, and the time were measured. The cardiac output was estimated from data correlating ventilation rates with cardiac outputs (Asmussen and Nielsen, 1952; Spector, 1956). In the calculations, λ was equal to 205 for exposures from 550 to 570 ppm, 141 for exposures from 900 to 1500 ppm, and 82 for exposures from 1720 to 2180 ppm. Before the experiment, no attempt was made to control smoking and therefore the initial carboxyhemoglobin levels varied from 0 to 8.9%. The subjects were also tested under various conditions from rest to hard work.

Table 1 lists the experimental parameters and the predicted and measured carboxyhemoglobin levels. The average deviation was 1.84% carboxyhemoglobin, and was similar under all conditions tested. Therefore, the equation was able to accurately predict carboxyhemoglobin levels over a wide range of exposure conditions.

Perhaps the best measurements of carboxyhemoglobin levels during experimentally controlled carbon monoxide exposures are those of Peterson and Stewart (1970) and Stewart (1970). Peterson made many determinations of carboxyhemoglobin levels in inactive nonsmoking young men, during and following long exposures to carbon monoxide from 25 to 100 ppm. Figure 1 shows the uptake of carbon monoxide during exposures to 50, 100, 200 and 500 ppm. The lines are the predicted carboxyhemoglobin levels and the points are the levels actually measured by Peterson. The predicted carboxyhemoglobin levels were calculated for a standard inactive, nonsmoking man with an alveolar ventilation of 6.0 L/min, a blood volume of 5.5 L, and a cardiac output of 7.5 L/min. The partition coefficient was equal to 365. As can be seen, the equation is able to predict accurately the level of carboxyhemoglobin attained under the conditions tested.

TABLE 1. UPTAKE OF CARBON MONOXIDE IN WORKING AND RESTING MEN

Subject[a]	Air CO	Time	Min. Vol.	Blood Vol.	Initial Blood COHb	Final Blood COHb	Predicted	Deviation
PIT	1720	15	18.4	6.09	3.3	23.0	20.6	-2.4
FED	1870	15	22.3	5.85	7.2	26.5	28.7	+2.2
WAG	2120	15	18.7	5.28	4.2	33.8	29.3	-4.5
SHA	1420	20	24.9	6.39	6.4	26.1	30.6	+4.5
CAT	1410	20	17.8	5.67	5.4	26.1	25.6	-0.5
JAM	1290	20	21.1	5.28	0.8	24.8	24.9	+0.1
WIS	900	30	17.5	5.04	2.8	24.7	22.3	-2.4
LEG	900	30	18.5	5.64	8.9	23.6	25.1	+1.5
HIN	940	30	18.1	5.28	8.9	29.5	27.6	-1.9
SPE	550	45	15.6	5.04	3.6	19.3	19.9	+0.6
WAR	560	45	17.4	4.83	3.0	25.4	21.9	-3.5
REI	570	39	20.2	5.61	1.8	20.0	19.4	-0.6
WAT	920	30	9.4	5.31	1.8	12.1	13.7	+1.6
MAR	2000	20	9.6	6.18	2.2	14.5	17.2	+2.7
COV	2000	20	6.6	5.61	1.6	11.2	13.3	+2.1
KLI	2000	20	6.1	5.76	0.0	10.7	10.9	+0.2
HOL	2000	20	5.8	4.98	4.0	13.0	15.4	+2.4
BLA	2000	20	17.9	5.37	0.4	34.6	29.3	-5.3
KRU	2000	20	22.4	6.03	1.3	32.3	32.3	0.0
TUS	2000	20	24.2	6.09	3.3	34.9	34.8	+0.1
MCB	2000	20	22.8	6.24	2.9	33.7	32.7	-1.0
SCE	2000	20	19.1	5.49	3.6	34.9	32.1	-2.8
BIT	1500	24	30.1	7.08	1.7	35.8	35.5	-0.3
SCA	1500	30	23.5	5.49	3.7	28.9	32.4	+3.5
DEM	1000	30	17.6	5.22	6.9	27.0	26.3	-0.7
DIE	1000	30	18.9	5.49	2.4	26.1	23.9	-2.2
ERI	1000	30	17.1	5.16	5.0	24.8	24.8	0.0

[a]Data of Pace et al Am. J. Physiol. 147:352, 1946.

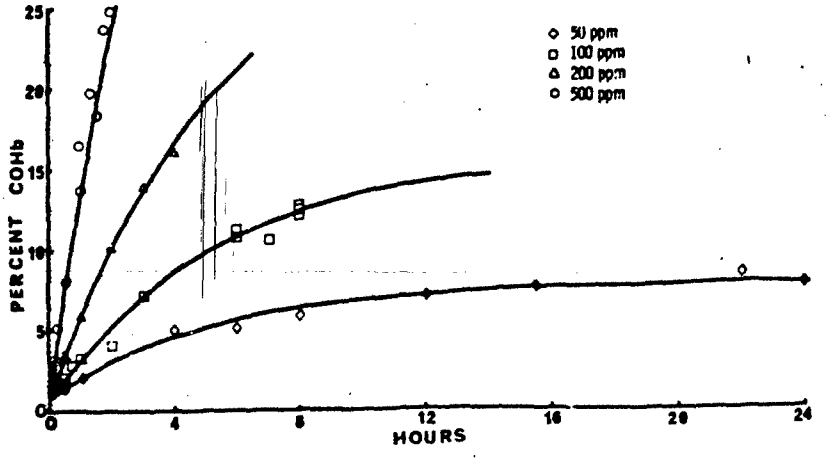

Figure 1. Uptake of carbon monoxide as a function of time.

In summary, the inert gas equation can predict carboxyhemoglobin levels following exposures to carbon monoxide from 25 to 2180 ppm, for a five-fold increase in ventilation, for smokers as well as nonsmokers, and for exposures from 15 to 1440 minutes in duration.

Since the equation derived could predict carboxyhemoglobin levels under all conditions tested, the equation was used to predict carboxyhemoglobin levels in situations in which data were limited or completely lacking.

The 50 ppm threshold limit value for carbon monoxide is not a ceiling limit but a time-weighted average exposure with a maximum permissible concentration of one and one-half times the limit value (ACGIH, 1971). No data were available on carboxyhemoglobin levels during a time-weighted average exposure to carbon monoxide in smokers or nonsmokers. These levels were calculated for three different time-weighted average exposure schedules to 50 ppm of carbon monoxide:

a. 75 ppm (4 hr), 25 ppm (4 hr)
b. 75 ppm (2 hr), 25 ppm (2 hr), 75 ppm (2 hr), 25 ppm (2 hr)
c. 25 ppm (4 hr), 75 ppm (4 hr).

The calculations were based on an average man, with a blood volume of 5.5 L, an alveolar ventilation of 6 L/min, and a cardiac output of 7.5 L/min. The initial carboxyhemoglobin level was taken as 1.4% for the nonsmoker, and 7.3% for the smoker. These were the carboxyhemoglobin levels actually measured by McIlwaine (1969) throughout a work day in two subjects not occupationally exposed to carbon monoxide. The carboxyhemoglobin levels resulting from exposures to the three time-weighted average schedules were calculated, and the results of these calculations are shown in Figure 2. For the nonsmoker the carboxyhemoglobin levels vary markedly with the exposure schedule. The biggest difference observed was between exposures to schedule a and c. For the first four hours, the exposure to schedule a produces about twice the carboxyhemoglobin levels as exposure to schedule c, while during the last four-hour exposure, the carboxyhemoglobin levels are nearly the same for the two schedules. Therefore, the total carboxyhemoglobin level during the day varies greatly depending on the exposure schedule, even though the time-weighted average is exactly the same.

The upper three curves represent the exposures of a heavy smoker to the same three time-weighted average schedules. As expected, the level of carboxyhemoglobin is much higher for the smoker; however, the carboxyhemoglobin level is not affected greatly by the time-weighted schedule but is similar for all three exposure schedules.

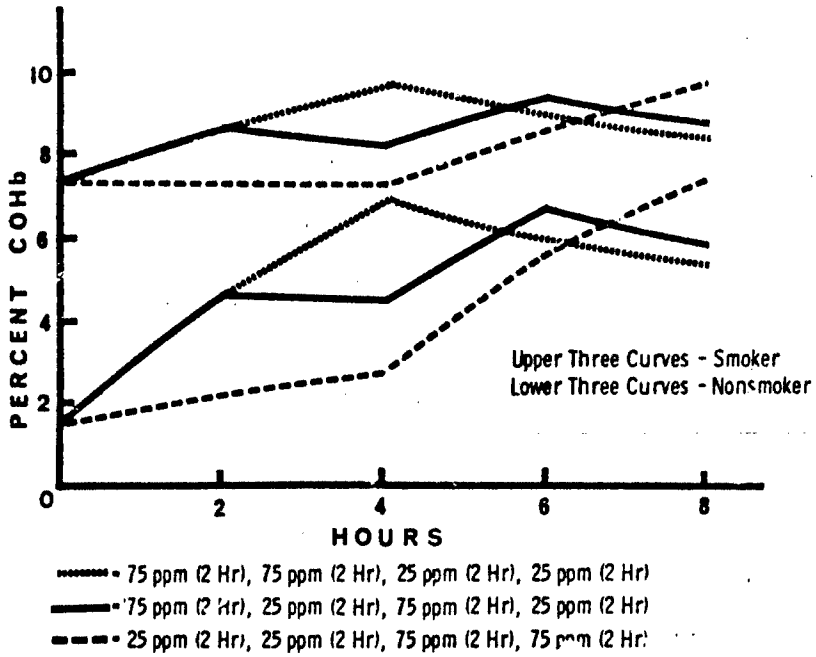

Figure 2. Eight-hour exposure to a time-weighted average of 50 ppm carbon monoxide.

Recently NIOSH has published a criteria document on carbon monoxide recommending a change in the time-weighted average to 35 ppm and in the ceiling concentration to 200 ppm (NIOSH, 1972). Under this proposal an exposure to 200 ppm could continue for 1 hour as long as the concentration of carbon monoxide remained at 10 ppm throughout the rest of the day. This schedule would meet the 35 ppm time-weighted average standard.

In order to determine whether the new proposed standard was an improvement over the previously recommended one, the levels of carboxyhemoglobin were calculated for several hypothetical time-weighted average exposures to either 50 or 35 ppm carbon monoxide for a nonsmoker and a smoker during light activity (alveolar ventilation = 9.5 L/min). Table 2 is a summary of the calculated maximum carboxyhemoglobin levels attained during the eight-hour exposure. For the nonsmoker the maximum level of carboxyhemoglobin attained during a continuous exposure to 35 ppm carbon monoxide for eight hours if 4.02% and the maximum during a continuous exposure to 50 ppm is 7.31%. However, under a time-weighted average schedule to 35 or 50 ppm the maximum carboxyhemoglobin levels attained are nearly identical, 8.58% for the 50 and 8.48% for the 35. Under some

time-weighted average schedules the new standard will not afford a significant decrease in the maximum carboxyhemoglobin level.

TABLE 2. MAXIMUM CARBOXYHEMOGLOBIN LEVELS AFTER AN 8-HOUR TIME-WEIGHTED AVERAGE EXPOSURE TO 50 OR 35 PPM CARBON MONOXIDE

Time Weighted Average	Schedule ppm x Time, Hr	Maximum % Carboxyhemoglobin	
		Nonsmoker	Smoker
50	50 x 8	7.31	a
50	75 x 4, 25 x 4	8.58	10.47
35	35 x 8	4.02	a
35	10 x 7, 200 x 1	8.48	12.5
35	10 x 6, 100 x 2	6.99	11.0
35	10 x 7, 200 x 1	12.9*	14.1*

V = 9.5 Liter/Min. Light Activity
*V = 18.0 Liter/Min. Light Work

a = Maintains level of carboxyhemoglobin from smoking.

Even more significant is the observation that a smoker can attain much higher carboxyhemoglobin levels with the new standard than were possible under the old TLV of 50 ppm. Clearly, carboxyhemoglobin levels in excess of 12% are highly undesirable for a continued occupational exposure. It would be beneficial to add a provision to the criteria document recommending that the 200 ppm ceiling level be reduced, or that there be a time limit of approximately 5 minutes a day during which the ceiling level would be permitted.

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AMRL-TR-73-125

PAPER NO. 22

DICHLOROMETHANE INHALATION AND DRUG METABOLIZING ENZYMES. THE EFFECT OF CHEMICAL TREATMENT WITH MIXED-FUNCTION OXIDASE INDUCING AND INHIBITING AGENTS ON CARBOXYHEMOGLOBIN FORMATION

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INTRODUCTION

For some time, dichloromethane (DCM) has been classified as having a relatively low order of toxicity (Kutob and Plaa, 1962; Browning, 1965). Stewart et al. (1972) recently showed that humans who inhaled 500-1000 ppm DCM also had increased values of % carboxyhemoglobin (COHb). Using radio-labeled techniques, Miller et al. (1973) demonstrated that the carbon monoxide (CO) moiety in the COHb molecule is metabolically derived from the DCM molecule. In addition, the group here at Wright-Patterson Air Force Base (Bullock et al., 1971) has shown that continuous exposure to DCM vapors at 1000 and 5000 ppm was toxic to dogs, mice, rats, and monkeys. These recent studies have stimulated new interest in this compound.

Our initial intent was to determine if, and to what extent, the conversion of DCM to CO occurred in the lung. If such conversion occurred exclusively in lungs, the DCM metabolism might be used as a model for lung metabolism of chemicals analogous to the hexabarbital sleeping time model for liver metabolism. Animals could then be challenged with various chemicals and drugs to measure the effect of these materials on DCM metabolism. Preliminary studies convinced us that the metabolism of DCM to CO is occurring in the liver and thus our original hypothesis concerning the lung metabolism of DCM to CO was largely negated.

In addition, solvent toxicity and the modification of solvent toxicity through chemical treatment of animals have been investigated in our laboratory. Dechlorination reactions have been shown to be catalyzed by enzymes found in the hepatic endoplasmic reticulum (Van Dyke and Chenoweth, 1965; Van Dyke and Wineman, 1971). Thus, we continued our research with two

goals: to characterize the response of rabbits to exposure to DCM and to see if this response was altered by treatment with modifiers of hepatic mixed-function oxidase.

As Dr. Van Stee and others did yesterday, I hasten to mention that the authors of this paper are Dr. R. Roth, Dr. Ray Lo and myself and would like to acknowledge the support of my entire section for carrying out the various aspects of this study.

METHODS

Male, New Zealand white rabbits weighing 2.5-3.5 kg were used throughout this study. They were acclimatized in our animal quarters at 72 F, 50% RH for at least one week prior to use. A 12-hour-on, 12-hour-off lighting cycle was used. Exposures were carried out using a nose exposure system designed and built in our laboratory (Figure 1). During exposure to DCM rabbits were placed in restrainers and a latex rubber mask was placed over the face of the animal. This mask, having a central opening for nose breathing, also prevented chewing and served to calm the animals as the mask usually covered the eyes.

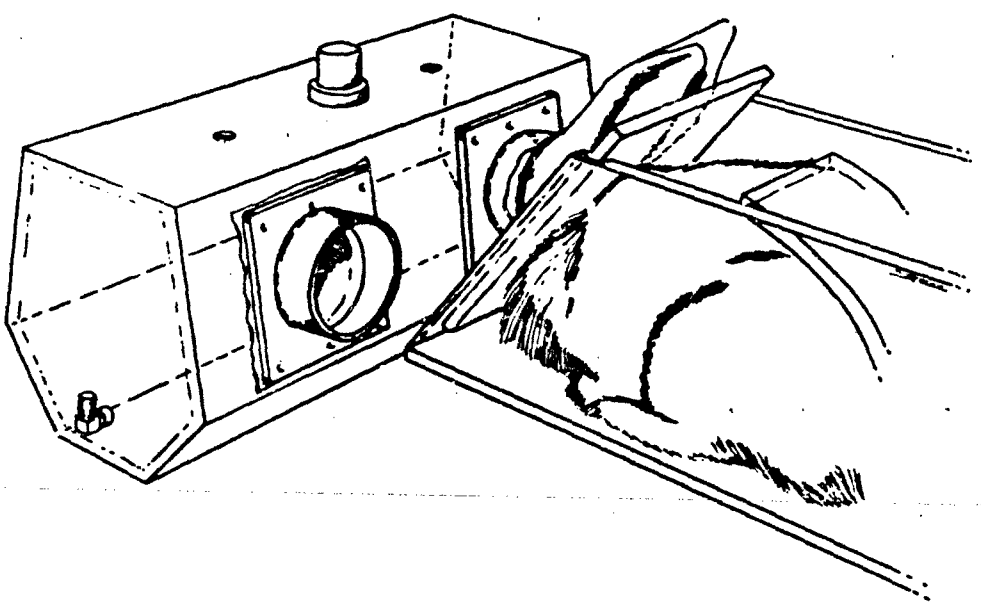

Figure 1. Nose exposure chamber for four rabbits.

DCM vapor was generated using a simple bubbler and then mixed with dilution air to the concentration desired. Concentrations within the nose exposure unit were monitored by sampling the atmosphere with a tandem bubbler system having 2 tubes containing carbon tetrachloride as the absorbing medium. Using this device we could calculate the sampling efficiency of each sample. The samples were measured on a Varian Aerograph gas chromatograph with a flame ionization detector.

Percent carboxyhemoglobin (% COHb) was determined by the spectrophotometric method of Amenta (1963) with some modifications. We measured absorbance at 3 wave lengths on a double beam recording spectrophotometer. Using blood equilibrated with either oxygen or CO as standards we could determine the % COHb in the unknown samples. Using various ratios of the 100% oxyhemoglobin and the 100% COHb, prepared ratios of COHb of 30% and below always gave measured values within 1% of that calculated. It is important to note that this procedure measures the ratio of oxyhemoglobin or % COHb and not hemoglobin or carboxyhemoglobin concentration.

The second part of this presentation deals with the effects of treatment with chemical modifiers of hepatic enzyme activity. These agents when used were given IP with the study usually being done on the morning after the last treatment. The enzyme studies reported here were always done with washed microsomes prepared by the methods in use at our laboratory (Bend et al., 1972).

For the most part the data were analyzed using a paired t-test with $P<0.05$ considered significant and $0.05<P<0.1$ considered suggestive.

RESULTS

We first characterized the increase in % COHb in rabbits after a single exposure to DCM vapor. Figure 2 shows the typical increase in % COHb in blood of a single rabbit after a 20-minute exposure. The % COHb continues to rise after the exposure is stopped, has a relatively broad peak, usually is decreasing by 4.5 hours after cessation of the exposure and returns to baseline values within about 8 hours. Since considerable time is required for COHb analysis, we felt that sampling at 1.5, 3, and 4.5 hours would give a peak value. This procedure allowed us to expose 4 rabbits at one time and has been used in subsequent studies. The % COHb value reported is the maximum value obtained at any of the sampling times.

The peak Δ% COHb as a function of the DCM concentration for a 20-minute exposure is shown in Figure 3. These data as well as all subsequent data represent the increase in % COHb after exposure to CO. The baseline values measured prior to the exposure (which averaged about 0.75% COHb but were occasionally above 1%) were subtracted from the values measured after exposure.

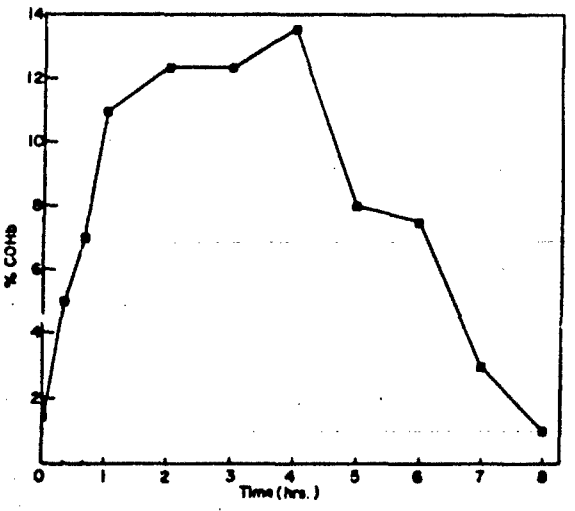

Figure 2. Carboxyhemoglobin response of one rabbit to a 20-minute exposure of 6850 ppm dichloromethane.

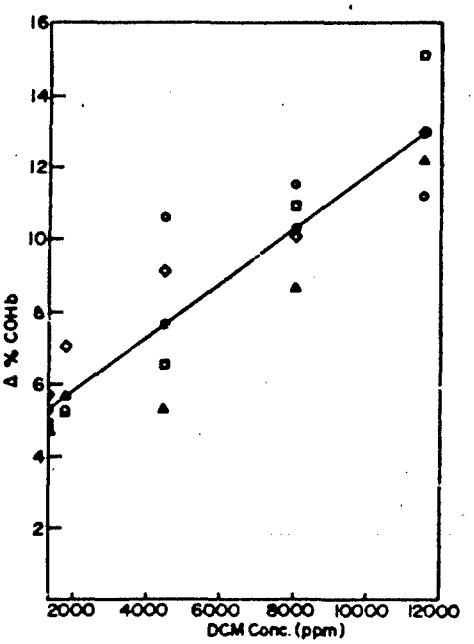

Figure 3. Dose-response curve for maximum Δ% COHb in blood after a single 20-minute exposure to dichloromethane.

Thus, we have established the response after a single 20-minute exposure and developed a dose-response curve for that particular exposure pattern. But what happens after prolonged exposures? We have exposed rabbits for up to 4 hours, measuring Δ% COHb during and after such prolonged exposures. The Δ% COHb after a 4-hour exposure to 7320 ppm DCM is shown in Figure 4. Since data at 3 and 4 hours were similar and we were reluctant to confine rabbits for longer periods, we stopped the exposures at 4 hours. The Δ% COHb began to fall at 5 hours and returned to preexposure values by the next morning.

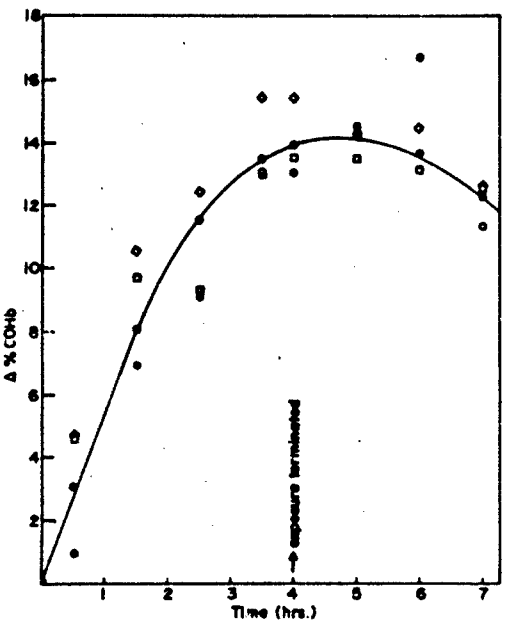

Figure 4. Δ% carboxyhemoglobin response to four hours of continuous exposure to 7320 ppm dichloromethane. Exposure was terminated at four hours followed by a recovery phase. Individual responses of the four rabbits, denoted by their respective symbols, are plotted along with the regression line.

The remainder of this presentation will describe some of our attempts to modify hepatic DCM metabolism. Prior to measuring effects of metabolic modifiers of DCM metabolism, we had to determine whether DCM itself was an inducer or inhibitor of its own metabolism to CO, i.e., if previous exposure to DCM altered Δ% COHb seen after subsequent exposures. Rabbits were exposed to DCM and the Δ% COHb was measured on days 1, 4, and 7. As can be seen (Table 1) there are no differences in the response and thus exposure to DCM does not appear to modify its own metabolism. This

experiment was necessary because prior to treatment with modifiers of hepatic MFO activity a baseline response to a DCM challenge was measured. Our procedure was to expose 4 rabbits to a given concentration of DCM, measure the resultant Δ% COHb, treat the animal for the prescribed time with the proposed modifier of DCM metabolism and then repeat the DCM challenge.

TABLE 1. EFFECT OF REPEATED 20-MINUTE EXPOSURE TO DICHLOROMETHANE VAPORS ON Δ PERCENT CARBOXYHEMOGLOBIN

Day	DCM Conc. (ppm)	Δ% COHb[a]
1	1230	6.0 ± 0.3[b,c]
4	1100	4.8 ± 0.7
7	1240	6.5 ± 0.6

[a] Maximum value minus preexposed value

[b] Mean ± SE, N = 4

[c] There are no significant differences between any of the three means.

The effects of treatment of animals with 4 different metabolic modifiers are shown in Table 2. Contrary to our expectations, treatment of rabbits with phenobarbital or pregnenolone 16α carbonitrile (PCN) (Lu et al., 1972) did not increase the Δ% COHb in blood. In fact, phenobarbital treatment significantly diminished the Δ% COHb after DCM exposure as compared to the Δ% COHb in the same rabbits prior to phenobarbital treatment. Carbon tetrachloride treatment of rabbits markedly reduced Δ% COHb whereas 2-diethylaminoethyl-2, 2, -di-phenyl-valerate hydrochloride (SKF 525-A) had no effect on Δ% COHb after DCM exposure.

TABLE 2. EFFECT OF MODIFIERS OF HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASES ON DICHLOROMETHANE-PRODUCED CARBOXYHEMOGLOBIN

	Before treatment		After treatment	
	DCM concentration	Δ% COHb	DCM concentration	Δ% COHb
Sodium phenobarbital[a]	1380	6.9 ± 0.4[e]	1520	4.4 ± 0.6[f]
Pregnenolone 16 -carbonitrile[b]	1770	7.1 ± 0.5	1730	5.4 ± 0.7[g]
Carbon tetrachloride[c]	2910	9.6 ± 1.5	2930	1.4 ± 0.4[f]
SKF 525-A[d]	3820	7.8 ± 1.1	4290	8.7 ± 0.8[h]

[a]40 mg/kg in saline (IP) 120, 96, 72, 48, 24 hours prior to exposure.
[b]60 mg/kg in corn oil (IP) 48 and 24 hours prior to exposure.
[c]0.5 ml/kg in corn oil (IP) 24 hours prior to exposure.
[d]30 mg/kg in saline (IP) 0.5 hours prior to exposure.
[e]Mean ± SE, N=4 except for carbon tetrachloride treatment where N=3.
[f]Significantly lower than Δ% COHb before treatment.
[g]Suggestive of decrease in Δ% COHb production ($P>0.05$ but <0.1).
[h]Not significantly different from Δ% COHb production prior to treatment.

In order to confirm that the xenobiotic metabolism modifiers were causing the expected alterations in hepatic MFO activity, in vitro studies were performed with liver microsomes from control versus treated rabbits (Table 3). As expected, treatment of rabbits with phenobarbital induced hepatic microsomal MFO activity as measured in vitro and CCl_4 treatment of rabbits inhibited hepatic microsomal MFO activity. The data from groups treated with CCl_4 as reported here are from animals given a single dose of CCl_4 with sacrifice and enzyme assay 24 hours later. In that experiment we looked at 3 different dose levels and 3 different time periods after CCl_4 treatment and thus used only 2 animals per group. Rabbit treatment with CCl_4 inhibited hepatic microsomal metabolism at all dose levels and time periods studied. However, because there are only 2 animals per group, the effects of CCl_4 reported here are not statistically significant at $P<0.05$. Treatment of rabbits with the other compounds, SKF 525-A and PCN, had no effect on MFO activity. We also treated rabbits with 3 methyl-cholanthrene (3MC) and this had no effect on MFO activity of rabbit liver microsomes. When 3MC treated rabbits were exposed to DCM the results were equivocal.

TABLE 3. INTRAPERITONEAL CHEMICAL PRETREATMENT AND HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE ENZYMATIC ACTIVITY IN RABBITS

Treatment	Substrate	Activity (Nmoles/min/mg protein) Treated	Control
SKF 525-A[a]	Benzphetamine	6.44 ± 0.73[f,g]	5.65 ± 0.16
3 Methylcholanthrene[b]	Aniline	3.18 ± 0.38[g]	2.62 ± 0.53
3 Methylcholanthrene[b]	Benzpyrene	0.97 ± 0.09[g,j]	1.66 ± 0.40
3 Methylcholanthrene[b]	Aminopyrene	5.06 ± 0.34[c]	4.86 ± 0.69
Carbon Tetrachloride[c]	Benzphetamine	1.96 ± 0.14[h]	5.28 ± 0.97
Pregnenolone 16α-carbonitrile[d]	Ethyl Morphine	4.38 ± 0.59[g]	5.00 ± 0.62
Sodium Phenobarbital[e]	^{14}C Benzene	6.94 ± 0.25[i]	2.00 ± 0.20
Sodium Phenobarbital[e]	Benzphetamine	16.55 ± 0.78[i]	6.09 ± 0.69

[a] 30 mg/kg in saline 2 hours prior to sacrifice.
[b] 20 mg/kg in corn oil 72, 48, and 24 hours prior to sacrifice.
[c] 0.5 ml/kg in corn oil 24 hours prior to sacrifice.
[d] 60 mg/kg in corn oil 48 and 24 hours prior to sacrifice.
[e] 50 mg/kg in saline 72,48, and 24 hours.
[f] Mean ± SE, N=4 except for CCl_4 treatment where N=2.
[g] Not significantly different from control.
[h] Suggestive of inhibition; P>0.05 but <0.1.
[i] Significantly different from control.
[j] Units metabolized/min/mg microsomal protein.

The lack of response to SKF 525-A was unexpected since many investigators have reported the inhibitory effects of this drug on hepatic xenobiotic metabolism. Our treatment protocol called for IP injection of 30 mg SKF 525-A/kg and exposure to DCM 30 minutes later. We also looked for inhibition of some typical mixed-function oxidases in vitro using microsomes at 2, 3.5, and 5 hours after rabbit treatment with SKF 525-A to coincide with our blood sampling times. Again there was no effect of the SKF 525-A treatment on MFO activity even at higher doses of 50 and 100 mg/kg. On the assumption that we may be washing any SKF 525-A from our microsomes during preparation and before assay of enzyme activity, we repeated the experiment using 9000 x g supernatant fraction from homogenized livers of SKF 525-A treated rabbits. Again the results were negative; treatment with SKF 525-A did not lead to inhibition of MFO activities as assayed in vitro. Finally we did significantly decrease the activity of benzphetamine N-demethylase from 6.1 to 4.5 nmoles metabolized/min/mg protein by adding SKF 525-A (10^{-3} molar) directly to the microsomal suspension prepared from control rabbits.

DISCUSSION

These experiments have characterized changes in % COHb in the blood of rabbits as a result of various inhalation exposures to DCM. Treatment of rabbits with CCl_4, a strong hepatotoxic agent, reduces the Δ% COHb in blood resulting from exposure to DCM. This fact argues strongly in favor of DCM metabolism to CO occurring for the most part in liver.

In order to see if this increased production of COHb was mediated by microsomal mixed-function oxidase systems, the Δ% COHb in blood after DCM exposure was measured before and after treatment of rabbits with several modifiers of hepatic mixed-function oxidase activity. Two of these compounds, PCN, an inducer and SKF 525-A, an inhibitor of hepatic MFO activities, neither altered the Δ% COHb in blood after DCM exposure nor changed the hepatic MFO marker enzyme activities at the concentrations used. After treatment of rabbits with 3MC, the Δ% COHb after DCM exposure was perhaps slightly increased, but there was no change in the in vitro hepatic microsomal MFO activity as measured by 3 substrates, aniline, benzpyrene or aminopyrene. Only pretreatment of rabbits with CCl_4 and phenobarbital altered the Δ% COHb after a DCM challenge.

After an exposure to 2900 ppm DCM, the increase in the % COHb in blood of rabbits was 9.6%. Intraperitoneal injection of rabbits with CCl_4 prior to DCM exposure reduced the Δ% COHb to 1.4%. The effect of animal treatment with sodium phenobarbital before DCM inhalation was not as great as with CCl_4 treatment. Exposure of rabbits to 1380 ppm DCM resulted in a Δ% COHb of 6.9%. After these same animals were treated with phenobarbital a DCM exposure to 1520 led to a Δ% COHb of only 4.4%; this is less than the Δ% COHb prior to phenobarbital treatment in spite of the DCM concentration being slightly higher (1380 vs. 1520 ppm).

The fact that phenobarbital treatment reduces the Δ% COHb in blood of rabbits after a DCM challenge as compared to the Δ% COHb resulting from a similar challenge prior to the phenobarbital treatment does not necessarily imply that the MFO systems are not converting DCM to CO. Phenobarbital treatment of rabbits does increase the hepatic microsomal cytochrome P-450 levels which may, in turn, increase the rate of CO production from DCM. However, CO is an inhibitor of cytochrome P-450 mediated reactions and thus the breakdown of DCM to CO may be self limiting with a resultant decrease in the Δ% COHb. Montgomery and Rubin (1971) exposed rats to CO and showed that at 60% COHb, hexabarbital sleeping times were significantly prolonged in exposed versus control animals. Zoxazolamine paralyses times were prolonged at COHb levels of 20%. In their studies CO must go from the air via the hemoglobin to the site of action. In our experiments the CO can be produced right at the area where it may act to inhibit metabolism. Thus the same degree of inhibition of MFO activity could occur with much lower % COHb values in the blood.

Finally, phenobarbital may induce an alternate pathway for metabolism of DCM which does not produce CO. If this were the case, the amount of DCM metabolized after an exposure could actually increase in phenobarbital treated rabbits; but the amount of CO produced would be less than in untreated rabbits. Thus rabbits treated with phenobarbital and then challenged with DCM would have blood with Δ% COHb values lower than the Δ% COHb values in blood from untreated rabbits.

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DISCUSSION

DR. CULVER (University of California, Irvine): Why did you just use the nasal exposure for the rabbits in your experimental set up?

DR. DREW (National Institute for Environmental Health Sciences): Well, to be quite frank, I was interested in developing a nose exposure system. But the real reason was that I thought we were going to be measuring dichloromethane breath levels during and after exposure when we first started the experiment. And in the one experiment, the prolonged exposure, we did look at breath levels during and after exposure. Since we had the exposure technique and it was operational, we never changed.

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PAPER NO. 23

TOXICITY OF PROPELLANTS*

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INTRODUCTION

When aerosols were first introduced in the 1950's, refrigerants were examined for possible use as propellants. The Underwriters' Laboratories in the United States had developed a classification of gases and vapors based on their comparative hazards to experimental animals (Nuckolls, 1959). The hydrocarbons of high toxicity belonging to groups 1 to 4 were excluded from consideration but those of low toxicity belonging to groups 5 and 6 were accepted as safe for use as propellants in aerosol products. These are listed in Table 1 by their chemical names and fluorocarbon numbers, the latter indicating the position of the halogen substitution.

In the 1960's, fatalities were attributed to the use and/or abuse of aerosols, particularly those used in the treatment of bronchial asthma (Taylor and Harris, 1970; Thompson, 1971). In light of such findings, it has become necessary to reexamine the toxicity of the propellants. This review covers completely the information on the respiratory and cardiovascular effects of propellants on animals. The observations in humans are few and conjectural and have been omitted. They will be reviewed at a later date when additional reports become available.

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TABLE 1. CLASSIFICATION OF COMPARATIVE LIFE HAZARD OF GASES AND VAPORS

Group Number And Definition	Not Used As Propellants	Used As Propellants: Low Pressure	Used As Propellants: High Pressure
Group 1: Gases or vapors which in concentrations of 0.5 to 1.0% for durations of exposure of 5 min are lethal or produce serious injury	Sulfur dioxide		
Group 2: Gases or vapors which in concentrations of 0.5 to 1.0% for duration of exposure of 1/2 hour are lethal or produce serious injury	Ammonia Methyl bromide		
Group 3: Gases or vapors which in concentrations of 2.0 to 2.5% for duration of exposure of 1 hr are lethal or produce serious injury	Carbon tetrachloride Chloroform Methyl formate		
Group 4: Gases or vapors which in concentrations of 2.0 to 2.5% for duration of exposure of 2 hr are lethal or produce serious injury	Dichloroethylene Methyl chloride Ethyl bromide		
Between Groups 4 and 5: Gases or vapors less toxic than Group 4	Ethyl chloride	Trichloroethane[a] Methylene chloride Trichlorotrifluoroethane (FC 113) Dichlorofluoromethane (FC 21)	Vinyl chloride[a]
Group 5a: Gases or vapors much less toxic than Group 4 but more toxic than Group 6	Carbon dioxide	Trichlorofluoromethane (FC 11) Monochlorodifluoroethane (FC 142b)	Monochlorodifluoromethane (FC 22)
Group 5b: Gases or vapors which available data indicate would classify as either Group 5 or Group 6	Ethane	Isobutane	Propane
Group 6: Gases or vapors which in concentrations up to at least 20% for duration of exposure of 2 hr do not appear to produce injury	Chlorotrifluoromethane (FC 13)	Dichlorotetrafluoroethane (FC 114) Octafluorocyclobutane (FC C-318)	Dichlorodifluoromethane (FC 12) Difluoroethane (FC 152a) Chloropentafluoroethane (FC 115)[a]

[a] Not tested by Underwriters' Laboratories but estimated to belong in the group

PROPELLANTS USED IN AEROSOLS

This laboratory has collected information from manufacturers of 168 aerosol products regarding their use of the propellants. A summary of product composition follows:

Inhalants containing bronchodilator drugs: 11 products
FC 11 = 5 products; FC 12 = 11 products; FC 114 = 9 products

Mouth products: 6 products
FC 12 = 5 products; FC 114 = 5 products; FC 142b = 1 product

Vaporizers: 2 products
FC 11 = 2 products; FC 12 = 2 products; trichloroethane = 1 product

Hair products: 62 products
FC 11 = 53 products; FC 12 = 54 products; FC 114 = 4 products; FC 152a = 5 products; methylene chloride = 8 products; vinyl chloride = 1 product; propane = 3 products; isobutane = 32 products

Women's personal hygiene products: 22 products
FC 11 = 5 products; FC 12 = 20 products; FC 114 = 6 products; isobutane = 2 products

Deodorants and antiperspirants: 38 products
FC 11 = 13 products; FC 12 = 37 products; FC 114 = 8 products; FC 142b = 1 product; propane = 1 product; isobutane = 1 product

Foot products: 9 products
FC 11 = 8 products; FC 12 = 9 products; FC 114 = 1 product

Miscellaneous products for personal use:
FC 11 = 7 products; FC 12 = 15 products; FC 114 = 3 products; isobutane = 2 products; propane = 2 products

The propellant composition of each of the above 168 aerosol products is summarized in Table 2. Six have low vapor pressure (up to 30 psig at 20 C); four high high vapor pressure (above 30 psig at 20 C). There are additionally included in Table 2, three low pressure propellants and 2 high pressure propellants which are presently being considered for commercial use.

This review covers the 15 hydrocarbons listed in Table 2. While toxicity of the fluorinated compounds has been reviewed by Clayton (1966, 1967, 1968), Elchardus (1953), and Waritz (1971), this is the first attempt to compare the toxicity of fluorinated and nonfluorinated hydrocarbons.

The propellants listed in Table 2 are newly classified according to their toxicity to the respiratory and circulatory systems. The low pressure propellants are divided into those of high toxicity and intermediate toxicity groups, whereas the high pressure propellants fall into the intermediate and low toxicity groups. It should be noted in the table that our new classification does not conform to the rating used by the Underwriters' Laboratories. The low pressure propellants of high toxicity include one propellant rated 5a and four rated between 4 and 5. The low pressure propellants of intermediate toxicity include one rated 5a, one 5b and two 6. The high pressure propellants of intermediate toxicity include one example of each group between 4 and 5, 5a, 5b, and 6. The high pressure propellants of low toxicity are both rated as 6. The difference between the two systems of classification can readily be understood by realizing that the earlier scheme was derived from observation of skeletal and nervous system involvement during exposure, and organ examination postmortem.

The new classification is based on parametric assessment of cardiac arrhythmia, heart rate, myocardial contractility, blood pressure, respiratory minute volume, pulmonary resistance and pulmonary compliance. Description of the 15 propellants conforms to the order in Table 2.

TABLE 2. NEW PROPOSED CLASSIFICATION BASED ON TOXICITY TO RESPIRATORY AND CIRCULATORY SYSTEMS

Low Pressure Propellants			High Pressure Propellants		
Section No. Name of Propellant Fluorocarbon No.	Underwriters' Laboratories Classification	Frequency Of Use In Aerosols	Section No. Name of Propellant Fluorocarbon No.	Underwriters' Laboratories Classification	Frequency Of Use In Aerosols
Low Pressure Propellants Of High Toxicity					
(3.1) Trichlorofluoromethane (FC 11)	5a	93			
(3.2) Dichloromonofluoromethane (FC 21)	4-5				
(3.3) Trichlorotrifluoroethane (FC 113)	4-5				
(3.4) Trichloroethane	4-5	1			
(3.5) Methylene Chloride	4-5	8			
Low Pressure Propellants Of Intermediate Toxicity			**High Pressure Propellants Of Intermediate Toxicity**		
(4.1) Dichlorotetrafluoroethane (FC 114)	6	36	(5.1) Dichlorodifluoromethane (FC 12)	6	153
(4.2) Monochlorodifluoroethane (FC 142b)	5a	2	(5.2) Monochlorodifluoromethane (FC 22)	5a	
(4.3) Isobutane	5b	37	(5.3) Propane	5b	6
(4.4) Octafluorocyclobutane (FC C-318)	6		(5.4) Vinyl Chloride	4-5	1
			High Pressure Propellants Of Low Toxicity		
			(6.1) Chloropentafluoroethane (FC 115)	6	
			(6.2) Difluoroethane (FC 152a)	6	5

LOW PRESSURE PROPELLANTS OF HIGH TOXICITY

There are 9 propellants used in aerosol products which exert a low vapor pressure. Five of them are highly toxic to the respiratory and circulatory systems. Trichlorofluoromethane (FC 11), the most toxic, induces cardiac arrhythmia, tachycardia, myocardial depression, hypotension, respiratory depression, bronchoconstriction, and decreased pulmonary compliance. The remaining 4 provoke some but not all of these adverse reactions.

Trichlorofluoromethane: Fluorocarbon 11

(CCl_3F; Boiling Point $_{760}$ 23.8 C; Vapor Pressure at 20 C = 1.3 psig)

Trichlorofluoromethane is the most widely used and the most extensively investigated low pressure propellant. Since it is used as the prototype for comparison with the other 14 propellants, details of its toxicity are discussed in the following paragraphs.

Acute Inhalational Toxicity

Guinea Pig

Nuckolls (1959) reported the first investigations of the acute toxicity of FC 11. Twelve guinea pigs divided into 4 groups of 3 each were exposed for 5 minutes, 30 minutes, 1 hour and 2 hours, respectively. Exposure to 2.5% for 30 minutes caused occasional tremors and chewing movements; rate of breathing became irregular. Exposure to 10% for 1 hour resulted in unconsciousness. The guinea pigs exposed to this concentration for 2 hours were sacrificed 8 days later. While their lungs were found to contain a mottled area of congestion, other organs showed no pathological conditions. Scholz (1962) reported that exposure to a concentration of 20% for 1 hour was lethal. According to Caujolle (1964) a concentration of 25% for 1/2 hour was lethal in half the guinea pigs tested. A concentration of 3% inhaled for 2 hours, though not fatal, caused unconsciousness.

Rat

Lester and Greenberg (1950) exposed male rats to FC 11 in concentrations ranging from 5 to 50% for 30 minutes. While a concentration of 5% caused no symptoms of intoxication, 6 and 7% concentrations caused a loss of postural reflex, 8% caused a loss of righting reflex and 9% caused complete unconsciousness. The following concentrations were lethal: 10% for 20-30 minutes; 15% for 8 minutes; 20-30% for 4 minutes; and 50% in 1 minute. Scholz (1962) reported a lethal concentration of 10% for 90 minutes.

Mouse

Caujolle (1964) determined the lethal concentration in mice. In an atmosphere containing 15% FC 11, mice succumbed in a few minutes.

Cat

Scholz (1962) reported that inhalation of 10% FC 11 for 1 hour was lethal to a cat.

Chronic Inhalational Toxicity

Two investigations show the development of pathological lesions following exposure to FC 11. Nuckolls (1959) reported that guinea pigs exposed to 10% FC 11 for 2 hours, when sacrificed 8 days later, showed pulmonary congestion and hemorrhage. Morrison (cited by Waritz, 1971) exposed rats to 1.2% concentrations of FC 11 and observed the appearance of pulmonary emphysema and edema, liver vacuolization, increased splenic hematopoiesis and neuronal edema.

The following investigators did not report having found pathological lesions after chronic exposure of animals:

Scholz (1962) 1.25 or 2.5% FC 11 for 3.5 hours for 20 days using guinea pigs, rats and cats.

Nick (cited by Waritz, 1971) 0.4% FC 11 for 6 hours for 28 days in guinea pigs, rats, rabbits and monkeys.

Jenkins et al. (1970) 0.1% FC 11 inhaled continuously for 90 days, 1.025% FC 11 8 hours daily for 30 days in guinea pigs, rats, dogs and monkeys.

One investigation of the oral toxicity of FC 11 in the rat wherein a mixture of 1:1 in liquid paraffin was administered at a dose level of 5 ml/kg found no signs of liver toxicity in the treated rats. Other rats treated with carbon tetrachloride suffered from hepatotoxicity (Slater, 1965).

Acute Cardiovascular Toxicity

Fluorocarbon 11 can influence the circulatory system in a number of ways, all of which are potentially dangerous. This propellant induces cardiac arrhythmia, depresses myocardial contractility, and provokes hypotension.

Cardiac Arrhythmia in the Mouse

The recent interest in cardiotoxicity of propellants started with the observations of Taylor and Harris (1970), using aerosols released from a bronchodilator pressure unit containing three propellants including FC 11.

Experiments relating to the inhalation of FC 11 in gaseous form have been conducted in our laboratory. Mice under pentobarbital anesthesia did not show any cardiac arrhythmia following inhalation of 2 or 5% FC 11. However, inhalation of 10% FC 11 caused second degree atrioventricular block and inhalation of 5% FC 11 caused the appearance of atrioventricular block following a concurrent intravenous injection of epinephrine.

Cardiac Arrhythmia in the Dog

Reinhardt et al. (1971) reported sensitization of the heart to epinephrine in the unanesthetized dog. The inhalation of 0.35 to 0.61% of FC 11 for 5 minutes caused ventricular fibrillation and cardiac arrest following the injection of epinephrine. Though inhalation of lower concentrations (0.09 to 0.13%) did not sensitize the heart, administration of higher concentrations (0.96 to 1.[illegible]%) resulted in a higher frequency of cardiac arrhythmia. When exposure to noise prompted the release of endogenous epinephrine, cardiac arrhythmia was observed in dogs inhaling a mixture of 80% FC 11 and 20% oxygen. Exercising on a treadmill, likewise known to effect release of endogenous epinephrine, did not induce arrhythmia in dogs inhaling 0.5 to 1.0% FC 11 (Mullin et al., 1972).

Clark and Tinston (1972) investigated the interaction between FC 11 and sympathomimetic drugs in unanesthetized dogs. Inhalation of 1.25% FC 11 sensitized the heart to epinephrine but not to isoproterenol.

In our laboratory, the inhalation of 1% FC 11 caused acceleration of heart rate and a fall in aortic blood pressure in the anesthetized dog. The minimal concentration necessary to influence the cardiovascular system was not determined. Instead, concentrations up to 10% were administered to determine standards for use in a comparison with other fluorocarbons.

Myocardial Depression in the Dog and Monkey

The inhalation of 10% FC 11 depressed the ventricular function curve in the canine heart-lung preparation. These observations indicate that the effect is a direct one that is not mediated through the central nervous system. In the anesthetized monkey, there was a depression in myocardial contractility during the inhalation of 5 or 10% FC 11. That this effect was accompanied by a fall in aortic blood pressure suggests that the former contributed to the latter.

Acute Bronchopulmonary Toxicity

Inhalation of fluorocarbon 11 causes death by the depression of respiration. Inhalation of sublethal concentrations significantly depresses tidal volume in the rat but not in the dog. These species react differently not only in their respiratory response but also in the bronchomotor effect of FC 11.

Bronchodilation in the Monkey

In the anesthetized monkey the inhalation of 2 to 10% FC 11 caused a reduction in pulmonary resistance which is interpreted as bronchodilation (Belej and Aviado, 1973). The mechanism for the effect was identified by the administration of a beta-blocking agent. The bronchodilator response to the inhalation of FC 11 was prevented by a prior injection of the blocking agent indicating that FC 11 was directly stimulating the adrenergic beta receptors in the smooth muscles of the airways.

Bronchodilation in the Dog

In the anesthetized dog, a reduction in pulmonary resistance accompanied an increase in pulmonary compliance and a decrease in respiratory minute volume following the inhalation of 1, 2.5, 5, or 10% FC 11. These effects were similar to those encountered in the monkey and described in the preceding paragraph.

Bronchoconstriction in the Rat

The inhalation of FC 11 in the rat caused an increase in pulmonary resistance which is the exact opposite of the response in the dog and the monkey. There was also a reduction in pulmonary compliance, respiratory rate and in heart rate. The differences in response of pulmonary resistance and of heart rate among various animal species are under investigation.

Summary of Toxicity of Fluorocarbon 11

Fluorocarbon 11 is used as the prototype for comparison with the other propellants. In the remainder of this article, there are summary tables comparing the cardiovascular and bronchopulmonary effects of a specific propellant with FC 11.

Dichloromonofluoromethane: Fluorocarbon 21

($CHCl_2F$; Boiling Point $_{760}$ = 8.9 C; Vapor Pressure at 20 C = 84 psig)

Although FC 21 is not currently used in aerosols, it has been suggested as a substitute for FC 11. The results of toxicity tests are

inconclusive. While FC 21 is more toxic than FC 11 with regard to unconsciousness and death in the guinea pig, mouse and rat, respiratory depression in the monkey, and bronchoconstriction in the dog, FC 21 is less likely to elicit tachycardia and cardiac arrhythmia in the mouse, dog and monkey than is FC 11. From the standpoint of chemical structure, it should be noted that addition of the chlorine atom has increased bronchopulmonary toxicity but reduced cardiovascular toxicity.

The comparative toxicities of FC 21 and FC 11 are summarized in the following columns. The index of toxicity is expressed as a ratio where 1 represents the prototypical activity of FC 11. The relative toxicity of FC 21 is expressed by a number less than 1 (<1) to mean higher potency or more than 1 (>1) to indicate lower potency or lesser toxicity. Dissimilar effect is identified as opposite and lack of activity of one propellant by 0.

Toxicity Test Species	Dichloromonofluoromethane (FC 21) $CHCl_2F$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Guinea pig	5% for 30 min = unconscious	0.5	10% for 30 min = unconscious
Guinea pig	5% for less than 2 hr = lethal	0.25	25% for a few min = lethal
Guinea pig	50% for 6 min = lethal		
Mouse	10% = lethal	0.5	15% for a few min = lethal
Rat	10% for 1 hr = lethal	0.5	10% for 2 hr = lethal
Acute Cardiovascular Toxicity			
Mouse	10% = cardiac arrhythmia	1.0	10% = cardiac arrhythmia
Dog	5% = tachycardia with hypotension	2.0	2.5% = tachycardia with hypotension
Monkey	10% = tachycardia with hypotension	2.0	5% = tachycardia with hypotension

Toxicity Test Species	Dichloromonofluoromethane (FC 21) $CHCl_2F$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Bronchopulmonary Toxicity			
Monkey	5% = bronchodilation with depression of respiration	0.5	10% = bronchodilation with slight depression of respiration
Dog	2.5% = bronchoconstriction without early depression of respiration	(opposite)	2.5% = bronchodilation with early depression of respiration
Rat	19% = no bronchoconstriction and no early reduction in tidal volume	0	20% = bronchoconstriction with early reduction in tidal volume

Trichlorotrifluoroethane: Fluorocarbon 113

(CCl_2F-$CClF_2$; Boiling Point $_{760}$ = 47.6 C; Vapor Pressure at 20 C = -9.2 psig)

Fluorocarbon 113 is a fluorinated ethane that has been recommended for use as a low-pressure propellant. Tests of acute inhalational toxicity indicate that FC 113 is about half as toxic as FC 11. Like FC 11, FC 113 induces a cardiac arrhythmia in the mouse and sensitizes the heart to epinephrine-induced arrhythmia in both the mouse and dog. Both propellants also depress myocardial contractility in the dog. The only important difference in their bronchopulmonary effects is that while FC 11 is a bronchodilator, FC 113 causes bronchoconstriction.

Toxicity Test Species	Trichlorotrifluoromethane (FC 113) CCl_2F-$CClF_2$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Guinea pig	20% for 2 hr = marked narcosis	2	20% for 1 hr = lethal
Guinea pig	1% = signs of distress; higher concentrations = lethal		3-6% = signs of distress; 15% = lethal in 30 min

Toxicity Test Species	Trichlorotrifluoromethane (FC 113) CCl_2F-$CClF_2$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Rat	10% for 4 hr = lethal		10% for 2 hr = nonlethal
Rat	1% for 2 hr = equilibrium disturbance	2	10% for 1-1/2 hr = lethal
Mouse	toxicity of subcutaneously administered propellant enhanced by piperonyl butoxide		
Mouse	6% for 2 min = anesthesia; 25% for 1.5 min = lethal		
Chronic Oral Toxicity			
Rat	55 mg/kg daily for 3-9 days = lethal		
Acute Cardiovascular Toxicity			
Dog	0.5-1.0% = sensitized heart to epinephrine	1.5	0.3-0.6% = sensitized heart to epinephrine
Dog	2% = tachycardia; 10% = myocardial depression	2	1% = tachycardia 10% = myocardial depression
Mouse	40% = cardiac arrhythmia	4	10% = cardiac arrhythmia
Acute Bronchopulmonary Toxicity			
Dog	4% = bronchoconstriction	(opposite)	2.5% = bronchodilation

Trichloroethane

(CH_3CCl_3; Boiling Point $_{760}$ = 74.1 C; Vapor Pressure at 20 C = -12.5 psig)

Stewart (1968) and Torkelson et al. (1958) have reviewed the toxicity of trichloroethane. Adams et al. (1970) reported 50% survived in rats exposed to 1.8% for 3 hours or to 1.4% for 7 hours. Since Lester and Greenberg (1950) reported the lethal concentration of FC 11 to be 10%, it appears that trichloroethane is the most toxic of the low-pressure propellants.

There are some comparative reports on the cardiovascular toxicity of trichloroethane. In the anesthetized mouse, inhalation of 40% trichloroethane induces cardiac arrhythmia whereas only 10% of FC 11 has been found necessary to elicit a similar effect; both are equally potent in depressing myocardial contractility in the dog heart-lung preparation. Trichloroethane causes hypotension (Krantz et al., 1959). In the anesthetized dog, trichloroethane causes bronchoconstriction and respiratory stimulation while exposure to FC 11 results in bronchodilation and respiratory depression.

Toxicity Test Species	Trichloroethane CH_3CCl_3	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Cardiovascular Toxicity			
Dog	0.5% = sensitized heart to epinephrine	1.6	0.3% = sensitized heart to epinephrine
Mouse	2.5% = tachycardia; myocardial depression without hypotension	2.5	1% = tachycardia; myocardial depression with hypotension
Acute Bronchopulmonary Toxicity			
Dog	2.5% = bronchodilation with respiratory stimulation	2.5	1% = bronchodilation with respiratory stimulation

Methylene Chloride

(CH_2CL_2; Boiling Point $_{760}$ = 40.1 C; Vapor Pressure at 20 C = -7.3 psig)

Methylene chloride has conflicting toxicity values compared to FC 11. While its toxicity in the guinea pig and rat is higher, its cardiovascular toxicity is less than that of FC 11. There are no published reports on the

bronchopulmonary effect of methylene chloride. Stewart et al. (1972) have observed an elevation of carboxyhemoglobin level in the blood in human subjects who have been exposed to methylene chloride.

Toxicity Test Species	Methylene Chloride CH_2CL_2	<1 = More Toxic 1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Guinea pig	5% for 30 min = unconscious	0.5	10% for 1 hr = unconscious
Rat	5% for 2 hr = lethal	0.5	10% for 2 hr = nonlethal
Dog	0.01-0.02% for 2 hr = detected absorption and excretion		
Acute Cardiovascular Toxicity			
Mouse	50% = sensitized heart to epinephrine; 40% = cardiac arrhythmia	4	5% = sensitized heart to epinephrine 50% = cardiac arrhythmia
Dog	2% = heart not sensitized to epinephrine	4	0.5% = sensitized heart to epinephrine

LOW PRESSURE PROPELLANTS OF INTERMEDIATE TOXICITY

The low pressure propellants of intermediate toxicity have the following effects when inspired by these species:

Rat:	> 20% concentration is lethal
Guinea pig:	> 5% concentration is lethal
Dog:	> 1.0% sensitizes the heart to epinephrine
Mouse:	< 40% does not induce arrhythmia but does sensitize the heart to epinephrine.

The members of this group have varied chemical structures and include two fluorinated ethanes, one fluorinated butane, and one nonfluorinated butane.

Dichlorotetrafluoroethane; Cryofluorane: Fluorocarbon 114

($CClF_2$-$CClF_2$; Boiling Point = 3.8 C; Vapor Pressure at 20 C = 12.9 psig)

Dichlorotetrafluoroethane is the most widely used propellant of intermediate toxicity. It has been investigated extensively in various animal species. A discussion of the results of these investigations follows.

Acute Inhalational Toxicity

The lethal concentration of FC 114 in the mouse has been determined by Paulet and Desbrousses (1969) who concluded that a concentration of 70% FC 114 inhaled for 30 minutes was lethal to 50% of the animals tested. Caujolle (1964) found rats to be more sensitive, however, and reported that while a concentration of 0.1% could be tolerated for as long as 17 days, the animals succumbed when exposed to 3% concentrations of FC 114. The sensitivity of guinea pigs to FC 114 is between that of the two other animal species. In experiments with this species, Nuckolls (1959) administered FC 114 at a concentration of 4.7% for 2 hours and noted occasional chewing and retching movements but no loss of consciousness.

Chronic Inhalational Toxicity

The first investigation of the chronic inhalational toxicity of FC 114 was performed in the guinea pig by Yant et al. (1933). Exposure to a 20% concentration of FC 114 caused immediate and continued marked generalized tremors and convulsions. While recovery after a single 8-hour period of exposure was complete, exposure for periods of from 16-24 hours caused death in 1 to 3 days. Exposure to 20% concentrations of FC 114 for 8-hour sessions on 4 consecutive days did not seriously affect guinea pigs.

Vieillefosse et al. (1962) administered FC 114 to the rat. While exposure to 2% concentrations for 100 hours neither reduced the animals' weight nor produced any pathological effects on the lungs, kidneys, or liver, a reduction in polymorphonuclear and an increase in mononuclear cells in some animals did, however, occur.

In experiments with mice, Paulet and Desbrousse (1969) noted that while no pathological lesions resulted from exposure to 10% FC 114 for 2-1/2 hours daily for 2 weeks, exposure to 20% FC 114 for a similar period caused pulmonary congestion. Caujolle's study (1964) of the oral toxicity of FC 114 in the rat found a daily ingestion of 2 g/kg FC 114 for 23 to 33 days did not produce any pathological lesions.

Acute Cardiovascular Toxicity

Mouse

The difference between the effects of FC 114 and FC 11 in the anesthetized mouse is conspicuous. For FC 114, though inhalation of 20% sensitized the heart to epinephrine, a concentration as high as 40% failed to induce cardiac arrhythmia. By comparison, inhalation of FC 11 at a concentration of 5% sensitized the heart to epinephrine and 10% caused arrhythmia.

Dog

Reinhardt et al. (1971) reported sensitization of the heart to injected epinephrine in the unanesthetized dog inhaling from 2.5 to 5% concentrations of FC 114. Sensitization to endogenous epinephrine occurred with the inhalation of concentrations of 5 to 10% FC 114 (Mullin et al., 1972). While Van Poznak and Artusio (1960) found that exposure of the anesthetized dog to concentrations of 25 to 50% FC 114 resulted in tachycardia, other work suggests that lesser concentrations (5 to 20%) are sufficient to provoke both tachycardia and a slight fall in aortic blood pressure.

Monkey

Taylor et al. (1970) administered 9% concentrations of FC 114 to anesthetized monkeys and reported the appearance of premature ventricular beats, bigeminy, and/or tachycardia 39 seconds after inhalation was begun.

Hypotension in Various Species

Vieillefosse et al. (1962) reported a fall in mean aortic blood pressure in the anesthetized rat and guinea pig following the inhalation of a concentration of 30% FC 114. A similar hypotension was encountered in the anesthetized dog inhaling 10 to 20% concentrations of FC 114.

Acute Bronchopulmonary Toxicity

The inhalation of FC 114 caused bronchoconstriction in two species. In the anesthetized dog, inhalation of 5% FC 114 effected respiratory stimulation, an increase in pulmonary resistance, and a decrease in pulmonary compliance. In the anesthetized rat, there was both an acceleration of the respiratory rate and bronchoconstriction followed by depression of respiration at lethal concentrations of FC 114. These effects represent the most outstanding difference between FC 114 and FC 11.

Summary of Comparative Toxicity

Two important differences exist between FC 114 and FC 11. First, from experiments on both the mouse and dog, fluorocarbon 114 would appear to have only 1/5 the predisposition to cardiac arrhythmia of FC 11. Second, FC 114 is a respiratory stimulant and bronchoconstrictor in both the rat and dog, whereas FC 11 is a bronchoconstrictor in the rat, a bronchodilator in the dog, and does not stimulate respiration in either species.

Toxicity Test Species	Dichlorotetrafluoroethane (FC 114) $CClF_2$-$CClF_2$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Guinea pig	4.7% for 2 hr = no loss of consciousness		10% for 1 hr = unconscious
Rat	60% for 2 hr = deep narcosis	6	10% for 1 hr = deep narcosis
Rat	11% = lethal		
Mouse	9.5% = lethal		
Guinea pig	12% = lethal		
Acute Cardiovascular Toxicity			
Mouse	20% = sensitized heart to epinephrine; 40% = no cardiac arrhythmia	4	5% = sensitized heart to epinephrine; 40% = cardiac arrhythmia
Mouse	Changes in ECG similar to asphyxia		
Dog	2.5% = sensitized heart to epinephrine	5	0.5% = sensitized heart to epinephrine
Dog	5% = tachycardia	5	1% = tachycardia
Acute Bronchopulmonary Toxicity			
Dog	5% = bronchoconstriction	(opposite)	5% = bronchodilation

Toxicity Test Species	Dichlorotetrafluoroethane (FC 114) $CClF_2$-$CClF_2$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Rat	30% = bronchoconstriction with respiratory stimulation	(opposite)	20% = bronchoconstriction without respiratory stimulation

Monochlorodifluoroethane: Fluorocarbon 142b

(CH_3-$CClF_2$; Boiling Point $_{760}$ = -10 C; Vapor Pressure at 20 C = 29.1 psig)

While fluorocarbon 142b has the same relative toxicity as FC 114, minor differences in their pharmacologic actions have been encountered in tests with two animal species. Unlike FC 11, FC 142b does not sensitize the heart in the mouse nor does it reduce pulmonary compliance in the dog. On the basis of tests of acute inhalation toxicity in the rat and the detection of cardiotoxicity in the dog, mouse, and monkey, it may be concluded that FC 142b is less toxic than FC 11. The only adverse effect of FC 142b, reported bronchoconstriction in the dog, has not been replicated in experiments with monkeys.

Toxicity Test Species	Monochlorodifluoroethane (FC 142b) CH_3-$CClF_2$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Rat	20% = postural reflex disappears 30% = unconscious 50% = lethal	5	6% = postural reflex disappears 9% = unconscious 10% = lethal
Acute Cardiovascular Toxicity			
Mouse	60% = no cardiac arrhythmia and heart not sensitized to epinephrine	0	5% = sensitized heart to epinephrine 10% = cardiac arrhythmia
Monkey	10% = no tachycardia 20% = myocardial depression	2 4	5% = tachycardia 5% = myocardial depression

Toxicity Test Species	Monochlorodifluoroethane (FC 142b) CH_3-$CClF_2$	< 1 = More Toxic > 1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Dog	5% = sensitized heart to epinephrine	10	0.5% = sensitized heart to epinephrine
Dog	20% = tachycardia with hypotension	20	1% = tachycardia with hypotension

Isobutane

[$CH(CH_3)_3$; Boiling Point $_{760}$ = -10.2; Vapor Pressure at 20 C = 30 psig]

Isobutane is the only nonhalogenated propellant of low pressure and intermediate toxicity. Like FC 114, isobutane sensitizes the heart to epinephrine induced arrhythmia, increases airway resistance, and decreases pulmonary compliance. They differ in that while isobutane does not reduce blood pressure but does depress respiration, FC 114 has the opposite action.

Comparisons of isobutane and FC 11 indicate lower cardiovascular toxicity in the former. Both reduce tidal volume and pulmonary compliance in the rat.

Toxicity Test Species	Isobutane $CH(CH_3)_3$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Rat	27% = lethal	1.5	19% = lethal
Acute Cardiovascular Toxicity			
Mouse	20% = sensitized heart to epinephrine 40% = no cardiac arrhythmia	4	5% = sensitized heart to epinephrine 10% = cardiac arrhythmia
Dog	10-25% = sensitized heart to epinephrine		
Dog	10% = tachycardia without hypotension	10	1% = tachycardia with hypotension

Toxicity Test Species	Isobutane $CH(CH_3)_3$	< 1 = More Toxic > 1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Dog	5-10% = sensitized heart to epinephrine	10	0.5% = sensitized heart to epinephrine
Acute Bronchopulmonary Toxicity			
Rat	< 27% = bronchoconstriction with reduced compliance and tidal volume	2	< 20% = bronchoconstriction with reduced compliance and tidal volume
Dog	2.5% = bronchoconstriction with respiratory depression	(opposite)	2.5% = bronchodilation with respiratory depression

Octafluorocyclobutane: Fluorocarbon C-318

(C_4F_8; Boiling Point 760 = -5.8 C: Vapor Pressure at 20 C = 25.4 psig)

Fluorocarbon C-318 is the least toxic of the low-pressure propellants. Inhalation of FC C-318 at concentrations as high as 60 to 81% fails to depress the central nervous system. Its effect on the bronchopulmonary system, bronchoconstriction, is the opposite of the bronchodilation FC 11 has been found to produce in the dog and monkey. To the extent that FC 11 reduces blood pressures while FC C-318 does not, FC C-318 may be considered the less cardiotoxic.

Toxicity Test Species	Octafluorocyclobutane (FC C-318) C_4F_8	< 1 = More Toxic > 1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Rat	48% = lethal	2.5	19% = lethal
Rat and Mouse	80% = transient effect on respiration		
Rat and Guinea pig	60% = no effect		

Toxicity Test Species	Octafluorocyclobutane (FC C-318) C_4F_8	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Chronic Inhalational Toxicity			
Rat, Mouse, Rabbit, and Dog	10% for 6 hours daily for 9 days = no harmful effects		
Acute Cardiovascular Toxicity			
Mouse	40% = no cardiac arrhythmia 20% = sensitized heart to epinephrine	4	10% = cardiac arrhythmia 5% = sensitized heart to epinephrine
Dog	20% = no tachycardia	(inactive)	1% = tachycardia
Dog	20% = slight depression of ventricular function	2	10% = marked depression of myocardial function
Monkey	10% = no tachycardia		
Acute Bronchopulmonary Toxicity			
Monkey	10% = bronchoconstriction without depression of respiration	(opposite)	10% = bronchodilation with depression of respiration
Dog	2.5% = bronchoconstriction	(opposite)	2.5% = bronchodilation
Rat	< 48% = bronchoconstriction with decreases in compliance and tidal volume	2.5	<20% = bronchoconstriction with decreases in compliance and tidal volume

HIGH PRESSURE PROPELLANTS OF INTERMEDIATE TOXICITY

The high pressure propellants, in contrast to those classified as low pressure, have a boiling point below -11 C and a vapor pressure (at 20 C) above 30 psig. The four propellants belonging in this group have the following range of toxicity:

Rat: 20 to 40% concentrations are lethal
Dog: 5 to 10% sensitizes the heart to epinephrine.

Dichlorodifluoromethane: Fluorocarbon 12

(CCl_2F_2; Boiling point $_{760}$ = -29.8 C; Vapor Pressure at 20 C = 70.2 psig)

The chemical properties and toxicity of Fluorocarbon 12 when used as a refrigerant have been reviewed by both Midgley and Henne (1930) and by Thompson (1932). The most widely used of the high pressure propellants, it was present in 153 of the 168 aerosol products examined.

Acute Inhalational Toxicity

Guinea Pig

According to Nuckolls (1959), while exposure to FC 12 at concentrations ranging from 2 to 30% for 2-hour inhalation periods did provoke tremors, retching movements and deeper breathing, no loss of consciousness occurred.

Rat

Lester and Greenberg (1950) studied the effects on rats of exposure to 20 to 80% concentrations of FC 12. Muscular twitching and tremors were observed at concentrations of from 30 to 40%; at 70 and 80% concentrations, corneal reflexes were absent and the animals were in deep anesthesia. A few rats exposed to 80% FC 12 for 4-hour and 6-hour periods appeared to suffer no permanent effects.

Scholz (1962) exposed rats to concentrations ranging from 20 to 60% and noted that inhalation of 60% FC 12 for 2 hours produced narcosis.

Mouse

Leeuwe's study (1962) of the effects on mice of exposure to various concentrations of FC 12 found that though a concentration of 16% could be safely tolerated for as long as 5 hours, inhalation of 60% FC 12 in oxygen

caused narcosis. Carpenter (1954) reported that a concentration of 25% FC 12 protected mice from electrical-induced convulsions.

Shugaev (1963) calculated that inhalation of 62% FC 12 constituted a lethal dose and, further, that while inhalation of 10% depressed conditioned reflexes, a 5% concentration did not.

Cat and Dog

Brenner (1937) administered a concentration of 70% FC 12 to cats and observed the appearance of rapid, coarse tremors of the head and extremities which he explained as a "release" phenomenon attributable to the propellant's acting primarily on the regulatory motor centers. Inhalation of 15 to 50% concentrations of FC 12 produced convulsions in the dog (Van Posnak and Arrusio, 1960).

Chronic Inhalational Toxicity

Sayers et al. (1930) exposed dogs, monkeys, and guinea pigs to mixtures of 20% FC 12 for 7 to 8 hours daily, 5 days a week for 12 weeks. The dogs and monkeys developed tremors initially, but showed a tolerance to successive exposures; no gross pathology could be attributed to exposure to the propellant. Yant (1932, 1933) exposed monkeys and dogs to 20% concentrations of FC 12 continuously for 121 hours; the animals developed tremors but did not lose consciousness. Caujolle (1964) observed that exposures to 5% FC 12 did not produce any immediate effect on the guinea pig. Inhalation of concentrations as high as 20% have not been found to disturb reproduction.

Prendergast et al. (1967) exposed rats, guinea pigs, dogs, rabbits, and monkeys to 2 ppm of FC 12 continuously for 90 days and to 9 ppm for 8 hours repeatedly for 30 exposures; neither deaths nor any visible signs of toxicity were reported.

Acute Cardiovascular Toxicity

Cardiac Arrhythmia in the Mouse

Azar et al. (1971) exposed anesthetized mice to 100% FC 12 and noted a reduction in heart rate not unlike that resulting from the inhalation of pure nitrogen. Inhalation of 20 to 40% concentrations failed to induce cardiac arrhythmia, nor did it sensitize the heart to epinephrine.

Cardiac Arrhythmia in the Dog

Reinhardt et al. (1971) examined the effects of FC 12 on the unanesthetized dog. Inhalation of a 5% concentration sensitized the heart to epinephrine-induced arrhythmia, and inhalation of 80% FC 12 in oxygen

sensitized the heart to endogenous epinephrine. Mullin et al. (1972) subsequently observed that the inhalation of FC 12 at a concentration of 10% caused ventricular arrhythmia in a dog exercising on a treadmill. In the anesthetized dog, inhalation of 5 to 20% FC 12 caused tachycardia but no fall in aortic blood pressure, whereas inhalation of 5 and 10% concentrations of FC 12 caused both tachycardia and a fall in aortic blood pressure in the anesthetized monkey.

Myocardial Depression

Kilen and Harris (1972) reported depression of myocardial contractility in the rat heart; in the anesthetized monkey, inhalation of 5 or 10% concentrations of FC 12 depressed contractility.

Acute Bronchopulmonary Toxicity

Guillerm et al. (1960) administered 80% concentrations of FC 12 in oxygen to the anesthetized dog and noted no effect on the clearance of mucus from the trachea. In our laboratory, however, we found a concentration of from 10 to 20% FC 12 sufficient to increase pulmonary resistance in the same species. Bronchoconstriction was also encountered in the anesthetized rat and in the anesthetized monkey.

Comparative Toxicity

Fluorocarbon 12 differs structurally from FC 11 in the substitution of a chloride ion with a fluorine and has been found to have a lower acute inhalational toxicity in the guinea pig and rat. FC 12 is about 5 to 10 times less cardiotoxic than FC 11 in the mouse, dog, and monkey and, unlike FC 11, does not provoke hypotension in either the dog or monkey. The two propellants have opposite effects on the airways of the dog: FC 11 is a bronchodilator whereas FC 12 causes constriction. In the rat, FC 11 is a bronchoconstrictor and FC 12 is inactive.

Toxicity Test Species	Dichlorodifluoromethane (FC 12) CCl_2F_2	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Guinea Pig	30% for 2 hr = no loss of consciousness		10% for 1 hr = unconscious
Rat	50% = loss of postural reflex 60% = loss of righting reflex 70% = deep anesthesia	8	6% = loss of postural reflex 8% = loss of righting reflex 9% = unconscious 10% = lethal

Toxicity Test Species	Dichlorodifluoromethane (FC 12) CCl_2F_2	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Rat	60% for 2 hr = narcosis	6	10% for 90 min = lethal
Rat	36% = lethal	2	19% = lethal
Chronic Inhalational Toxicity			
Rat, guinea pig, dog, rabbit, and monkey	0.0002% continuous exposure and 0.0009% repeated exposure = nonlethal with no signs of toxicity		
Acute Cardiovascular Toxicity			
Mouse	40% = no cardiac arrhythmia and no sensitization	(inactive)	5% = sensitized heart to epinephrine
Dog	5% = sensitized heart to epinephrine	5	0.5% = sensitized heart to epinephrine
Dog, exercising	10% = cardiac arrhythmia	10	1% = cardiac arrhythmia
Dog	1% = tachycardia without hypotension	10	10% = tachycardia with hypotension
Monkey	10% = myocardial depression without hypotension	2	5% = myocardial depression with hypotension
Acute Bronchopulmonary Toxicity			
Rat	<36% = no bronchoconstriction and no reduction in tidal volume	0	<19% = bronchoconstriction with reduction in tidal volume and compliance
Dog	10% = bronchoconstriction	(opposite)	2.5% = bronchodilation

Toxicity Test Species	Dichlorodifluoromethane (FC 12) CCl_2F_2	< 1 = More Toxic > 1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Monkey	10% = bronchodilation	(opposite)	10% = bronchoconstriction

Monochlorodifluoromethane: Fluorocarbon 22

($CHClF_2$; Boiling Point $_{760}$ = -40.8 C; Vapor Pressure at 20 C = 121.4 psig)

Investigation of FC 22 has been largely toxicologic in nature. There is no information on its bronchopulmonary effects. In the dog and mouse, the concentration of FC 22 which initiates cardiac arrhythmia is 8 to 10 times higher than that of FC 11.

Toxicity Test Species	Monochlorodifluoromethane (FC 22) $CHClF_2$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Guinea Pig	10% for 1 hr = tremors 20% for 30 min= convulsive movements	2	5% for 1 hr = tremors 10% for 1 hr = unconscious
Guinea Pig	40% for 2 hr = tremors		
Rat	20% for 2 hr = nonlethal		10% for 2 hr = nonlethal
Rat	10% for 1 hr = excitation 20% for 45 min = excitation with respiratory stimulation followed by narcosis	3	3.5% = excitation 6% = narcosis
Rat	5% for a few min = injury		
Chronic Inhalational Toxicity			
Rat, guinea pig, dog and cat	5% for 3-1/2 hr for 20 days = no pathological change		

Toxicity Test Species	Monochlorodifluoromethane (FC 22) $CHClF_2$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Rat	13 ppm for 6 hr daily for 10 mo = neurologic signs		
Acute Cardiovascular Toxicity			
Mouse	40% = sensitized heart to epinephrine	8	5% = sensitized heart to epinephrine 10% = cardiac arrhythmia
Dog	5% = sensitized heart to epinephrine	10	0.5% = sensitized heart to epinephrine
Dog	25-70% = tachycardia with depression of ST segment		

Propane

(CH_3-CH_2-CH_3; Boiling Point $_{760}$ = -42.2 C; Vapor Pressure at 20 C = 110.3 psig)

Only very limited information is available on propane. Although propane is more toxic than FC 11 in causing stupor in the guinea pig, it is less potent in sensitizing the heart to epinephrine-induced arrhythmias.

Toxicity Test Species	Propane CH_3-CH_2-CH_3	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Guinea Pig	5% for 1 hr = stupor	0.5	10% for 1 hr = unconscious
Acute Cardiovascular Toxicity			
Mouse	20% = sensitized heart to epinephrine	4	5% = sensitized heart to epinephrine

Toxicity Test Species	Propane $CH_3-CH_2-CH_3$	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Dog	10-25% = sensitized heart to epinephrine		
Dog	10% = sensitized heart to epinephrine	20	0.5% = sensitized heart to epinephrine

Vinyl Chloride

(CH_2 = CHCl; Boiling Point $_{760}$ = -13.9 C; Vapor Pressure at 20 C = 31.5 psig)

Like all the other high-pressure propellants of intermediate toxicity, vinyl chloride sensitizes the dog heart to epinephrine-induced arrhythmia. It is, moreover, the only example in the group that induces arrhythmia in the mouse not given an injection of epinephrine.

Toxicity Test Species	Vinyl Chloride CH_2 = CHCl	< 1 = More Toxic > 1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Chronic Inhalational Toxicity			
Rat	3% for 4 hr daily 5 days a wk for 12 mo = degeneration of skeleton and cancerous lesion		
Acute Cardiovascular Toxicity			
Mouse	10% = sensitized heart to epinephrine 20% = cardiac arrhythmia	2	5% = sensitized heart to epinephrine 10% = cardiac arrhythmia
Dog	5% = sensitized heart to epinephrine	10	0.5% = sensitized heart to epinephrine
Dog	10-25% = sensitized heart to epinephrine		

HIGH PRESSURE PROPELLANTS OF LOW TOXICITY

The remaining two propellants are both fluorinated ethanes and are the least toxic of the hydrocarbons under consideration.

Chloropentafluoroethane: Fluorocarbon 115

($CClF_2$-CF_3; Boiling Point $_{760}$ = -38 C; Vapor Pressure at 20 C = 103 psig)

Fluorocarbon 115 was originally introduced as a propellant for use in food products and is presently not contained in pharmaceutical and personal products. Fluorocarbon 115 produces no effects on the central nervous system when inhaled at a concentration of 6%.

Toxicity Test Species	Chloropentafluoroethane (FC 115) $CClF_2$-CF_3	< 1 = More Toxic > 1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Rat	20% = no effect		
Rat	43% = lethal	2.2	19% = lethal
Rat and Guinea Pig	60% = no effect		
Guinea Pig	20% for 2 hr = no effect		
Chronic Inhalational Toxicity			
Rat, guinea pig, dog, and cat	20% = no effect		
Rat and dog	10% for 6 hr for 90 exposures = no effect		
Acute Cardiovascular Toxicity			
Mouse	20% = sensitized heart to epinephrine 40% = no cardiac arrhythmia	4	5% = sensitized heart to epinephrine 10% = cardiac arrhythmia

Toxicity Test Species	Chloropentafluoroethane (FC 115) $CClF_2$-CF_3	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Dog	15-25% = sensitized heart to epinephrine	30	0.5% = sensitized heart to epinephrine
Dog	20% = tachycardia with hypotension 20% = depressed ventricular function	20 2	1.0% = tachycardia with hypotension 10% = depressed ventricular function
Acute Bronchopulmonary Toxicity			
Rat	<43% = bronchoconstriction with increase in respiratory rate	2.2	<19% = bronchoconstriction without increase in respiratory rate
Dog	5% = bronchoconstriction without respiratory depression	(opposite)	5% = bronchodilation with respiratory depression

Difluoroethane: Fluorocarbon 152a

(CH_3-CHF_2; Boiling Point $_{760}$ = -24.7 C; Vapor Pressure at 20 C = 63 psig)

Fluorocarbon 152a is the least toxic of the 15 propellants. It does not induce arrhythmia in the mouse, nor produce tachycardia, hypotension or respiratory depression in the dog. It neither increases pulmonary resistance nor reduces pulmonary compliance. Although it sensitizes the dog heart to epinephrine-induced arrhythmia, a concentration of 15% is needed for FC 152a as compared to 0.3% for FC 11. It is not currently used in any aerosol propellant but deserves consideration as a substitute for the more toxic pressure propellants.

Toxicity Test Species	Difluoroethane (FC 152a) CH_3-CHF_2	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Acute Inhalational Toxicity			
Rat	20% = loss of postural reflex 25% = loss of righting reflex	5	6% = loss of postural reflex 8% = loss of righting reflex

Toxicity Test Species	Difluoroethane (FC 152a) CH_3-CHF_2	<1 = More Toxic >1 = Less Toxic 0 = Inactive	Trichlorofluoromethane (FC 11) CCl_3F
Rat	45% = unconscious 50% = lethal		9% = unconscious 10% = lethal
Chronic Inhalational Toxicity			
Rat	10% for 6 hr daily for 60 days = no pathological change		
Acute Cardiovascular Toxicity			
Mouse	50% = no cardiac arrhythmia and no sensitization of heart to epinephrine	(inactive)	5% = sensitized heart to epinephrine 10% = cardiac arrhythmia
Dog	15% = sensitized heart to epinephrine	30	0.5% = sensitized heart to epinephrine
Dog	20% = no effect on heart rate and no fall in blood pressure	0	2.5% = tachycardia with a fall in blood pressure
	20% = depressed ventricular function	2	10% = depressed ventricular function
Monkey	10% = depressed contractility without hypotension	2	5% = depressed contractility with hypotension
Acute Bronchopulmonary Toxicity			
Dog	10% = bronchoconstriction without respiratory depression	(opposite)	2.5% = bronchodilation with respiratory depression
Monkey	10% = no effect on pulmonary resistance and no respiratory depression	0	10% = bronchodilation with respiratory depression

SIGNIFICANCE OF THE PHARMACOLOGIC ACTION OF PROPELLANTS

So far, this review has been concerned with classifying the propellants according to their comparative toxicities expressed as either high, intermediate, or low. This first step is essential if we are to be able to discriminate the less desirable propellants from those that can be used with greater safety. It can be accepted as a general principle that among the low pressure propellants, those of low toxicity are to be preferred over those of intermediate toxicity.

Within a group of propellants at any given level of toxicity the selection is more complex and will depend on several factors which, though beyond the scope of this report, can be outlined as follows. First, the comparative toxicity of the aerosol containing both the propellant and the pharmaceutical product must be assessed. Second, the bioavailability of the propellant - i.e., its absorption, metabolism and excretion - must be determined. And third, the toxicity of the aerosol and of the propellant in man must be gauged. As this last factor includes consideration of the influence of disease of the heart and lungs on the sensitivity of these organs to the propellants, it is pertinent to conclude this review with a discussion of the significance of the known actions of the propellants on respiration and circulation. Cardiac arrhythmia and/or myocardial depression would, of course, be undesirable in a patient with coronary heart disease. Likewise, a depression in respiration, bronchoconstriction and/or a reduction in compliance would be hazardous if a patient with pulmonary disease were exposed to a particular propellant. Table 3 summarizes the toxicity findings for the propellants studied.

Cardiac Arrhythmia

It can be concluded that the inhalation of propellants sensitizes the heart to epinephrine-induced arrhythmia. This effect was consistently found for 14 of the 15 propellants administered to the unanesthetized dog. In the anesthetized mouse, 13 sensitize the heart to epinephrine and 5 are sufficient to induce arrhythmia without a concurrent injection of epinephrine. The mechanism involved in the arrhythmia is not well understood. Yet it has been demonstrated that the blockade of neither the cholinergic nor the adrenergic receptors interferes with the induction of arrhythmia. In addition to a direct action by the propellant on the heart muscle, other participatory mechanisms may include the release of epinephrine from the adrenal medulla and the stimulation of receptors in the nasal and pharyngeal mucosa when the propellants in aerosol form are inhaled. Such administration has been shown (Aviado, 1971) to elicit the Kratschmer reflex as well as the resultant increase in vagal tone known to trigger the appearance of arrhythmia. As there is no information now available on the interplay of these factors in cardiac arrhythmias associated with the inhalation of propellants, it is not possible to determine if the fatalities resulting from the use of aerosols are due to the arrhythmia-inducing potential of the aerosol.

TABLE 3

SUMMARY OF PATTERN OF EFFECTS ON CIRCULATORY AND RESPIRATORY SYSTEMS

Inhalation Of Propellants: ++=response at lower concentration; +=response at higher concentration; 0=absent or opposite response

Name of Propellant Fluorocarbon No.	Cardiac Arrhythmia Induce Mouse	Cardiac Arrhythmia Sensitize Mouse	Cardiac Arrhythmia Sensitize Dog	Tachycardia Dog	Tachycardia Monkey	Myocardial Depression Dog	Myocardial Depression Monkey	Hypotension Dog	Hypotension Monkey	Early Respiratory Depression Rat	Early Respiratory Depression Dog	Early Respiratory Depression Monkey	Bronchoconstriction Rat	Bronchoconstriction Dog	Bronchoconstriction Monkey	Decreased Compliance Rat	Decreased Compliance Dog	Decreased Compliance Monkey
Low Pressure Propellants Of High Toxicity																		
Trichlorofluoromethane FC 11	++	++	++	++	++	++	++	+	++	++	+	+	++	0	0	++	0	0
Dichlorotetrafluoroethane FC 21	++	++		++	+		+	+	+	+	0	++	0	+	0	0	+	0
Trichlorotrifluoroethane FC 113	++	++	++	++		+		+			0			+			+	
Trichloroethane	+	+	++	+		+		0			0			0			0	
Methylene Chloride	+	+	++								0							
Low Pressure Propellants Of Intermediate Toxicity																		
Dichlorotetrafluoroethane FC 114	0	+	+	+				+		0	0		+	+		+	+	
Monochlorodifluoroethane FC 142b	0	0	+	+	0		+	+	0		0	0		+	0		0	0
Isobutane	0	+	+	0				0		++	+		++	+		++	+	
Octafluorocyclobutane FC C-318	0	+	+	0	0	+	0	0	0	++	0	0	+	+	+	++	+	0
High Pressure Propellants Of Intermediate Toxicity																		
Dichlorodifluoromethane FC 12	0	0	+	+	0		+	0	0	+	+	+	0	+	0	+	+	0
Monochlorodifluoromethane FC 22	0	+	+															
Propane	0	++	+															
Vinyl Chloride	++	++																
High Pressure Propellants Of Low Toxicity																		
Chloropentafluoroethane FC 115	0	+	+	+		+		+		0	0		++	+		+	+	
Difluoroethane FC 152a	0	0	+	0	0	+	+	0	0		0	0		0	0		0	0

Tachycardia

Inhalation of propellants causes tachycardia in the monkey and dog and bradycardia in the rat. As the mode of response is species-dependent, it is not yet possible to predict the effect in humans. While the mechanism responsible for the change in animals heart rates has not been identified, it seems probable that the tachycardia is due to either a release of epinephrine or an increase of sympathetic innervation to the heart. Approximately half the propellants tested induce tachycardia and would thus be particularly dangerous to an individual suffering from coronary heart disease. An understanding of the propellants' effects on coronary circulation awaits systematic investigation.

Myocardial Depression

The information on the propellants' effects on myocardial contractility is incomplete. All of the 6 propellants so far tested in the dog show depression. In the monkey, all the propellants tested, with the exception of octafluorocyclobutane (FC C-318), were found to provoke depression. It can be hypothesized that the mechanism involved is probably direct and not unlike that characteristic of most inhalation anesthetics, e.g., halothane and chloroform.

Hypotension

The hypotension resulting from the inhalation of propellants is the least well understood of the responses noted. No measurements of cardiac output and systemic vascular resistance during propellant administration are yet available. To the extent that hypotension can occur independently of myocardial depression, the propellants would appear to decrease vascular resistance directly.

Respiratory Minute Volume

The nature of respiratory response is especially important in differentiating among the various propellants. While some propellants are clearly depressants, others are stimulants. Among the latter are trichlorotrifluoromethane (FC 113), dichlorotetrafluoroethane (FC 114), chloropentafluoroethane (FC 115) and trichloroethane. That these are all halogenated ethanes suggests that respiratory stimulation may be characteristic of this group. The mechanism involved has been identified as sensitization of the stretch receptors in the parenchyma of the lung.

Pulmonary Resistance and Compliance

The effect of the propellants on lung function is relevant to understanding the possible harm that aerosols may exert in the asthmatic individual. Unfortunately, there is no uniformity of response across species to these propellants. A substance which causes bronchodilation in the dog may be inactive in the monkey and act as a bronchoconstrictor in the rat. The explanation for this and a similar species difference in heart rate appears to hinge on the relative predominance of the autonomic vis a vis the sympathetic nervous system in each of the species studied, i.e., bronchoconstriction and bradycardia in the rat suggest autonomic (vagus) dominance while the opposite effects in the dog confirm sympathetic preeminence. While pulmonary compliance is usually altered with pulmonary resistance, it is difficult to exclude the possibility that the propellants act directly on the pulmonary capillaries to reduce compliance. The dearth of information on the effects of propellants on pulmonary circulation must be remedied. Particularly deserving of attention are the deaths attributed to abuse of aerosols where postmortem examination has revealed pulmonary hemorrhage.

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OPEN FORUM

MAJOR VAN STEE (Aerospace Medical Research Laboratory): I would like to take about 2 or 3 minutes of your time to offer some comments on Dr. Aviado's criticism of my data yesterday. I went back to my lab and examined it at some length. A little bit earlier today I sat out at the desk and prepared a handmade viewgraph which I think is ready for projection. I might add that I did detect an error in my reference values, and I'm not certain exactly how the errors crept in, but for these I apologize. On the other hand, for my data I do not apologize and I offer these in support of my data. These data, comparative values, are taken from a paper by Whereat and Chan published in 1972. The title of the paper is "The Effects of Hypoxemia and Acute Coronary Occlusion on Myocardial Metabolism in Dogs." This work was done at the Cardiovascular Pulmonary Division of the Department of Medicine at the University of Pennsylvania. The values at the top of the slide represent control values taken directly from this paper. These experiments were performed on closed-chest dogs in which alpha chlorolose was used as the anesthetic. There were no hemoglobin values given. For purposes of this argument, I assumed a value of 15 grams of hemoglobin per 100 ml of blood. There were 3 samples and the size of each sample is given under the column "N", to the left. This first group contained 6, the second contained 6, and the third contained 9 dogs. They measured the coronary sinus blood PO_2 and got the values you see there of 27.7, 24.25 and 30.6. He reported the percentage saturation for the hemoglobin in those same samples as 42.8, 36.2 and 47.0. Now, if one calculates the oxygen content of these coronary sinus blood samples based upon the ability of hemoglobin to bind 1.34 ml of oxygen per gram of hemoglobin, one comes out with calculated values in my units which were in ml/L, 89, 75.9 and 97.6 translated to volumes percent 8.9, 7.6 and 9.8. If we pool these data to give us a sample size of 21 for which he has a mean PO_2 of 28, a saturation of 42.7 and a calculated O_2 content of 89 mg/L, at the bottom of the slide you will see equivalent data from our studies for a sample size of 73 and in the lower right hand corner, the oxygen content of the coronary sinus samples compared very favorably. Ours averaged 83.4, his averaged 89. This I offer as support of the validity of our measurements of coronary sinus samples.

DR. AVIADO (University of Pennsylvania): I'm still on the same theme as I was yesterday. The old studies that were quoted by you then, by Rushmer and by many others who used the Van Slyke technique showed lower values than yours. I gather Whereat didn't use the Van Slyke, did he? Did he use the indirect technique of measuring oxygen content based on PO_2?

MAJOR VAN STEE: He doesn't give a complete methods of analyses in his paper and he didn't even cite the hemoglobin values which necessitated making this assumption. I don't know precisely how he arrived at his final figures. He just stated what they were without going into it further.

DR. TOTH (University of Nebraska Medical Center): I wanted to ask two questions from Dr. Shaffer, but since he is not here, I hope Mr. Wands may be willing to reply. First it was mentioned that several people were voluntarily exposed to MMH and I'm just wondering whether these people were told that MMH is a carcinogen. In addition to the acute effect, it has an irreversible long-term effect. The second question is that he mentioned that he classified the hydrazines as a class of chemicals which do not induce tumors de novo; rather they are tumor promoters or co-carcinogens. But this is simply not true. I would like, if you would, Mr. Wands, to substantiate this.

MR. WANDS (National Academy of Sciences): Dr. Toth, I'm totally unable to answer that question. I'm merely quoting from the published literature and that particular experiment was performed here at the Wright-Patterson Air Force laboratories so I'll have to turn that over to Dr. MacEwen or Dr. Thomas or some of their staff to answer that question.

DR. BACK (Aerospace Medical Research Laboratory): No they weren't told that MMH was carcinogenic. The people who were exposed were not told that the compound was carcinogenic.

DR. TOTH: I'm asking you whether you told the people that they were facing an acute effect which is irreversible usually, which may cause cancer since this compound produced tumors in two species.

DR. BACK: They were not told that this compound was carcinogenic. I'm just making a flat statement.

MR. WANDS: I would like to ask Dr. Toth if he feels that MMH or any of these other hydrazines is going to be carcinogenic by a single short exposure?

DR. TOTH: I haven't done that experiment but I can assure you that these compounds, by repeated exposures, induce tumors in mice and in hamsters.

DR. CULVER (University of California, Irvine): Then we do not have any indications that it is carcinogenic in man. Is that true?

MR. HAUN (University of California, Irvine): I just want to comment on Dr. Toth's remark. These human experiments were done three or four

years ago. This was before his work, I believe, and at that time we were not aware of any information that suggested that MMH was carcinogenic.

DR. SCHEEL (National Institute of Occupational Safety and Health): In the carcinogenicity area, we have to be extremely careful in trying to extrapolate what we see in animals to man. The best example, at the moment, where we have adequate human examination data is the beryllium case registry where we have examined the people in the whole industry, and even though we can produce tumors in rabbits, in rats and in monkeys, we don't get them in man. So I think in this case we have to be very careful about how we make a statement about what is or is not carcinogenic in man.

DR. CULVER: I would certainly like to second what Dr. Scheel says. We have to be extremely careful in this whole area of carcinogenesis in man. We don't need any more confusion than we already have in this area.

DR. THOMAS (Aerospace Medical Research Laboratory): Let me add one thing to this discussion. The DOD (Department of Defense) might be the smallest user of hydrazines. The industrial use of hydrazines is much larger in industry than in the Air Force. Hydrazines have traditionally been used for cleaning out boilers and I would be glad to discuss any epidemiological data on the carcinogenicity of plain hydrazine. If anybody has some, please tell us about it.

DR. HENDERSON (Olin Corporation Research Center): We operate a hydrazine plant at Lake Charles, Louisiana. That plant started operating in 1954. Prior to going into operation, we had a pilot plant at Niagara Falls, New York. At the present time, we have, I believe, 10 people who have worked continuously with hydrazine since 1953, including being in the pilot plant. We have another 10 that did work with it originally and are presently in other areas of the corporation. We do periodic physical exams on our people and as far as I can tell from any of our records, we haven't seen any evidence of carcinogenicity in these human subjects who have worked with it now for approximately 20 years.

DR. CULVER: I'd like to add a datum point on the subject, too. At the time I joined Aerojet-General Corporation in 1958, the company had just sustained an episode in which 4 people were seriously exposed to dimethylnitrosoamine to the degree that they were hospitalized with acute hepatotoxicity. Two of those cases went on to develop a significant reduction in hepatic reserve. This was back in 1957 when the exposure occurred. All of these people are still alive, and they were recently restudied for any evidence of cancer or of other tumors. So far, anyway, there's been no indication of such having occurred. This I again repeat, is dimethylnitrosoamine, not hydrazine.

MAJOR MC NUTT (Aerospace Medical Research Laboratory): I'd like to ask Dr. Goldstein to please elucidate on the possible mechanisms of the decreased resistance of animals after exposure to ozone and nitrogen dioxide. I wondered if there was any information on the number of alveolar macrophages that respond to the bacterial stimulus, whether this has changed, or whether it is a decreased ability of these cells to kill the bacteria?

DR. GOLDSTEIN (University of California, Davis): There is a considerable body of information concerning this done by many, many people. All of what I will say now was reported by other research laboratories. The first is that the number of macrophages has been calculated by Dr. Brane at Harvard Medical School. He can wash out more than one million from the lungs of a rat, and he is washing out only a small percentage of the total. There are many, many more. Therefore, even though the numbers of bacteria that were present in this system, being over a million bacteria per animal, looked like a lot, the numbers of macrophages were greatly in excess. Even this astronomical challenge really is a small insult to an animal's defense mechanism. Secondly, there is a large body of data which shows that these macrophages are drawn chemotactically towards bacteria and that they can be drawn towards them very readily, very rapidly. Their cell membranes are invaginated and the bacteria brought into the macrophage. The ingestion process has been very well studied by Drs. Anvil, Cohen, Hersch and others. It's also known that there are primary lysosomes that are located within the macrophages containing many different enzyme systems. I'm not a good enough biochemist to discuss which ones are most important, but you know about the Klebanoff mechanisms for the killing of bacteria. They can be shown with electron microscopy and with in vitro techniques in which alveolar macrophages are grown in cell culture. In the invaginated bacteria which are ingested, a primary lysosome comes up to a bacterial vacuole which is called a phagosome and you get a phagolysosome, the two attach, and then the lysosome contents are actually injected into the phagosome. You get one big, huge vacuole. It's believed that this is the mechanism for killing. There are in vitro studies showing that these cells do kill in tissue culture by these mechanisms. Which of the enzymes is actually involved in the lysis of these bacteria, I really wouldn't want to discuss because I'm not that good at it.

MAJOR MC NUTT: The thing that I was wondering about is where in this series of steps does ozone or nitrogen dioxide interfere? I was intrigued that the number of bacteria per macrophage was enormously increased after the ozone exposure, I believe. That increase seems to me to indicate that possibly the number of macrophages responding to the challenge would be less in that particular regard, so a given macrophage would have to ingest more bacteria.

DR. GOLDSTEIN: No, I don't think that's what's really occurring. Ozone has been shown to inactivate proteins and to destroy macrophages.

Remember those were high concentrations of ozone that you were shown. Ozone also inactivates many sulfhydryl enzyme systems. It also involves lysosomal destruction of the enzyme lysozyme. So what I think is actually occurring is that the enzymatic systems within the macrophage itself are impaired by exposure to ozone. There's considerable evidence for this mechanism. Now, I do not know of any comparable evidence for nitrogen dioxide. But there's considerable biochemical evidence for phagocytic systems that the enzymes within the alveolar macrophage are probably being destroyed by exposure to ozone. I think, Dr. Stokinger, that you probably have more information on this than I do. You've written books on it.

DR. STOKINGER (National Institute of Occupational Safety and Health): I think you've said it well enough.

DR. THOMAS: I've got a philosophical question for Dr. Nettesheim. The first day of this conference we were talking about the relevance of exposure conditions as they pertain to the Toxic Substances Act. I understand your reasons for saying that inhalation exposures are very costly procedures and that that's not the way to proceed in research if you can avoid it. However, there are certain things which come to mind. One is that Dr. Weibel has shown that the total mass of the surfactant in the lung is about 7.8 grams. I might be wrong on this point but it was published 3 years ago in our proceedings. If you use a pellet and implant it in the lung, the ratio of contact area of the pellet to the total lung surface is minimal. You can very easily visualize that depositing a pellet is not a very realistic way of exposure when you present a dose, to an area of a few square millimeters, which would equal a body burden of about 10,000 times more than that which you could get by an inhalation exposure.

DR. NETTESHEIM (Oak Ridge National Laboratory): I never said that these were very realistic exposures. Obviously, I've never heard of anybody inhaling pellets. But, I think I tried to point out that this question about how realistic your exposure is, regardless of whether you're using inhalation or something else, is a matter of what you're trying to do. All I was trying to point out is that there are many experiments where you do not have to copy the physiological situation as closely as possible, and there are others where it is absolutely essential that you do. Of course, to insert a paraffin pellet into the pulmonary parenchyma is not a physiological way of encountering it. There's no way of comparing that to inhalation. What it does is give you a pretty good idea how much tissue mass is exposed to a given amount of carcinogen over a given amount of time. That's a tremendous advantage over any inhalation method if you want to have accurate dose studies because with the inhalation method, you can never calculate, no matter how good a computer system you have, how much carcinogen was deposited in the left primary bronchus, per square micron, and how long it

was there. That is just to point out one of the problems with inhalation studies. I have a large inhalation facility at Oak Ridge where I'm doing a lot of inhalation experiments and I fully realize that there are certain experimental questions which cannot be answered other than with inhalation methods.

DR. STEMMER (University of Cincinnati, Kettering Laboratory): I find it very illogical to consider the use of an implanted pellet to establish that something is carcinogenic especially if you look at the dose used. You pointed out yourself that it's extremely difficult to extrapolate from rat to human if you don't use the physiological way man or animal is exposed to pollutants. All the data you actually collect from implanted pellets or any other short cut methods have no meaning whatsoever in establishing carcinogenic hazard.

MR. WANDS: I have two comments. First of all for Dr. Nettesheim. We did discuss this question earlier today, and I think that you made in your formal presentation the statement that negative data are of no value. I would agree with you as long as you are trying to determine data that would be useful in a publication or a doctoral thesis where the student who gets negative data for his doctorate is in deep trouble. On the other hand, when you are trying to get data that relate exposure to hazard rather than to toxicity, negative data are, indeed, extremely valuable, just as they are also valuable when you are having to deal with a regulatory body such as FDA on a food additive where you have taken a good hard look by any number of techniques and come up with negative data. Then these negative data are of value. And I think that ties in with the answer you gave Dr. Thomas. And now if I may, Dr. Culver, I would like to ask a question of Dr. Goldstein or do you want to continue exploring this?

DR. CULVER: There are several people sitting on the edge of their chairs still that I think are trying to get involved.

DR. BRUBAKER (Environmental Protection Agency): In view of the need for information on inhalation carcinogenicity in the agency which I am with, I would like to ask Dr. Nettesheim if the factors that he outlined could be arranged in some hierarchial manner that could give us at least a fix on relative carcinogenicity, and how well do they lend themselves to either standardization or quantitation?

DR. NETTESHEIM: It depends on what kind of prejudgments you have made. As the gentleman before remarked, if you have decided that any data you get that do not involve inhalation techniques are meaningless, then obviously there is no hierarchy to be had. I'm not about to argue that point because that's a statement I cannot understand. I think there can be very meaningful data derived from methods other than inhalation exposures

to carcinogens. Now if you don't take that kind of point of view, other approaches can be taken. I want to know whether a particular pollutant or mixture of pollutants is carcinogenic or not, it's as simple as that. Not how or under what circumstances. The primary question is, is it carcinogenic or is it not carcinogenic? I would either start with the intrabronchial or intrapulmonary pellet technique or with intratracheal injection methods depending on which one is more feasible because of the physical/chemical characteristics of the material. I believe you can save yourself a lot of time, a lot of trouble and a lot of money if you find with any one of these techniques that your material is noncarcinogenic. Then I think the likelihood that with any other method it will turn out to be carcinogenic is extremely low. We're talking about respiratory tract epithelium, that's all we're talking about. I would think that is a first approach, if you are confronted with a pollutant that hasn't been around before, or one that you don't know whether it is or is not carcinogenic. It is a first approach to determine whether there is any carcinogenic hazard to the living. I personally would go that way. If the results are negative, I would feel comfortable with them and I would say I don't have to do anything further. Now, if it's positive, then you have a lot of work on your hands, and I think then, ultimately, you will have to run some kind of inhalation test to establish at what concentrations and under what conditions it is carcinogenic. But I think we can save our nation a tremendous amount of money by this approach. I think we are talking about several orders of magnitude in terms of the cost difference between properly run inhalation experiments and the pellet method. A small number of laboratories can test a great number of suspect compounds by screening methods but if you are talking about chronic inhalation studies, how many can you do per year?

DR. CROCKER (University of California, Irvine): I didn't wish to add anything to what Dr. Nettesheim has said. I think he answered for himself in a perfectly adequate way and that is that the more rigorous the test and the more simple answer you wish, the simpler the system you may use, namely, by installation or by pellet implantation. But if you wish to begin to work with those levels of potential inhalant carcinogenesis at which you may wish to make species comparisons, and I don't mean now to study mechanisms, but still to approach something about the safety limits, then inhalation exposure under realistic conditions does continue to be an issue. I don't think Dr. Nettesheim would deny that because he would say that at that point you were approaching a question from the point of view of the specific potential for the causation of lung cancer in man. What has been touched upon during today, and I would like to see if we could generate some benefit from, is the matter of interspecies comparisons by which we would then be able to convert inhalation values taken in animals to human exposures. A good part of this may be achieved by the comparisons by which we would then be able to convert inhalation values taken in animals to human exposures. A good part of this may be achieved by the comparison between a rodent and a monkey, for example, although I'm not sure of data fully sufficient to say that

monkey and man are interchangeable in terms of their pulmonary defense mechanisms; they are at least a good deal more like one another in terms of their anterior airways and in terms of their potential for alternate mouth and nose breathing while rodents are obligatory nose breathers with very much more effective anterior airways for filtration. The point that Dr. Hodge was beginning to make, and I know he can make it much more completely, has to do with the effective dose measurement upon delivery to a system. This lies not only in the concentration of contaminant in the air but taking account of that and then concentrations in blood and rates of removal ultimately reaching a composite balance by which to establish some kind of relevance between the air exposure concentration and the capacity of the animal to deposit or remove the carcinogen or other material and that at the point at which this is in equilibrium, you could compare one animal with another reaching by some rational means a better method of dosimetry than merely speaking of an air concentration. To that end, I would wonder if Dr. Hodge would care to comment about the extension of such approaches beyond CO to particulates, dust or any of the other materials that might be relevant to inhalation exposure.

DR. HODGE (University of California, Irvine): I'm not prepared to discuss that. I really hadn't tried to think that whole proposition through. It certainly is appealing. We should try to use good information, if and when we develop it, on the relation between intake and exposure, using kinetic data. Dr. Stokinger, in the past, hadn't we relied greatly on descriptive pathology, the effects that we can recognize by any means whatsoever and used these as our criteria; first establishing the presence of an adverse effect then reducing the concentration to the point where we no longer can detect it?

DR. NETTESHEIM: Excuse me, but I think the problem is much more complex than that. I understand that there are a lot of inhalation toxicologists in the room that are speaking por domo, but I think we have to be fair here. Granted, from the inhalation toxicologist's point of view, there's only one appropriate method of exposing animals to inhalation hazards and that is by inhalation. However, when you look at the problem from the carcinogenecist's point of view, and I think that is something you gentlemen may be overlooking, then you will find that 95% of all tumors that have been induced in nonhumans, to date, by inhalation are not bronchogenic in origin. Now, this is a very important thing because practically all are bronchiolar/alveolar in origin. Now that means that the mechanism involved in this species is completely different from the mechanism involved in cancer induction in the human where the bronchogenic carcinoma is the prevailing tumor. We're talking about events happening in the bronchial tree, in the major bronchi, and the accumulation and clearance in that area is different than the accumulation and clearance of the alveolar ducts and the respiratory bronchioles. Now, you know better than I do that the clearance mechanism, the clearance kinetics of those two areas are completely different. So what is happening in animals is a carcinogen accumulating in an area and becoming an effective

carcinogen where in humans it rarely occurs. What I'm saying is that the inhalation carcinogenesis study superficially looks like a very physiological way of going about it because it's very similar to what has happened in man. But I think that is only a sleeping pill because in reality it is not the case. It doesn't do the job. That's what I'm trying to get across.

MR. SULKOWSKI (Koppers Company, Inc.): Dr. Nettesheim, the data indicated, and I think this was part of the argument, that BAP was non-carcinogenic alone but when absorbed on iron oxide produced lung tumors. Therefore, it appears you wanted to prove BAP positive so you injected a large particle that was not respirable and then you got the result that you wanted. Now, what happens when you inject a pellet of iron oxide dust alone that is not respirable in the same manner? Would you get the same result?

DR. NETTESHEIM: First, these were not my data. These were data I collected from the literature to present to you. You asked me whether iron oxide causes cancer when put into a pellet?

MR. SULKOWSKI: Would it do it in the same way? Do you know for sure? Has any evidence been gathered one way or the other?

DR. NETTESHEIM: All I can say is what has been done. Benzpyrene has been injected in the form of very small crystals of about one micron median diameter suspended in gelatin. When you give it alone, you do not get tumors unless you use absolutely huge quantities. When you mix it with ferric oxide and inject it intratracheally, you do get ample tumor yield at considerably lower doses. If you inject ferric oxide by itself, in large quantities you do not get any tumors. Now, to my knowledge, this thing has not been done with pellets. I'm not aware of any study where iron oxide pellets have been implanted. As far as intratracheal injection is concerned, you cannot induce pulmonary tumors in hamsters with large amounts of ferric oxide. I think that's the only animal that's been tested in this manner.

MR. WANDS: Dr. Goldstein, you spoke of your work with ozone and with oxides of nitrogen independently. We're becoming more and more concerned about the problems associated with mixed exposures. Have you either done or do you have plans for doing experiments with combinations of ozone and NO_x? Are you going to go then either further on with even more pollutants added to the exposure mixture? Sooner or later we do have to deal with this question of mixed exposures. We're seeing this most clearly now with the latest concerns about revising or even totally withdrawing some air quality standards that EPA has issued for common air pollutants because they were developed independently and without consideration of the tremendous interactions possible. I hope you're leading the way into this area of interaction with your studies.

DR. TERRILL: I see, because we know of a case where the material turned out to have 10% carbon tetrachloride in it. And I read a rough draft by David Blake who indicates that there is much, much less metabolism of fluorocarbon 11 than you found. It will be interesting to see how we can correlate these two different results.

DR. AVIADO: Well, I'm willing to concede even though there may be a contamination of 10%, there's still 10% that's been expelled in the air as carbon dioxide tagged with carbon 14.

DR. TYLER (University of California, Davis): I'd like to ask Dr. Aviado a question. First, I would like to compliment him on the very excellent and extensive job of physiological toxicology. Dr. Aviado stated yesterday that he was going to answer my rudimentary questions about the influence of these compounds on the lung and I submit he did not do that. The questions I would like answered are: What is their influence on things like diffusion capacity? What is the influence on the numbers and disposition of macrophages and their functions as Dr. Goldstein has shown these differences? What is the difference in the type 2 cells? What is the influence of these compounds on the clear cells? All of these things could very well be altered by these compounds and they have not been investigated in any of these studies that we have seen. That's the question that I think should be asked relative to some of these fluorocarbons which are used as propellants. Maybe I've missed it in the literature.

DR. AVIADO: Well, I thought you would be happy with the pulmonary resistance the pulmonary compliance data but let me go one step further. I do not have any information on bacterial clearance similar to what Dr. Goldstein has been doing and I don't think there's any information known about that subject. I have not examined clear cells, which as you know, can only be detected by the ultra microscope. I have some values on phospholipid, if you would like to know about the surfactant activity, which chemically can be measured by phospholipid. There is a certain amount of reduction of that activity.

DR. TYLER: If there's a reduction in the amount of surfactant and if this is measured not by phospholipid, is it dipalmityl lecithin you're actually measuring?

DR. AVIADO: Yes, that's right.

DR. TYLER: These are standard extraction procedures?

DR. AVIADO: Yes.

DR. TYLER: Well then, if this is true, either there's an increased turnover of this material or there is a decrease in the number of cells producing it. Isn't that true?

DR. AVIADO: That's right.

DR. TYLER: And this decrease in the number of cells producing it could be fairly significant over a long period of time. Your exposures were relatively short so that I think this question should remain open. You've done a beautiful job of physiological toxicity but essentially none of morphological toxicity and only a limited amount on biochemical toxicity. The question should still remain open as to the toxicity of these substances as they are commonly used by both healthy and diseased people. I'd like to comment once more that I'm really very pleased that you are going to use these diseased animals in toxicological experiments which, hopefully, will be transferrable then to human information, and I'd like to chat with you privately about that if I could, please.

DR. CROCKER: This question is for Dr. Tyler and it may connect with his emphasis on a function of lung that is different, let's say, from those functions related to nerve supply and smooth muscle activity and related instead to cellular defense mechanisms, both of epithelial tissue and of macrophages. It goes back to the point that was stated by Dr. Nettesheim when he referred to the incomparability of many animal model systems for epithelial alterations in comparison with man. For example, just to expand Dr. Goldstein's point: Goldstein finds in the rodent that the chief clearance mechanism is macrophage mediated, but in the monkey or man, the chief clearance mechanism is mucociliary. The chief mode of removal of particulates inhaled by man is by the mucociliary mechanism and it is very rapid. Thus, there indeed may be some failures in comparison and Dr. Tyler is an outstanding comparative anatomist of experimental and domestic animals in terms of respiratory systems and might comment, not only on the carcinogenesis studies, but even the mechanisms of defense as these may differ across the species in our effect to reach animal versus human correlations.

DR. TYLER: Thank you very much. I wish that I had this information available. We don't have it available. We do know that most of the laboratory rodents, especially, have lungs which are extremely different from those of man. I'm presently at a primate center, and I regret to say that our preliminary look at primates of three species of macaques indicate that their lungs are also significantly different from those of man. At this time we do not have, really, a suitable laboratory animal with lungs that are similar to man in critical areas, namely the distal airways, either terminal or respiratory bronchial and in some of these clearance mechanisms. We just don't have the animal that we're all searching for, that will let us directly extrapolate this data. We, like most of you, are taking data from one or more rodent species, then we're picking either the dog or the nonhuman primate as an intermediate animal and trying to extrapolate to man, which I think, is very hazardous and I wish that we had a better next point along the line. There are still quite a few nonhuman primates that we want to look at and see if they are really a better model for the human being than any that we've looked at to date.

DR. AVIADO: Could I ask a question of Dr. Hodge? Since I am a cigarette smoker, I was interested, Dr. Hodge, in those predictions of carboxyhemoglobin levels in which the calculations for a nonsmoker are different from the predictions of a smoker. I gather the smoker has a steady level in spite of exposure to additional carboxyhemoglobin whereas a nonsmoker would have considerable fluctuation. Which do you think would be more desirable, Dr. Hodge?

DR. HODGE (University of California, San Francisco): Well, the point I was making was that the initial carboxyhemoglobin level was one of the important points in discussing relation between carbon monoxide in the air and the biological effect.

DR. BUCHWALD (Department of National Health & Welfare, Canada): I'd like to make an observation on the misuse of our normal time-weighted average concentration concept as used together with the threshold limit value tables. In particular, I would point out to the exposure where Dr. Hodge referred to 10 ppm CO for 7 hours and then 200 ppm for 1 hour both under light activity and light work. I'd like to quote to you from the booklet of the Threshold Limit Values for this year. It says, "The degree of permissible excursion is related to the magnitude of the threshold limit value of a particular substance as given in the appendix." It also states, "The relation between the threshold limit and permissible excursion is a rule of thumb and in certain cases may not apply." And in Appendix D, it does suggest that "The excursion factor for a substance of a threshold limit value between 1, 10 and 100 ppm should be not more than 1.5." I see in the particular case of time-weighted average of 35 ppm that the 200 ppm quoted in Dr. MacGregor's paper is greatly in excess of that 1.5 factor that is recommended here. I might add that any self-respecting industrial hygienist wouldn't tolerate a situation of 200 ppm for 1 hour when a threshold limit value of 35 ppm was operative. This sort of situation wouldn't normally exist in a supervised workplace. Although I do accept that this particular value that Dr. Hodge quoted was used as an example primarily, I just want to make the observation that the well-trained industrial hygienist wouldn't tolerate a situation of this kind.

DR. HODGE: I won't argue with your point. The slide that I showed you had the 1.5 factor of the present TLV of 50 ppm as its basis and I showed you that for the nonsmoker, there was a considerable difference where the time-weighted average was calculated from 50% above the TLV for 4 hours and 50% below for 4 hours versus what the 50 ppm limit by itself for the 8 hour period would be. This was within the rules that you just quoted. Now the reason for the extraordinary lopsided example of 7 hours at 10 ppm CO and 1 hour at 200 ppm was that in the NIOSH criteria document where the new value of 35 ppm is proposed, it is stated that an exposure of 200 ppm is the ceiling concentration. We simply took that number and said suppose we had 200 ppm for an hour and all the rest of the time were at a low enough

DR. GOLDSTEIN: Thank you very much for this question. I have to leave shortly and this is a fine question to end with because we have done this. We have exposed animals to ozone in combination with NO_2. We were hoping very much for a synergistic interaction from a personal basis, but also there's some evidence that N_2O_5 is formed when you add the two together and some of the English authors had claimed that N_2O_5 was more toxic than ozone. Therefore, if you had ozone and NO_2 and you got N_2O_5, you would have a more severe effect. As it turned out, we did not. The response of animals to the mixture looked like an independent response to the one which had the higher concentration. The principal finding was that when you added ozone and NO_2 together, the most precise measurement wasn't our bactericidal measurement, but it was the bronchoconstrictive effect. Ozone causes more bronchoconstriction than interference with bactericidal activity, but you could notice a failure in inhalation uptake of the bacteria which reflected the bronchoconstrictive activity of ozone. So I would say the two together are at most additive and probably are acting somewhat independently. I might also say that there are some other data in the literature which state that N_2O_5 is not more toxic than ozone. If I could have a minute to write an equation on the board I could show you where we went wrong. In hindsight, it's an interesting way of learning something that we should have learned in the beginning. In the process of making N_2O_5, the O_3 is consumed, and therefore, there cannot be a combined effect of N_2O_5 and ozone. We were losing our ozone and, therefore, the biologic effect of the mixture was less than or similar to ozone. You're losing ozone one mole for one mole so that one should not have anticipated a greater biologic effect. We had been aware of the British literature, and subsequently became aware of the American literature and the fact that this concept had been under attack but we were unaware of this when we first did the experiment. We had anticipated synergy but we did not get it. This work will soon be published in the Archives of Environmental Health.

DR. TERRILL (E. I. duPont de Nemours and Company, Inc.): I'd like to ask Dr. Aviado about the metabolism work he referred to, just briefly, and what the outcome was. He more or less said there was metabolism of some specific fluorocarbon. I'd be very interested to know which one it was.

DR. AVIADO: The propellant that we had been working on was number 11. About 20% of the ingested tagged fluorocarbon 11 was excreted in expired air as carbon dioxide which we interpreted as the breakdown product.

DR. TERRILL: Did you check the radiochemical purity?

DR. AVIADO: Yes, this was checked for us by the manufacturer, and the manufacturer assured us profusely that the amount of contaminant was certainly less than 20%. It was probably about 2 to 3%.

concentration so that the time-weighted average still was 35, what would this do to the person's carboxyhemoglobin percentage? And it showed that it could be quite a lot higher than the ordinary fluctuations around 50. Dr. MacGregor was trying to emphasize the usefulness of looking at kinetic data when a new TLV is being considered. If you will concede that it would be useful to look at kinetic data in setting TLV's, I'll concede anything you want about the self-respecting industrial hygienist.

DR. MC GAUGH (University of California, Irvine): This question has to do with Dr. Weir's statement that he would like to lay the ghost of the notion that the carbon monoxide and alcohol in those levels affected driving practice. I just wonder if the circumstances under which this was assessed weren't so ideal as to make the human subject muster every effort he could to behave properly, much like the 16-year-old taking the driving course or the person taking the driving exam and doing everything quite properly. That is to say that subjects sitting in a car where everything is being observed may really be behaving in the best way that they can. Now, I wonder if this applies to the circumstances which I think are more ordinary where we don't behave in the best way that we can while we're driving. How many more risks are taken when the camera isn't there as opposed to the camera being there? How much attention is a person in that experiment really paying to the job of driving as opposed to the ordinary attention that you and I would pay when we're driving? Is that a factor in these experiments?

DR. WEIR (General Motors Technical Center): That certainly deserves consideration. We cannot experimentally control such factors as attention and desire to drive properly or your behavior at Irvine on a Saturday night after a party. During recess, one of the participants stated that when he drinks at lunch and drives in the afternoon, it's a much different situation than when he drinks again after supper and then tries to drive home after that. We have several situations involved. We can only get these kinds of data by instrumenting the subjects as we did and we had to use fairly well-trained subjects to get reproducible data. These subjects, all of them, were exposed in an experimental system at least 6 times in addition to the training runs. They were fairly routine experiments for them in the series.

DR. MC GAUGH: I can understand that. Obviously, these are difficult and time-consuming experiments, but I wonder, nonetheless, if that doesn't really seriously limit the generalization and not really lay the ghost. If the circumstances were changed ever so slightly, isn't it possible where the subjects aren't being monitored, aren't being watched, isn't it possible that they would keep their eyes closed for a longer period of time, for example, if they didn't have somebody in the back seat watching their every movement?

DR. WEIR: Well, then you would never have the contribution of any other factor. We were only trying to assess the one condition and if you change any factor in an experiment, then you can't extrapolate this to any other experiment aside from one with the particular conditions that you have.

DR. MC GAUGH: I'm not concerned about extrapolating to another experiment.

DR. WEIR: If you add another factor, then it's a different experiment. I'm trying to say that the physiology under those circumstances rules out the contribution of alcohol or alcohol and carbon monoxide.

DR. MC GAUGH: And thus seriously limits the generalization and keeps the ghost around for a while.

DR. WEIR: I don't agree.

MR. WANDS: I wanted to comment on the basic issue that's been raised here of motivation as it pertains to any behavioral toxicology experiment whether it's with humans or with rodents, or the monkey, or any of our other behavioral tests. This is a basic problem for all behavioral response studies and one way in which this can be overridden is to superimpose upon the basic experiment, such as Dr. Weir has described for his studies, a stress situation where you give the individual 2 or 3 other tasks to perform to distract his attention from the basic one. Under those kinds of stress conditions, his performance of a critical test will begin to drop off depending upon the degree of stress and also the degree of interference with the chemical toxicant under study. These have been particularly well shown with studies on alcohol and its effects on driving or alcohol and its effects on star tracing. This is really the answer.

DR. MC GAUGH: I fully believe the results of this experiment. There's no question about that, but I have an equal amount of disbelief that the generalizations apply to any circumstances in which this thing has changed. For example, what would happen if a diesel truck pulled out in front of a car? Would this same ability to maintain high driving performance continue at a moment of critical stress? I simply don't know from the data. Now it doesn't mean that your conclusions are necessarily wrong. The subject may perform equally well under that degree of stress, but I'm saying that your circumstances are so contrived in the restricted sense and there's such pressure put on the subjects to behave properly that it seems unlikely that they are going to perform badly under routine circumstances in the same sense that it is highly unlikely that most of us are going to fail the driving examination because we'll behave perfectly, both hands on the wheel, and so on. So, all I'm questioning is the generalizability of the results, not the accuracy of the experimentation as you reported it.

DR. TERRILL: I would like to ask Dr. Weir about higher carboxyhemoglobin levels and possibly higher blood alcohol levels. This pertains to a question that concerns esc pe of possible fire victims and the role of blood alcohol. I'm interested in learning about the possible synergism of these two in terms of people's ability to reason under duress and under extreme stress conditions. These would not be normal highway driving conditions.

DR. WEIR: Our research would not get into that area because when we were doing 20% carboxyhemoglobin levels, we were not authorized to test alcohol levels that high. When we were doing driving experiments, we were not permitted to investigate concentrations of alcohol higher than .05 mg % on the highway. So under those circumstances, I can't answer that question. I'm trying to make the point that at threshold levels of alcohol effect, you do not see incremental changes due to increases in carboxyhemoglobin levels up to 12% of such as you might expect to find in some of the people in this room or in an automobile. I don't know how far these data can be extrapolated. It is apparent, however, that these data and the data that I've not yet completely analyzed suggest that carbon monoxide is not acting as a classical CNS depressant. It does not produce the effects that a classical CNS depressant gives when you increase the dose. Alcohol and CO are not, in our experiment, acting additively or, worse than that, synergistically.

DR. BACK: I want to make a statement about some research that Dr. Weir reported on in New York. At the high level of CO, of about 20% carboxyhemoglobin saturation, he also didn't see any driver error. So obviously, CO is not a factor in these experiments. Probably the factor that would cause any changes at all is the alcohol.

DR. STOKINGER: In regard to the possible interaction of alcohol and CO, there was a recent international behavioral toxicology conference held in Cincinnati a few months ago. It was the consensus of opinion of a number of speakers that the effect on motor activity of small amounts of alcohol, such as was used in these experiments, facilitated movement, not depressed it, and it only affected cerebral activity in a depressant way.

SESSION IV

TOXICOLOGY OF ROCKET FUELS

Chairman

James D. MacEwen, Ph.D.
Laboratory Director
Toxic Hazards Research Unit
University of California, Irvine
Dayton, Ohio

AMRL-TR-73-125

PAPER NO. 24

BEHAVIORAL METHODS FOR TOXICITY ASSESSMENT

James L. McGaugh, Ph.D.

University of California, Irvine
Irvine, California

Manuscript was not available for publication.

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AMRL-TR-73-125

PAPER NO. 25

CHRONIC INHALATION TOXICITY OF HYDRAZINE

Charles C. Haun
Edwin R. Kinkead

University of California, Irvine
Toxic Hazards Research Unit
Dayton, Ohio

INTRODUCTION

Because of their high reactivity, hydrazine, 1,2-dimethylhydrazine, 1,1-dimethylhydrazine, and monomethylhydrazine are used extensively as rocket fuels. Invariably because of their high energy properties, all of these compounds are quite toxic. Previous studies in our laboratory reported at this conference this year (1973) and in the last few years (1970, 1971) have defined rather well the chronic toxicity of monomethylhydrazine, which is the most toxic of this group of compounds. Because of increased production, military and commercial use of hydrazine with the associated health hazards, it was our task in this last year to study the long-term inhalation effects of this compound in order to evaluate the current industrial threshold limit value (TLV) of 1 ppm.

Clark et al. (1968) provided a detailed review of the toxicology and pharmacology of propellant hydrazines. Hydrazine is a strong convulsant at high doses but may cause central nervous system depression at lower doses. Animals may die acutely of convulsions, respiratory arrest, or cardiovascular collapse within a few hours of an acute exposure by any route of administration, or may die 2 to 4 days later of liver and kidney toxicity (Weir et al., 1964; Witkin 1956). Jacobson et al. (1955) reported the 4-hour inhalation LC_{50} value as 252 ppm (330 mg/m^3) for the mouse and 570 ppm (750 mg/m^3) for the rat. House (1964) exposed monkeys, rats and mice to a hydrazine concentration of 1.0 ppm continuously for 90 days. Though mortality was very high, some animals survived the experiment. Ninety-six percent of the rats and 98% of the mice died during the exposure while monkeys proved to be the most resistant species with only a 20% mortality. Comstock et al. (1954) exposed dogs, in separate experiments, to 5 and 14 ppm. Two dogs survived the repeated 6-hour exposures to 5 ppm hydrazine for 6 months, and 2 of 4 dogs lived after 194 6-hour exposures to 14 ppm. Two of four dogs died during the third and

fifteenth weeks in a debilitated condition. The dog that died during the fifteenth week had a severe convulsive seizure prior to death. Prior to death, both dogs showed signs of anorexia and general fatigue. Changing diets and forced feedings resulted in the survival of the remaining two dogs.

METHODS

In the study reported here, four concentration levels were selected to compare the effects on 4 animal species of repeated 6 hours per day, 5 days per week (industrial) exposures with continuous exposures of equivalent concentration. Table 1 shows the inhalation doses, type of exposure, and CT concentration per week. It can be seen that the first two exposures, the 1 ppm continuous and the 5 ppm intermittent, were very comparable with regard to CT's. Likewise, the weekly concentrations for the exposures conducted at the TLV level, 1 ppm intermittent and 0.2 ppm continuous, are equivalent to each other. The higher concentrations are 5 times the TLV doses.

TABLE 1. HYDRAZINE WEEKLY INHALATION DOSES

N_2H_4 Exposure	Condition	CT (Concentration x Time) ppm Hours/Week
1.0 ppm	Continuous	168
5.0 ppm	Intermittent	150
1.0 ppm	Intermittent	30
0.2 ppm	Continuous	33.6

The four exposed groups and a control group consisted initially of 8 male beagle dogs, 4 female rhesus monkeys, 50 male Sprague-Dawley rats, and 40 female ICR mice. This study was conducted for 6 months in the Thomas Domes. Temperature and RH conditions are automatically controlled. The chambers were operated at a slightly reduced pressure (725 mm Hg) to prevent any leakage of hydrazine into the laboratory room air. Nominal airflows of 40 CFM were used in all cases.

During daily maintenance of the domes, unused food was discarded and replaced with fresh supplies. Water was provided by lick actuated valves to prevent any possible hydrazine contamination.

Anhydrous hydrazine, 97% pure, was introduced through a stainless steel needle into a heated air stream by means of a single syringe feeder. Concentrations could be adjusted by either changing the speed of the syringe feeder or by adjusting the air flows through the domes or both. The concentration in each dome was monitored using an AutoAnalyzer® as described for MMH analysis (Geiger, 1967). The 2 continuous domes and the control dome were sampled for 40-minute periods in succession. The intermittent domes were each sampled for 30 minutes of each hour. The control dome was sampled during each cycle for baseline values since the baseline changed slightly with the buildup of animal waste in the dome.

Large numbers of measurements were made to determine the toxicity of hydrazine in this study. Signs of toxic stress were noted as well as mortality and times to death. Body weights of dogs, monkeys, and rats were taken on a biweekly schedule throughout the duration of the study, and post-exposure in some cases. Pathologic examinations were made on animals that died or were sacrificed during exposure, and on animals that were killed at termination and shortly thereafter. Organ weights were taken at sacrifice on dogs, monkeys, and rats.

Myeloid to erythroid ratios were obtained from bone marrow samples taken from 5 rats/group removed and sacrificed after 8, 16, and 26 weeks of exposure. Blood samples, by means of cardiac puncture, were also taken from these rats for HCT, HGB and RBC determinations. Bone marrow was sampled from 4 dogs per group at the end of the study.

Table 2 shows the various clinical tests performed on blood samples taken biweekly from dogs and monkeys during the course of the study. Additional tests included methemoglobin determinations, microscopic examination of erythrocytes for possible Heinz body formation, and RBC osmotic fragility measurements. These tests were made on all monkeys and 4 dogs per group prior to the start of the study, biweekly during the first month, and then monthly thereafter.

TABLE 2. CLINICAL TESTS PERFORMED ON HYDRAZINE EXPOSED AND CONTROL DOGS AND MONKEYS

Chemistry		Hematology
Sodium	BUN	Hematocrit
Potassium	Glucose	Hemoglobin
Cholesterol	Alkaline Phosphatase	RBC
Calcium	SGOT	WBC
Inorganic Phosphorus	SGPT	Differential
Total Bilirubin	Creatinine	Reticulocyte
Albumin/Globulin	Chloride	
Total Protein	Triglycerides	

Ten rats and 10 mice from each group were retained postexposure for lifetime observation of any possible carcinogenic effects. To determine recovery of blood parameters, 2 dogs each from the high concentration exposures were maintained for 6 weeks postexposure. Two dogs from the control group were also retained for comparison.

RESULTS

Signs of Toxicity

During the first few weeks of the study, and periodically thereafter, minimal eye irritation was observed in monkeys exposed to the two highest dose levels, that is the 1 ppm continuous and the 5 ppm intermittent exposures. During the first few weeks of exposure to these concentrations, the mice in these groups were obviously ill. They were lethargic and their fur was rough and yellowed. The most significant or noticeable signs of stress occurred in the case of the dogs exposed to 1 ppm continuously. Weight loss was very noticeable in these dogs, and although we did not measure food consumption, it was obviously reduced. Anorexia continued with progressive emaciation until about 16 weeks when some recovery occurred in the surviving dogs. One dog in this group experienced tonic convulsions on 3 separate occasions, once after 3 months of exposure, then once in the morning and once in the afternoon of the same day after 5 months of exposure.

Body Weights

Mice were not weighed and exposed monkey weights showed no significant changes when compared to control values. However, dog and rat weights were affected by hydrazine exposure.

Dog mean body weights are shown in Figure 1. The weights of the dogs exposed to the 2 lowest concentrations, the TLV levels, were not affected by hydrazine exposure. Their weights parallel those of the control group very closely throughout the study as can be seen in the top 3 curves in this graph. However, actual weight losses occurred in dogs exposed to the two highest concentrations. These changes are reflected in the bottom 2 plots. A maximum loss of about 3/4 of a kilogram for the 5 ppm intermittently exposed dogs is seen at 4 weeks, with recovery occurring thereafter. A 2 kilogram mean loss occurred at 6 weeks for dogs exposed to 1 ppm continuously, with a sharp recovery at 8 weeks, then a steady loss to 16 weeks. The weight loss between 8 and 16 weeks was due largely to the influence on the mean values of the severe weight loss of 2 dogs that died within a day or two after the 16 week weighings.

Rather sharp gains are noticeable for this group between 16 and 26 weeks, a definite trend to almost complete weight recovery as occurred in the dogs exposed to 5 ppm intermittently by the end of the study. Complete weight recovery occurred at 2 weeks postexposure for 2 dogs from each high concentration group that were retained for postexposure observation.

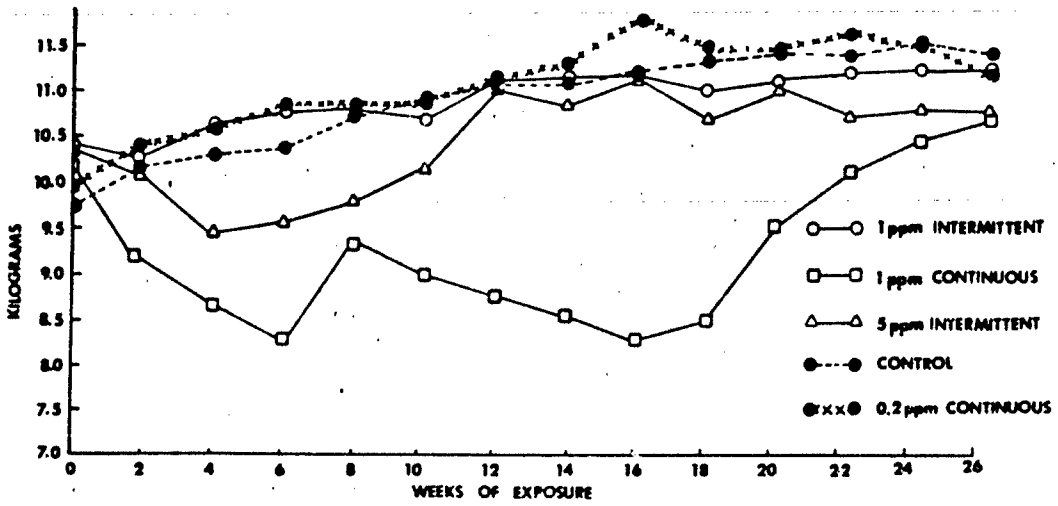

Figure 1. Mean Weight Changes in Dogs Exposed to Hydrazine.

Growth rates for rats are in Figure 2. It is important to list a few details in regard to this information:

1. There was a weight suppression in all exposed groups from the beginning to the end except for the 0.2 ppm continuous exposed rats whose mean weights were not statistically different from the controls after 10 weeks of exposure.

2. A reasonably clear dose response does exist.

3. The 2 highest concentrations produced the larger weight depression.

4. At 26 weeks, the end of the exposure, the largest weight difference is between the 5 ppm intermittent group and the control. This difference is approximately 35 grams.

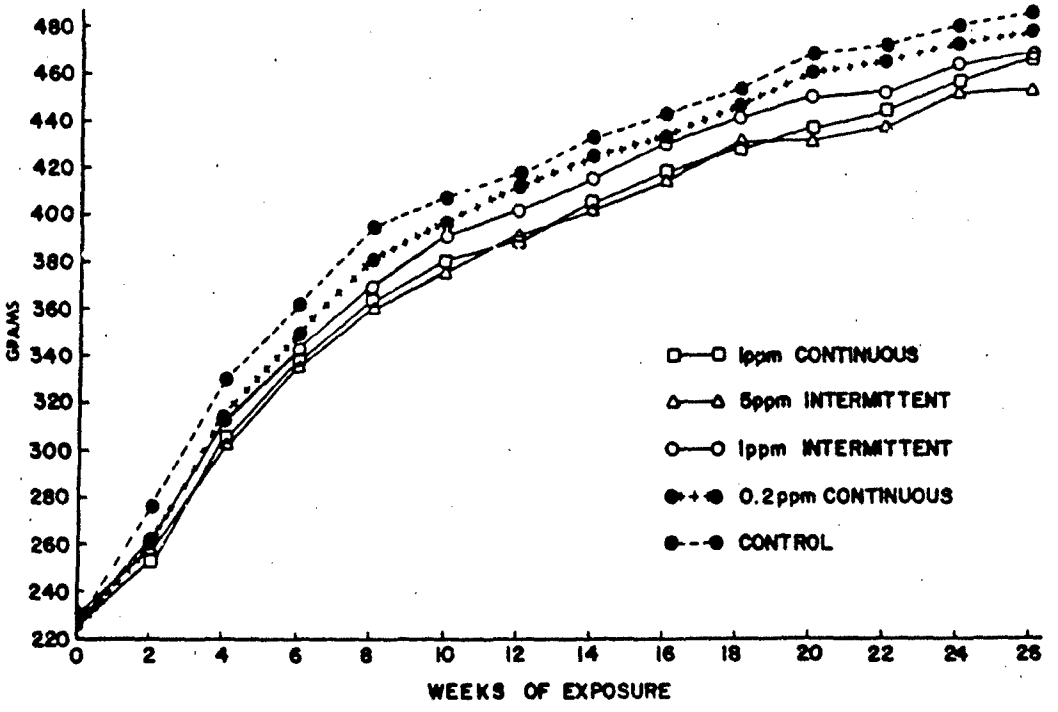

Figure 2. Mean Weight Changes in Rats Exposed to Hydrazine.

Mean weights of groups of exposed rats held over for observation were equivalent to the control weights by 2 weeks postexposure, except for rats exposed to 5 ppm intermittent. More will be discussed on this subject later in this report.

Mortality

There were no monkey deaths in any group, and only one rat death in the 0.2 ppm continuous exposure. This animal died during the first week of exposure but cause of death was not attributed to hydrazine toxicity. In the case of the mice, there is a far different picture, as seen in Table 3. This species is obviously susceptible to the lethal effects of hydrazine. The time to death pattern is interesting. The vast majority of the deaths occurred actually in the first 2-2 1/2 weeks of exposure regardless of concentration. This suggests an induction of tolerance to hydrazine. The results in the percent mortality column in the table reveal again, as mentioned previously, a direct relationship to hydrazine dose received.

TABLE 3. EFFECT OF HYDRAZINE EXPOSURE ON MOUSE MORTALITY (N = 40/GROUP)

N_2H_4 Exposure	Condition	Months of Exposure 1	2	3	4	5	6	Total Mortality	Percent Mortality
1.0 ppm	Continuous	16	0	0	1	0	5	22	55
5.0 ppm	Intermittent	13	1	0	0	0	0	14	35
1.0 ppm	Intermittent	3	0	0	0	0	0	3	7.5
0.2 ppm	Continuous	1	0	0	0	0	0	1	2.5
Control		0	0	0	0	0	0	0	0

Two of 8 dogs exposed to 1 ppm continuously died within a day or two after 16 weeks of exposure. The death of these dogs was not unexpected. Progressive deterioration was obvious during an 8-week period prior to death. There was no other dog mortality.

Clinical

In regard to the blood measurements, there were no abnormal changes in blood samples taken from the serially sacrificed rats during exposure or immediately after exposure. The m/e ratios from bone marrow samples were also completely normal. Monkey blood values were normal throughout the duration of the study.

However, as shown in Figures 3, 4, and 5, the hematocrit, hemoglobin and red blood cell values for dogs exposed to the 2 highest concentrations were definitely depressed during the course of the 6-month exposure to hydrazine. Figure 3 shows the mean HCT values for all dog groups. At 8 weeks, the values for both of the high dose groups are 11% below their respective baseline values. From 8 weeks forward in the case of the dogs exposed to 5 ppm, there is a recovery by 18 weeks followed by a decline to about a 40% HCT value at the last exposure sampling period. Values for dogs exposed to 1 ppm continuously show similar changes. The mean value, a sharp recovery at 18 weeks, is mostly due to absence of the blood values of the 2 dogs that died shortly after the 16-week blood samples were taken. However, a 20% reduction followed at 18 weeks, then a gradual trend to recovery by the end of the study. Most important here is the fact that the HCT values for the dogs exposed to the TLV concentrations, the low doses, were unchanged with respect to their preexposure baseline values.

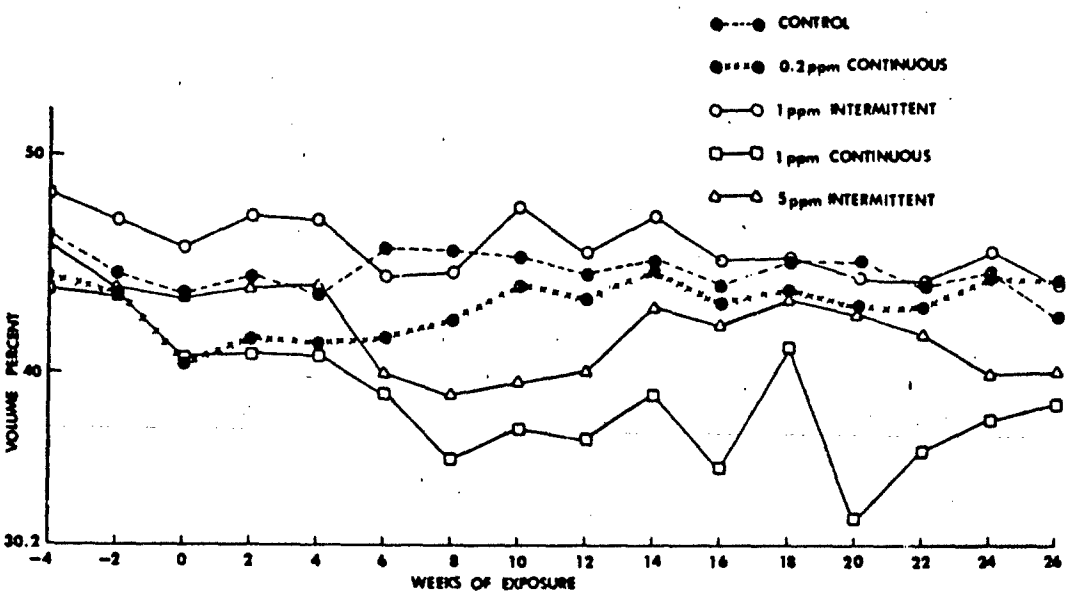

Figure 3. Effect of Hydrazine Exposure on Hematocrit Values in Dogs.

Figure 4 shows the dog HGB values. It presents a picture very similar to the HCT results. Both groups of dogs exposed to the highest hydrazine concentrations show definite hemolytic depressions, while those that received the TLV doses appear to be unaffected.

Figure 5 shows the red blood cell counts. The time and effect pattern is very similar to that seen in graphs of HCT and HGB values. RBC counts were depressed approximately 10% and 12% at 6 and 8 weeks for the dogs exposed to the two highest concentrations. Beyond these times, recovery occurred for the 5 ppm intermittently exposed dogs. In the case of the dogs exposed to 1 ppm continuously, a trend to recovery in the last 6 weeks of exposure was preceded by a sharp abnormal reduction in RBC's at 20 weeks. At that time, 20 weeks, reticulocyte counts were higher than normal for this group of dogs. This is the only time reticulocytosis occurred.

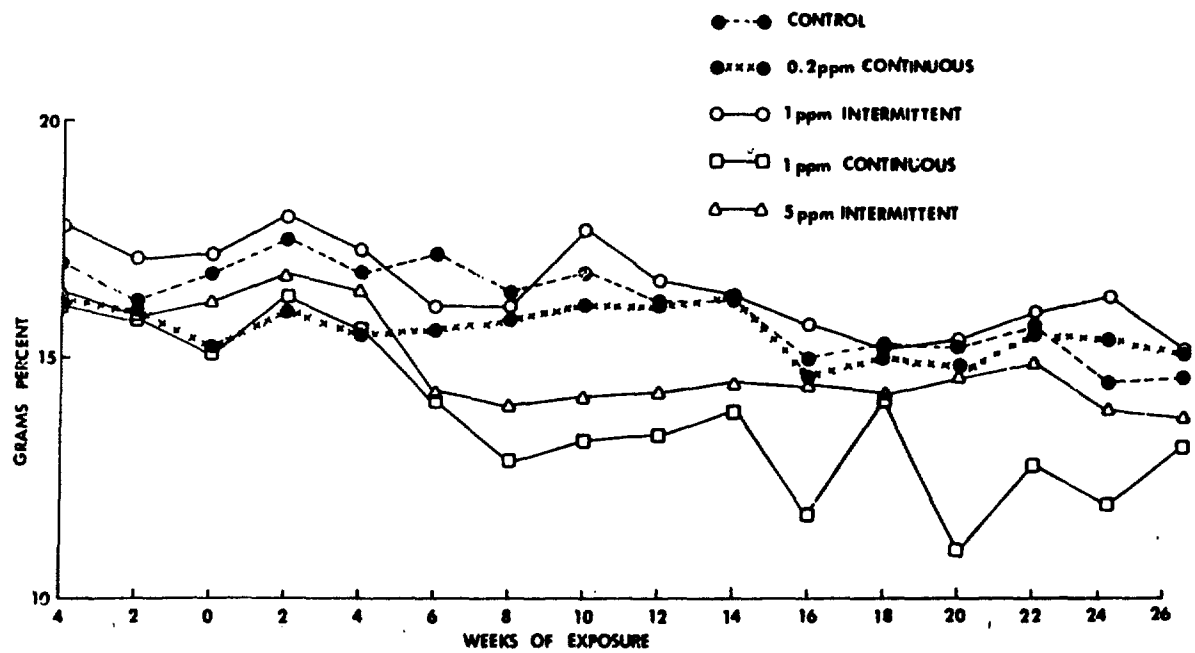

Figure 4. Effect of Hydrazine Exposure on Hemoglobin Values in Dogs.

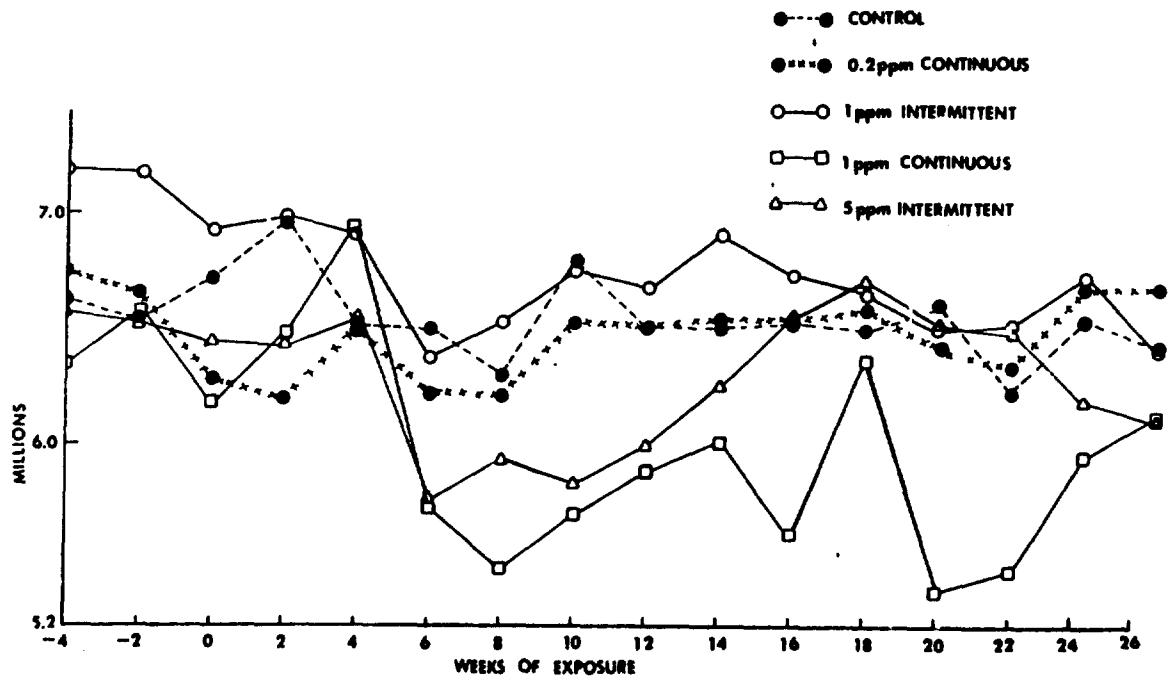

Figure 5. Effect of Hydrazine Exposure on Red Blood Cell Counts in Dogs.

At first glance, it was a little surprising that reticulocytosis did not occur earlier for both groups of dogs exposed to the highest concentrations; however, in consideration of the percentage depressions in all hematology measurements, particularly the RBC information, the lack of reticulocyte production was not an abnormal phenomenon. Some erythropoietic activity, however, did occur in the bone marrow of dogs exposed to 1 ppm continuously. Samples taken from 4 dogs at sacrifice showed slightly decreased m/e ratios. All in all, the during exposure hematologic effects from hydrazine inhalation by dogs exposed to the 2 highest doses can only be described as mild, perhaps moderate, but certainly real. However, hemoglobin, RBC, and HCT values returned to control levels within 2 weeks postexposure in 2 dogs from each of the high concentration groups that were held over for observation.

No measurable blood cell destruction occurred in dogs exposed to the TLV concentrations. Examination of clinical chemistry data, Heinz body counts, and methemoglobin values for all dogs and monkeys showed no significant differences between exposed and control groups.

The effect of hydrazine exposure on red blood cell fragility is interesting. The dog erythrocytes sampled after 2 and 4 weeks exposure and tested for osmotic fragility showed no abnormalities. At 8 weeks, there was some increased resistance to osmotic hemolysis in cells from dogs exposed to the 2 highest doses. Time and dose dependent effect appeared in that red cells from dogs exposed to the TLV concentrations showed similar resistance at 12 weeks. In both cases, this effect persisted to the end of the study. Two and 6 week postexposure blood samples from high concentration exposure dogs showed continuation of this resistance to osmotic pressure. Figure 6 shows an example of this effect. It presents percent hemolysis caused by various percent salt solutions for 2 groups of dogs at 16 weeks. The lower curve is for dogs exposed to 1 ppm continuously while the upper curve is for controls. At 0.4% salt solution, the RBC's of the control group show 50% hemolysis, while the exposed show only 10% hemolysis.

Pathology

The results of gross and histopathologic examination of mice that died during exposure showed that death was probably due to hydrazine hepatotoxicity, fatty livers. At sacrifice, moderate to severe fatty liver change was a consistent finding in mice from all exposure levels. Monkey livers showed slight to moderate fat accumulation. Perhaps compromising part of this information is the fact that control monkeys also showed some degree of fatty liver change. Malnutrition, the result of nonspecific hydrazine toxicity, caused the death of 2 dogs in the 1 ppm continuous exposure. At sacrifice, dogs exposed to the TLV concentration showed no abnormalities but dogs

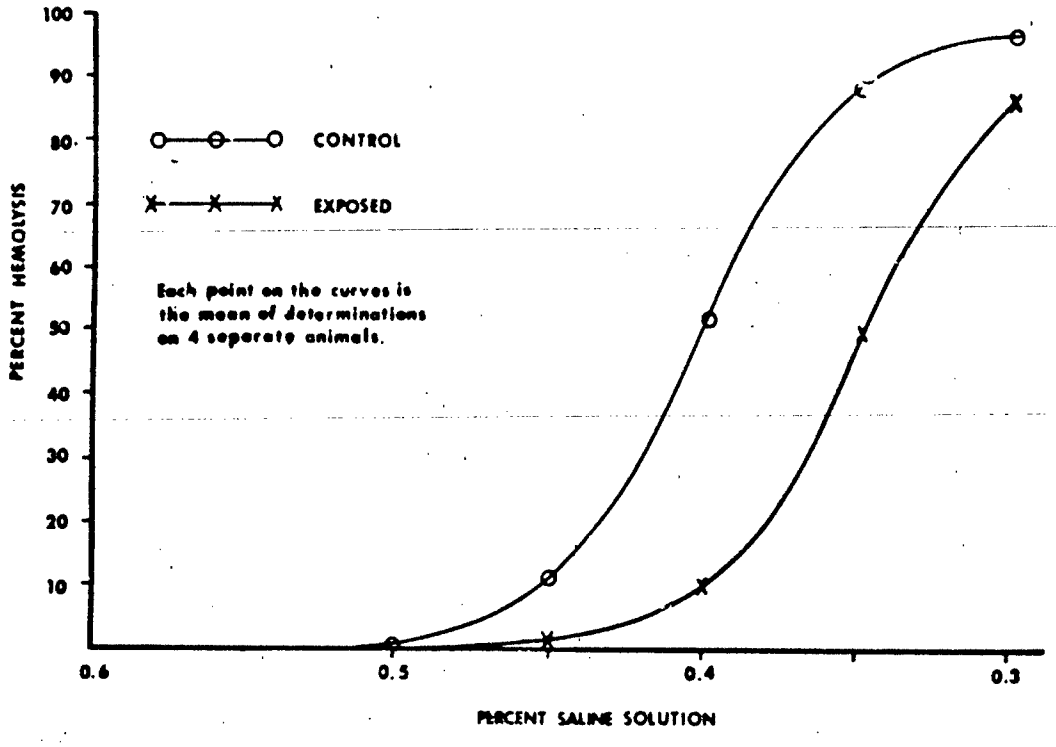

Figure 6. Sixteen Week Osmotic Fragility Values of Red Blood Cells from Dogs Exposed Continuously to 1 ppm Hydrazine.

from the high doses had fatty livers. Because one dog in the 1 ppm continuous exposure group convulsed during exposure, the brain of this dog and of 3 others in the same group were perfused at sacrifice. Histology examination revealed no CNS lesions. Two dogs each from the high concentration experiments were sacrificed at 6 weeks postexposure. All were described as being essentially normal animals.

There were no significant pathologic changes in rats except in the case of the 5 ppm intermittent exposure group. Of the 30 rats, 19 had chronic bronchopneumonia. Whether this condition was due to a hydrazine pulmonary irritation or pathogens present, or the former predisposing the rats to the latter, is difficult to say. The net effect, however, was that 10 rats from this group retained postexposure showed no weight recovery as demonstrated by the other exposed groups. Consequently, the infection, we reasoned, spread to all others during storage in the same space, and between 6-8 weeks following exposure termination 50% of the rats were dead. The number of deaths were distributed rather evenly in the exposed groups and in the controls as well.

Organ weights of exposed rats, monkeys, and dogs were not statistically different from control values. In the case of the rats, the depressed growth rates resulted in increased organ to body weight ratios to which no biological significance can be attributed.

In conclusion, the results of this study have described the toxic effects of hydrazine inhalation, particularly in regard to hematologic changes in dogs exposed to the highest concentrations used in this study. Unexplained, however, is the mechanism of RBC destruction. There is no firm answer at this time. Further experiments are planned to answer this question.

Most important, however, in regard to the safety factor in the current TLV, in the low dose levels tested, it should not be overlooked that hydrazine did kill mice, however few, and rather early in the intermittent and continuous experiments. Some degree of fatty liver change occurred in mice and monkeys as well.

Extrapolation of toxicity information from animal to man is difficult, especially when considering toxicants of high biological activity such as hydrazine. If man is measurably less sensitive than the mouse to the harmful effects of hydrazine, there is no problem. If he is not, this suggests that the 1 ppm TLV in itself is not safe.

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PAPER NO. 26

TUMOR INDUCTION STUDIES WITH SUBSTITUTED HYDRAZINES

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INTRODUCTION

The antituberculotic drug, isonicotinic acid hydrazide, is claimed to be metabolized in man and in the various animal species into a number of compounds such as isonicotinic acid, 1-acetyl-2-isonicotinoylhydrazine, n-methylisonicotinic hydrazide, isonicotinamide and hydrazine. In view of the fact that the derivatives of hydrazine are used in industry, agriculture, and even in medicine, we have begun investigating this class of chemicals to answer the following questions: a) Is there a relationship between the chemical structures of substituted hydrazines and tumor development at specific organ sites? b) Is the neoplastic reaction species dependent? c) Do these chemicals represent major environmental hazards? d) And finally, what are the mechanisms of action of these chemicals?

Prior to our investigation, the various hydrazines were already used in chemical carcinogenesis studies. The tumor inducing capability of hydrazine was shown originally in Italy (Biancifiori and Ribacchi, 1962). The methylhydrazine was studied for cancer producing effect by a number of investigators; their results were, however, negative (Roe et al., 1967; Kelly et al., 1969). The symmetrical dimethylhydrazine was shown to induce intestinal and liver tumors in mice, hamsters, and rats (Wiebecke et al., 1969; Osswald and Kruger, 1969; Druckrey et al., 1967), while the unsymmetrical dimethylhydrazine first was claimed to be a non-tumorigenic compound; later on, however, the same group of workers classified it as a cancer producing substance (Druckrey et al., 1961; Druckrey et al., 1967). Phenyl- and benzoyl-hydrazines were shown to be lung tumor inducing chemicals in mice (Clayson et al., 1966). Also, procarbazine elicited the development of breast and lung tumors in rats (Kelly et al., 1968), while 1, 2-diethylhydrazine gave rise to a variety of tumors such as gliomas, leukemias, liver cell carcinomas, etc. in rats (Druckrey et al., 1966).

MATERIALS AND METHODS

The experiments were conducted with randomly bred Swiss albino mice and randomly bred Syrian golden hamsters of this Institute. All animals were housed in plastic cages with granular cellulose bedding in groups of 10 (mice) and 5 (hamsters), separated according to sex, and given Wayne Lab Blox regular diet (Allied Mills, Inc., Chicago, Illinois) and tap water containing the chemicals ad libitum.

Toxicity Studies

With each compound toxicity studies were performed in both species. The chemicals were administered in the drinking water ad libitum at five dose levels for 35 days to five groups of animals each consisting of four females and four males and the controls. At the end, four parameters were taken into account: body weights, survival rates, chemical consumption figures and histological changes. In this way, the maximum tolerated dose was selected which was given for life. This toxicity method was developed in our laboratory recently (Toth, 1972).

Chronic Studies

The long-term tumorigenesis studies were done in two species, Swiss mice and Syrian golden hamsters. Each consisted of one hundred animals, fifty females and fifty males. The chemicals were given at the maximum tolerated dose orally in the drinking water daily ad libitum for life. The solutions were prepared three times weekly and the total water consumptions containing the chemicals were measured at the same intervals. All solutions were contained in brown bottles because of the possible light sensitivity of the chemicals.

In the long-term studies the following concentrations of chemicals were used: hydrazine, 0.001%; methylhydrazine (sulfate), 0.001% (mice), 0.01% (hamsters); 1, 2-dimethylhydrazine di HCl, 0.001% (mice and hamsters); 1, 1-dimethylhydrazine, 0.01%; phenylhydrazine HCl, 0.01% (mice); benzoylhydrazine, 0.01% (mice); ethylhydrazine HCl, 0.0125% (mice); 1-carbamyl-2-phenylhydrazine, 0.25% (mice); 2-hydroxyethylhydrazine, 0.015% (mice and hamsters). In addition, the following derivatives are under study: Benzyl-, n butylcarbamyl-, 1-carbamyl-2-phenyl-, n propyl-, n amyl-, n allylhydrazines, hydrazinophthalazine, β-phenylethyl-, trimethyl-, and 1, 1-diethyl-hydrazines.

The experimental animals were carefully checked and weighed at weekly intervals, and the gross observable changes were recorded.

For light microscopic examination the animals were either allowed to die spontaneously or killed with ether when they were found to be in poor condition. Complete necropsies were performed on all animals. All organs were examined macroscopically and were fixed in 10% buffered formalin. Histological studies were done on liver, spleen, kidney, bladder, thyroid, heart, pancreas, testis, brain, nasal turbinale and at least four lobes of the lungs of each animal as well as on those organs showing gross pathological changes. Sections from these tissues were stained routinely with hematoxylin and eosin. In addition, special stains were used when required and also certain tissues and tumors were studied with transmission and scanning electron microscopes.

RESULTS

The findings of tumorigenesis studies can be summarized as follows:

Tumor Inductions in Mice

In the hydrazine-treated, the lung tumor incidence was 51% (Toth, 1972); in the methylhydrazine sulfate-treated, the lung tumor incidence was 46% (Toth, 1972); in the symmetrical dimethylhydrazine di HCl-treated, the lung tumor incidence was 34%, and the blood vessel tumor incidence was 95% (Toth and Wilson, 1971); in the unsymmetrical dimethylhydrazine-treated, the lung tumor incidence was 71%, the blood vessel tumor incidence was 79%, the kidney tumor incidence was 10% and the liver tumor incidence was 6% (Toth, 1973); in the benzoylhydrazine-treated the lung tumor incidence was 65% and the malignant lymphoma incidence was 23% (Toth, 1972); in the ethylhydrazine HCl-treated, the lung tumor incidence was 75% and the blood vessel tumor incidence was 38% (Shimizu and Toth, 1973); in the 1-carbamyl-2-phenylhydrazine-treated, the lung tumor incidence was 73% (Toth and Shimizu, 1973).

In the untreated controls the types and incidences of tumors were: lungs, 11%; malignant lymphomas, 9%; blood vessels, 2%; kidneys and liver, 9%.

Tumor Incidences in Hamsters

In the methylhydrazine-treated, the malignant histiocytoma incidence was 43% and the cecal tumor incidence was 16% (Toth and Shimizu, 1973); in the symmetrical dimethylhydrazine-treated, the blood vessel tumor incidence was 85%, the cecal tumor incidence was 23% and the liver tumor incidence was 8% (Toth, 1972; Toth, 1972); in the 2-hydroxyethylhydrazine-treated, the liver tumor incidence was 8% (Shimizu and Toth, 1974).

In the untreated controls the types and incidences of tumors were: blood vessel tumors, 1%; liver, cecum and malignant histiocytoma incidences, 0%.

DISCUSSION

Our findings clearly show that seven chemicals including hydrazine, monomethyl-, the symmetrical and unsymmetrical dimethyl-, benzoyl-, ethyl-, 1-carbamyl-2-phenyl-hydrazines produced lung tumors in mice. In addition, the symmetrical dimethyl-, and ethyl-hydrazines evoked blood vessel tumors, the unsymmetrical dimethylhydrazine produced tumors of blood vessels, kidneys and livers, the benzoylhydrazine elicited the development of malignant lymphomas, while phenylhydrazine only induced blood vessel tumors. Strangely enough, the administration of 2-hydroxyethylhydrazine resulted in no detectable tumorigenic effect. In hamsters, monomethylhydrazine induced tumors of Kupffer cells and cecum, while the symmetrical dimethylhydrazine induced tumors of blood vessel, cecum and liver. 2-Hydroxyethylhydrazine had no tumorigenic effect in this species. As a matter of fact, it produced a few benign hepatomas but according to the statistical analysis they did not occur in significant incidences. Altogether it appears that the various derivatives had some sort of organ specific activity. Also, their tumorigenic effect to a certain extent is species dependent.

With regard to some of the usages of substituted hydrazines the following should be kept in mind. Hydrazine, monomethyl- and 1, 1-dimethylhydrazines are used in rocket fuel. The ethyl- and carbamyl-hydrazines as corrosive agents, while the benzoylhydrazine in thermoplastic molding process and the 2-hydroxyethylhydrazine in aluminum coating industry. In medicine, four substituents are known. Phenylhydrazine is used for polycythemia vera, 1-hydrazinophthalazine for hypertension and β-phenylethylhydrazine is used as an antidepressant. 1-Carbamyl-2-phenylhydrazine is known for antipyretic action, apparently, presently is not used in USA. In agriculture, two compounds are known. Hydrazine is indirectly involved since it is hydrolyzed from maleic hydrazide, a well-known herbicide. The other one is 2-hydroxyethylhydrazine which is used extensively in Hawaii as a pineapple flowering agent.

As far as the mechanism of action of hydrazines in tumorigenesis are concerned a few experiments which are underway may deserve mentioning. The first group consists of studies in which the early cellular and subcellular damage caused by the chemicals are investigated. In an earlier experiment we demonstrated cell damage and sequence of changes in the vascular tissue and hepatocytes of Swiss mice treated with 1, 2-dimethylhydrazine di HCl. The obvious question of whether there is any relationship

between the damage of a particular cell type and the eventual tumor which develops from it, however, was left unanswered. Right now, we are extending our interest in this area and using other hydrazines also. The second group of experiments involve the attempts to inhibit the toxicity and tumorigenicity of hydrazines. We are trying to achieve this by pregnenolone-16-α-carbonitrile which is a powerful stimulator of hepatic microsomal enzymes. This steroid in earlier experiments inhibited the toxicity of 7, 12-dimethylbenz(a)anthracene and dimethylnitrosamine. Also, in another study we are trying to find out whether there is a relationship between the tumorigenic activities of substituted hydrazines and their ability to inhibit monoamine oxidase enzyme. Finally, a group of experiments are underway with the object being to localize tissue-bound C14-labelled 1, 2-dimethylhydrazine di HCl in the various organs, cells and cell organelles of Swiss mice at the light and transmission electron microscopic levels. In addition, the effects of hydrazines on DNA synthesis are under study by using tritiated thymidine incorporation.

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PAPER NO. 27

MONOMETHYLHYDRAZINE - CHRONIC LOW LEVEL EXPOSURES AND 24-HOUR EMERGENCY EXPOSURE LIMITS

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INTRODUCTION

Monomethylhydrazine (MMH) is a highly reactive chemical which has been used increasingly as a rocket fuel component during the last decade. The acute effects of exposure to MMH and its analogs hydrazine and unsymmetrical dimethylhydrazine (UDMH) have been well defined in the literature (Comstock et al., 1954; Shook and Cowart, 1957; Rinehart et al., 1960; Weeks et al., 1963; Jacobson et al., 1966; Haun et al., 1970). Signs of toxicity following acute inhalation of MMH were found to be well defined and dose related (Haun et al., 1970). These symptoms in rats, mice, dogs and monkeys were irritation, emesis, ataxia and convulsions with animals either dying within 24 hours postexposure or surviving and recovering from the effects. The MMH was also found to cause a dose related hemolytic anemia which was reversible within a few weeks postexposure. Serial sacrifice of surviving dogs and monkeys also showed some persistent renal damage.

Chronic exposures to MMH have been evaluated extensively in our laboratory during the past few years (see Table 1). These have included both continuous and intermittent exposures to rats, mice, dogs and monkeys for 6 months duration. The present levels (continuous exposure to 0.1 and 0.04 ppm MMH) have been included in this table to show where they fall in relation to the rest of the studies.

TABLE 1. SUMMARY OF MMH WEEKLY DOSE EQUIVALENTS FOR ALL CHRONIC EXPOSURES CONDUCTED AT THRU

Chamber Concentration (ppm)	Type of Exposure	Weekly Dose (ppm-hours)
0.2	Intermittent*	6
0.04	Continuous	6.72
0.1	Continuous	17
1.0	Intermittent*	30
0.2	Continuous	33.6
2.0	Intermittent*	60
5.0	Intermittent*	150

*6 hours/day - 5 days/week

The previous chronic exposures to MMH have been described by Haun (1970) and MacEwen and Haun (1971), to evaluate the degree of safety of the current industrial threshold limit (TLV) of 0.2 ppm which was established by analogy to hydrazine and UDMH chronic toxicity. Those exposures included 4 intermittent exposures (6 hours/day - 5 days/week) of rats, mice, dogs and monkeys to 0.2, 1.0, 2.0 and 5.0 ppm MMH, and a continuous exposure of the same species to 0.2 ppm MMH. All of these exposures lasted for 6 months.

The MMH-induced hemolytic anemia seen following acute exposures (Haun et al., 1970) was seen as a dose-related response in the intermittent exposures as well. The hemolytic response was evidenced by dose related increases in methemoglobin formation, and decreased hematocrits, hemoglobin and red blood cell counts, as well as increased reticulocytes, serum alkaline phosphatase, total phosphorus and serum bilirubin levels. The hemolytic response appeared to result from the reaction between MMH and hemoglobin, forming methemoglobin. Methemoglobin formed during acute exposures is rapidly converted back to oxyhemoglobin while under chronic exposure conditions; the methemoglobinemia reaches an equilibrium level unique to the exposure concentrations, and is accompanied by Heinz body formation. Further indications of MMH-induced changes were dose related depression of the ratio of myeloid to erythroid elements of bone marrow, and a shift toward increased initial hemolysis in the RBC fragility determinations. In none of the intermittent exposures was a no-effect level found.

The animals exposed to 0.2 ppm MMH continuously for 6 months showed toxic alterations and hemolytic changes comparable to those observed in the intermittent exposure to 1.0 ppm MMH. These two exposure conditions were approximately equivalent in terms of total weekly dose in parts per million hours.

In addition to the hematologic changes noted here, one of the major toxic effects caused by chronic exposure of dogs to MMH was the production of severe renal damage. As mentioned above, evidence of renal damage was seen in the dogs acutely exposed to MMH (Haun et al., 1970). In the intermittent exposures (MacEwen and Haun, 1971), dogs exhibited hematuria, hemoglobinuria, methemoglobinuria and cast formation following exposure to subconvulsive levels of MMH. Histopathological examination of the kidneys revealed proteinaceous precipitates in the proximal tubules with occasional hemoglobin casts, moderate to severe degeneration of the proximal tubules with actual tubular necrosis present in many cases (Sopher et al., 1967). Van Stee (1965) reported a decrease in tubular excretion in dogs following MMH exposure, with a decrease in the glomerular filtration rate, which he attributed to decrease in the renal plasma flow rate. Monkeys exposed to MMH showed no change in renal function, but subcellular morphologic kidney changes were found by George et al. (1968). Kroe (1971) found periportal choleostasis, bile duct proliferation and hemosiderosis in the livers and renal tubular hemosiderosis in the kidneys of mice and dogs following 6 months chronic exposure to MMH. These effects were not seen at exposure concentrations below 1 ppm MMH in air.

Since a true no-effect level had not been reached in these previous chronic studies, the need arose for additional information about the effect of continuous exposure to MMH in confined spaces in order to establish specific safety standards for these conditions. A portion of the research program described in this report was designed to extend the previous continuous exposure studies to a level of insignificant effect on red blood cells. For this purpose atmospheric concentrations of 0.1 and 0.04 ppm MMH were selected and groups of dogs, monkeys, and rats were continuously exposed for 90 days in Thomas Dome chambers. Control groups of unexposed animals were maintained under identical environmental conditions in another Thomas Dome.

In addition to the need for information on chronic exposures to low MMH levels mentioned above, potential applications for future use of MMH in confined working areas suggest the need for Emergency Exposure Limit (EEL) values for periods up to 24 hours in duration. Consequently, animal experiments using the methodology and techniques by MacEwen et al. (1969) were also conducted.

Since dogs have been shown to be the most MMH sensitive species, preliminary experiments utilized these animals. Continuous 24-hour MMH exposures were conducted with groups of two beagle dogs each at 1, 2, 5 and 10 ppm. These animals were maintained for 30 days postexposure for comparison with two unexposed control dogs. Exposure at 2 ppm MMH produced a 5-7% hemolysis of red blood cells through the methemoglobin-Heinz body transformation mechanism. This effect was followed by reticulocytosis which brought hemoglobin, RBC and hematocrit values back to preexposure levels within three weeks. The same effects were seen to a greater degree after 24-hour continuous exposure to 5 and 10 ppm MMH.

No measurable decrease in the hematologic measurements was seen in the dogs exposed under the same conditions to 1 ppm MMH. Consequently, the 1 ppm MMH concentration was selected for a more comprehensive EEL experiment.

MATERIALS AND METHODS

Chronic Exposures

For the chronic exposures, each experimental group of animals, including the control group, consisted of 8 female beagle dogs, 4 female rhesus monkeys, and 80 male albino rats (Sprague-Dawley strain CFE). All animals were fed ad libitum during exposure, with old food being removed and replaced with fresh food during the daily chamber cleaning period.

At 45 and 90 days of exposure, 30 rats from each group were used to obtain blood samples for hematology measurements via cardiac puncture. The remaining 20 in each group were used for gross pathology observations with tissue specimens being collected for histologic evaluation by others.

Based on the findings from previous MMH exposures, a battery of tests was selected for evaluation of the effects of continuous MMH exposure on dogs and monkeys as shown in Table 2.

TABLE 2. CLINICAL TEST SCHEDULE

	Weeks of Exposure							
Test	0	2	4	6	8	10	12	13
HCT	x	x	x	x	x	x	x	x
HGB	x	x	x	x	x	x	x	x
RBC	x	x	x	x	x	x	x	x
WBC	x	x	x	x	x	x	x	x
Total Inorg. Phosphorus	x	x	x	x	x	x	x	x
Alkaline Phosphatase	x	x	x	x	x	x	x	x
Reticulocytes	x	x	x	x	x	x	x	x
Heinz Bodies	x	x	x	x	x	x	x	x
Body Weights (rats also)	x	x	x	x	x	x	x	x
RBC Fragility (dogs only)								x
Gross & Histopathology								x

Hematocrits were determined by drawing an aliquot of blood into a micropipette and sealing one end. The micropipette was then spun on a microcentrifuge and the hematocrit value was read directly from standard sedimentation charts.

Hemoglobin determinations were made by diluting a measured quantity of blood with water on a Labtek® autodilution machine and then reading the hemoglobin content with a Coleman Junior II® spectrophotometer. Red and white blood cell counts were done with a Coulter Counter® . Slides were made and stained with brilliant cresyl blue and observed under a light microscope for reticulocyte and Heinz body counts. The two blood chemistry determinations were made using a Technicon AutoAnalyzer II® and appropriate reagents for each specific compound. Red blood cell fragility was determined by diluting a measured quantity of blood with graded concentrations of saline and reading the optical density with a spectrophotometer.

MMH Introduction

The MMH introduction system is shown schematically in Figure 1.

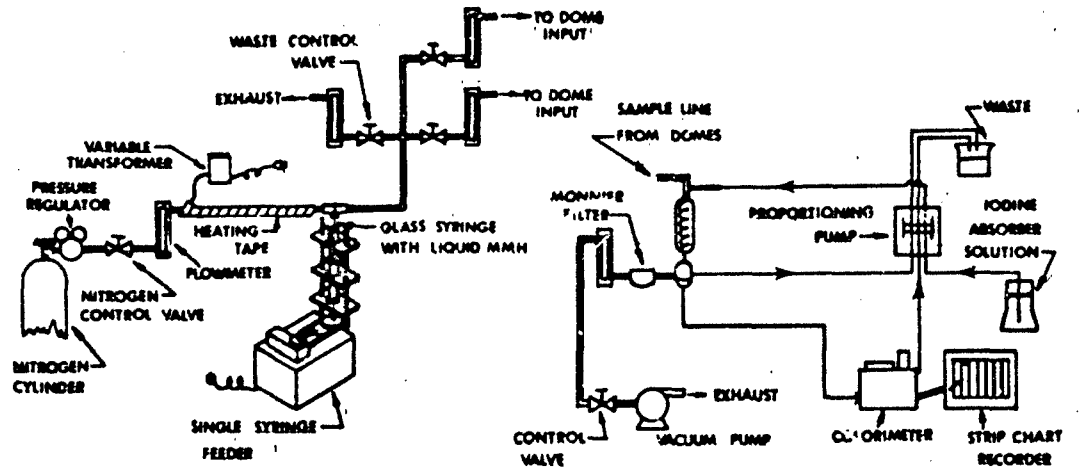

Figure 1. Schematic Diagram of MMH introduction and analytical systems.

Liquid MMH was expressed from a 20 ml glass syringe mounted on a single syringe feeder at a constant rate into a heated stainless steel tube containing a flow of 1 liter/minute dry nitrogen. The nitrogen stream with the MMH vapor was then split three ways, one for each of the two exposure domes, and one for venting the excess through a water scrubber. The nitrogen-MMH flow through each of these three paths was controlled by stainless steel valves and flowmeters. The contaminant flow lines then joined the chamber input air prior to entry in the domes. The syringe pump apparatus and all of the valves and flowmeters were housed in a fume hood for safety.

MMH Analysis

The MMH analysis used in this study was a modification of the method reported by Geiger and Vernot (1967). The analytical system is also shown schematically in Figure 1. A 2 liter/minute sample was drawn from the dome exhaust line through a teflon line to the top of a glass scrubber column which was filled with glass beads to increase the surface area for mixing the MMH vapor with the absorber solution. The absorber solution was prepared from distilled water by mixing 40 g/liter KI to prevent iodine volatilization, 20 g/liter Na_2HPO_4 and 6 g/liter KH_2PO_4 to maintain the pH of the buffer at pH 7.

While passing through the column, the MMH dissolved in the liquid and reacted with the iodine in a colorimetric reaction. The air and liquid were then separated in a glass separator, with the air being drawn through a Monnier filter to remove any water, and then through the vacuum pump. The buffer, containing the reacted MMH, was then pumped to a Technicon Auto-Analyzer colorimeter to determine the amount of MMH present in the sample by comparison to a standard having a known amount of MMH. The results were recorded continuously on a strip chart recorder. All transmission and pump tubing for the absorber was tygon and was pumped by a proportioning pump.

The analysis was calibrated with 200 liter mylar bag standards prepared by vaporizing liquid MMH slowly from a needle into dry air. The standard curve was always a straight line through the origin for concentration versus absorbance. The control dome was also sampled throughout the study as a baseline for the two experimental domes, since the animals in a normal dome load give certain known and unknown products which cause minor reaction with the iodine solution. Each of the three domes was sampled sequentially for 40 minutes around the clock, giving a 2-hour cycle for monitoring all three domes.

EEL Exposures

For the 24-hour EEL portion of this study, 8 beagle dogs, 8 rhesus monkeys, and 50 albino rats (Sprague-Dawley derived strain) were exposed to 1 ppm MMH for 24 hours in a Thomas Dome using the same introduction and analysis techniques described above. A similar group of control animals was not exposed and was kept for comparison with the experimental group. The animals were followed for 30 days following exposure during which time blood samples were drawn from dogs and monkeys on a biweekly basis for hematology, serum alkaline phosphatase, total phosphorus and red blood cell fragility. All animals, including rats, were weighed each week during the postexposure period for growth curve comparisons. The exposed and control

rats were divided into two groups of 25 each. One group was used in subgroups of two each three times weekly to obtain blood samples for hematology measurements while the other group was held the full 30 days for growth measurements and pathologic evaluation at the termination of the experiment.

EXPERIMENTAL RESULTS

Chronic Exposures

The concentration levels of MMH in the exposure chambers during the entire 90 days of the study remained remarkably constant as shown in Table 3. The overall means are shown at the end of the table and were very close to the desired nominal values.

TABLE 3. DAILY MEAN CONCENTRATIONS OF MMH (IN PPM)

Exposure Day	Nominal MMH Conc. (ppm) 0.10 Dome 1	0.04 Dome 2	Exposure Day	Nominal MMH Conc. (ppm) 0.10 Dome 1	0.04 Dome 2	Exposure Day	Nominal MMH Conc. (ppm) 0.10 Dome 1	0.04 Dome 2
1	0.121	0.067	32	0.099	0.043	63	0.099	0.040
2	0.100	0.640	33	0.102	0.043	64	0.107	0.038
3	0.088	0.031	34	0.098	0.037	65	0.089	0.041
4	0.099	0.027	35	0.093	0.036	66	0.093	0.043
5	0.101	0.038	36	0.101	0.038	67	0.091	0.049
6	0.103	0.044	37	0.104	0.033	68	0.112	0.050
7	0.107	0.033	38	0.110	0.035	69	0.101	0.038
8	0.098	0.040	39	0.108	0.046	70	0.113	0.042
9	0.101	0.043	40	--	--	71	0.101	0.045
10	0.100	0.038	41	0.083	0.042	72	0.086	0.033
11	0.096	0.042	42	0.108	0.038	73	0.098	0.033
12	0.096	0.052	43	0.115	0.045	74	0.099	0.039
13	0.094	0.037	44	0.110	0.040	75	0.096	0.032
14	0.108	0.040	45	0.110	0.038	76	0.089	0.035
15	0.101	0.043	46	0.098	0.038	77	0.091	0.039
16	0.102	0.035	47	0.094	0.037	78	0.093	0.035
17	0.110	0.035	48	0.101	0.034	79	0.090	0.033
18	0.097	0.037	49	0.085	0.036	80	0.100	0.032
19	0.105	0.042	50	0.111	0.045	81	0.100	0.033
20	0.108	0.038	51	0.091	0.043	82	0.104	0.038
21	0.104	0.035	52	0.086	0.045	83	0.107	0.038
22	0.107	0.040	53	0.104	0.049	84	0.122	0.045
23	0.096	0.040	54	0.102	0.047	85	0.107	0.037
24	0.115	0.042	55	0.098	0.044	86	0.087	0.037
25	0.108	0.045	56	0.102	0.044	87	0.096	0.049
26	0.090	0.033	57	0.110	0.041	88	0.086	0.036
27	0.081	0.041	58	0.105	0.042	89	0.086	0.041
28	0.094	0.042	59	0.105	0.043	90	0.110	0.045
29	0.092	0.042	60	0.108	0.043	91	0.101	0.031
30	0.088	0.038	61	0.099	0.044	92	0.107	
31	0.105	0.048	62	0.093	0.037			
						Overall Means:		
							0.100	0.0462

The continuous 90-day exposure of animals to an atmospheric concentration of 0.1 ppm produced measurable effects in dogs and rats. Rat growth rate was depressed as shown in Figure 2 and the difference between the exposed group and the controls was significant at the 0.1 ppm level until the last weighing period. Rat organ weights and organ to body weight ratios are shown in Table 4. Rat hematology values were slightly lower (Table 5) in both exposure groups, suggesting some hemolytic effect. This change was statistically significant after 45 days of exposure but was not significant at 90 days, although still present. Gross pathology examinations conducted on rats failed to show any significant differences.

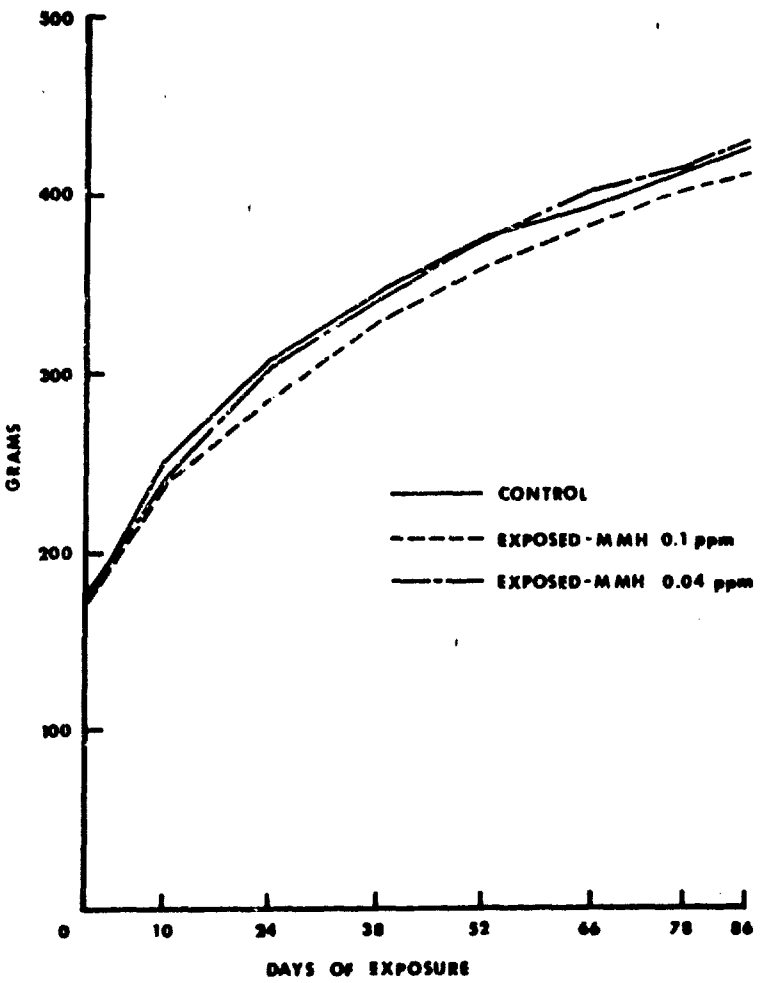

Figure 2. Growth rates of rats continuously exposed to MMH.

TABLE 4. EFFECT OF 90-DAY CONTINUOUS MMH EXPOSURE ON RAT ORGAN WEIGHT AND ORGAN TO BODY WEIGHT RATIOS

	Mean Organ Weights* (Grams)		
Organ	Unexposed Control	0.10 ppm Exposed	0.04 ppm Exposed
Heart	1.4	1.3	1.3
Lung	1.8	1.8	1.8
Liver	11.3	11.2	11.4
Spleen	0.8	0.8	0.8
Kidney	3.0	3.0	3.0
	Mean Organ to Body Weight Ratios* ($\times 10^{-2}$)		
Heart	0.338	0.335	0.319
Lung	0.432	0.456	0.437
Liver	2.763	2.823	2.755
Spleen	0.194	0.212	0.186
Kidney	0.735	0.747	0.718

*N = 20

TABLE 5. EFFECT OF CONTINUOUS MMH EXPOSURE TO RATS ON BLOOD MEASUREMENTS (MEAN VALUES)

	Control Group	Exposed Group 0.10 ppm	Exposed Group 0.04 ppm	
45 Days Exposure				
HCT (Vol %)	44	41**	42**	(1)
HGB (g %)	16.0	15.0**	15.6*	(1)
RBC ($\times 10^6$)	8.2	7.5**	7.6**	(1)
WBC ($\times 10^3$)	6.5	6.9	7.3	(1)
T. Phos. (mg %)	7.1	7.3	8.1	(2)
Alk. P'tase. (Int. Units)	154	146	142	(2)
90 Days Exposure				
HCT (Vol %)	44	43	44	(1)
HGB (g %)	16.7	15.4	15.7	(1)
RBC ($\times 10^6$)	7.0	6.1*	7.6	(1)
WBC ($\times 10^3$)	6.3	6.6	7.2	(1)
T. Phos. (mg %)	6.2	7.0*	6.7*	(2)
Alk. P'tase. (Int. Units)	117	117	117	(2)

* Significant at the 0.05 level

** Significant at the 0.01 level

(1) - N = 30

(2) - N = 10 pooled samples from 3 rats each

Beagle dogs showed significant increases in serum phosphorus and alkaline phosphatase levels during the exposure period as shown in Tables 6 and 7. Hematologic changes were seen only at the 0.1 ppm MMH concentration where significant hemolytic effects were noted as shown in Table 8 which presents the terminal blood measurements for dogs and monkeys. The red blood cells of the dogs exposed at the 0.1 ppm level demonstrated increased osmotic fragility when compared with controls. No significant change occurred at the 0.04 ppm level for this test. Gross pathologic changes were observed at the highest concentration (0.1 ppm MMH) only in dogs. The livers of the exposed dogs had a nutmeg appearance consistent with the passive congestion previously seen at higher dose levels. There were no gross differences between control and the MMH exposed monkeys.

TABLE 6. EFFECT OF CONTINUOUS MMH EXPOSURE ON SERUM TOTAL PHOSPHORUS LEVELS IN DOGS (MEAN mg %)

Exposure Weeks	Control Group	Exposure Group 0.10 ppm	Exposure Group 0.04 ppm
- 2	5.9	5.5	6.4
0	6.1	6.2	5.4
2	5.1	5.0	4.6
4	4.4	5.0	4.8
6	4.2	5.0*	4.7
8	4.4	5.1	4.6
10	4.2	5.2**	4.7
12	4.7	5.7*	5.1
13	4.0	4.9*	4.7

* Significant at the 0.05 level

** Significant at the 0.01 level

N = 8

TABLE 7. EFFECT OF CONTINUOUS MMH EXPOSURE ON SERUM ALKALINE PHOSPHATASE LEVELS IN DOGS (MEAN INTERNATIONAL UNITS)

Exposure Weeks	Control Group	Exposure Group 0.10 ppm	Exposure Group 0.04 ppm
- 2	89	103	85
0	131	180	144
2	101	211**	130
4	74	240**	101
6	67	354**	95
8	64	346**	99*
10	81	399**	118
12	63	302**	90
13	63	356**	85

* Significant at the 0.05 level

** Significant at the 0.01 level

N = 8

TABLE 8. EFFECT OF 90-DAY CONTINUOUS MMH EXPOSURE ON CLINICAL BLOOD MEASUREMENTS IN DOGS AND MONKEYS (MEAN VALUES)

	Dogs (N = 8)		
	Unexposed Control	0.10 ppm Exposed	0.04 ppm Exposed
HCT (Vol %)	49	44**	47
HGB (g %)	18.1	15.1**	17.0
RBC (x 10^6)	6.26	4.73**	5.41
WBC (x 10^3)	13.6	15.1	11.9
Reticulocytes (%)	0.7	2.4	2.1
	Monkeys (N = 4)		
HCT (Vol %)	40	38	38
HGB (g %)	13.8	13.8	12.7
RBC (x 10^6)	5.20	4.72	4.44
WBC (x 10^3)	8.2	7.2	6.8
Reticulocytes (%)	0.8	1.0	1.8

** Significant at the 0.01 level

One monkey in the 0.04 ppm exposure group died on the 10th day of exposure. At necropsy a preexisting condition of amyloidosis was observed. There was no evidence of any relationship of the MMH exposure to death, and the monkey was excluded from the experimental group.

The hematologic effects of continuous exposure to 0.10 ppm MMH were consistent with the dose response previously reported at higher exposure levels (MacEwen and Haun, 1971). However, continuous MMH exposure at an atmospheric concentration of 0.04 ppm did not significantly alter the hematology of the test animals and had no effect on rat growth. On the basis of this experimental data, we believe that 0.04 ppm monomethylhydrazine would be a safe threshold limit value for continuous exposure in confined working areas.

EEL Exposures

In animals exposed to the selected level of 1 ppm MMH for 24 hours, no significant changes were seen in any of the measured parameters of hematology, serum alkaline phosphatase, total phosphorus, rat growth rate, or red blood cell fragility. None of the three species tested (dogs, monkeys, and rats) was significantly different from preexposure values or from control animals of the same species during the 30-day postexposure observation period.

At the end of the 30-day postexposure period, all animals were necropsied and gross pathologic examinations performed. Tissue samples were harvested and sent to others for evaluation. There were no gross pathologic changes related to the exposure in any of the experimental animals.

The results of these experiments indicate that 1 ppm MMH is a safe concentration and it is proposed as the tentative 24-hour Emergency Exposure Limit.

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AMRL-TR-73-125

PAPER NO. 28

DEOXYGENATION OF ENVIRONMENTAL WATERS BY HYDRAZINE - TYPE FUELS

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INTRODUCTION

Hydrazine, methylhydrazine (MMH), and unsymmetrical dimethylhydrazine (UDMH) are used as fuels in several operational Air Force missile systems. Since these fuels are highly toxic to aquatic species (Hoover et al., 1964) as well as man (Oslake et al., 1961) (AFM 160-39), the release of these chemicals into aqueous ecosystems can result in substantial environmental degradation. In order to comply with the National Environmental Policy Act of 1969 (P.L. 91190), the Air Force must obtain quantitative data concerning the behavior of these fuels in nature. These data are necessary to evaluate the environmental impact resulting from the possible release of these propellants into the ecosphere as the result of missile system operations and associated accidents.

One convenient, and highly efficient, method of cataloging and using these data involves the use of mathematical ecosystem models. The models can initially help to identify the data required for characterization of the interaction of these propellants with the environment. When the proper data are available, the models can be used by a computer to simulate the environmental consequences of any hypothesized situation involving the release of these fuels to nature.

Many mathematical models have already been developed to describe the behavior of pollutants in aqueous systems including large bodies of water, flowing water, and ground water (O'Neill et al., 1970). The use of these models to describe the interaction of hydrazine, MMH, and UDMH with the environment requires physical and chemical data for these specific pollutants.

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A major deficiency in the available data appears to be chemical rate equations for the decomposition of these propellants in dilute aqueous solutions. The experimental development of mathematical correlations describing the aqueous degradation of hydrazine, MMH, and UDMH is the subject of this report.

THEORY

Most of the hydrazine, MMH, and UDMH decomposition studies to date have involved the pure liquid (Axworthy et al., 1968; Ross et al., 1970). There are few published data on the degradation of these propellants in dilute aqueous solutions and the available data is preliminary and qualitative (Hoover et al., 1964). The chemical degradation reaction which seems to be most important in terms of environmental effects involves reaction of the propellants with dissolved oxygen in basic solutions (Audrieth and Ogg, 1951; Hoover et al., 1964). The general equation for this reaction is

$$x \text{ Propellant} + y\ O_2 = \text{Products} \qquad (1)$$

where x and y are stoichiometric coefficients. Audrieth and Ogg (1951), who have described hydrazine chemistry in considerable detail, indicate that the major end products of hydrazine decomposition are nitrogen and water. The reaction goes to completion with 1:1 stoichiometry (Axworthy et al., 1967). The chemistry of MMH and UDMH has not been studied in detail, but it may be assumed that the same types of reactions occur with these derivatives. The gas phase oxidation of two moles of MMH by one mole of oxygen has been reported to give methane, nitrogen, and water (Ross et al., 1971). No definite information concerning UDMH oxidation end products or reaction stoichiometry was found in our literature survey. There is some evidence that UDMH may form rather stable intermediate degradation products which are toxic (Hoover et al., 1964).

Both a catalyst and dissolved oxygen are necessary for appreciable aqueous degradation of hydrazine, MMH, and UDMH (Hoover et al., 1964). The most effective catalyst appears to be Cu^{++}, but Co^{++}, Ag^{+}, and Hg^{++} also have catalytic properties (Audrieth and Ogg, 1951; Hoover et al., 1964). The reaction rate is temperature dependent, increasing with increasing temperature (Hoover et al., 1964). Thus, in addition to propellant concentration, the degradation rate equations should include temperature, dissolved oxygen concentration, and catalyst concentration as variables.

In addition to these variables, the degradation rate is also pH dependent (Audrieth and Ogg, 1951; Hoover et al., 1964). The degradation reaction proceeds rapidly only in basic solutions and almost stops in acidic solutions. In aqueous solution the propellants ionize according to the equation

$$P + H_2O = PH^+ + CH^- \qquad (2)$$

where P represents the non-ionized and PH^+ the ionized propellant. The ionization constant for hydrazine reported by Audrieth and Ogg is 8.5×10^{-7} at 25 C. Qualitative experiments in our laboratory have shown that the ionization constants for MMH and UDMH are in the same order of magnitude. The observed dependence of degradation rate on pH indicates that dissolved oxygen reacts only with non-ionized propellants. The effect of pH can be handled by using only non-ionized propellant concentrations in the rate equations. The non-ionized propellant concentration (P) can be calculated from the total propellant concentration (P_T), using equation 3 if the ionization constant, K_I, and the pH of the aqueous solution are known.

$$(P) = \frac{\frac{10^{pH-14}}{K_I}}{1 + \frac{10^{pH-14}}{K_I}} (P_T) \qquad (3)$$

EXPERIMENTAL PROCEDURE

A total of 144 degradation experiments were performed in this study. In preparation for each experiment, an Erlenmeyer flask containing two liters of glass distilled water (conductivity less than 5-umho/cm) was aerated with compressed air for 15 minutes. The aeration increased the dissolved oxygen concentration (DO) in the water to at least 90% of saturation.

After aeration, the flask was placed on a magnetic stirrer and a stirring bar, a pH electrode, and a DO/Temperature probe were submerged in the water. The DO/Temperature probe was connected through an oxygen meter to a strip chart recorder. The pH probe was connected to a pH meter. A measured amount of Cu^{++}, in the form of $CuSO_4$, was added to the reaction flask and the contents were mixed by the magnetic stirrer throughout the experiment.

A 5-minute mixing period was allowed, and then the temperature, DO, and pH of the water in the flask were recorded. If the DO was above 7.5 ppm, a measured amount of propellant was added to the flask and the DO was recorded continuously on the strip chart until it had dropped below 0.25 ppm. At this point the experiment ended and final temperature and pH values were recorded.

In the early experiments, pH values were recorded every 10 seconds during the experiment, but the pH change appeared to be a step function with flask mixing dynamics superimposed. Thus, in most of the experiments only the initial and final pH values were recorded. It was assumed that the pH changed instantaneously from the initial to the final value when the propellant was added.

The initial propellant and copper ion concentrations which were studied are shown in Table 1. The experimental conditions were selected to hold all variables, except DO, as nearly constant as possible. Since there was no apparent DO loss in control experiments with no copper ion, it was assumed that there were no interfering impurities in the glass distilled water. It was assumed that the copper ion concentration remained constant throughout the experiment. High propellant concentrations were used to justify an assumption that the propellant concentration changed during DO utilization was negligible. Calculations of the expected change in propellant concentration during degradation under the conditions in Table 1, based on 1:1 stoichiometry, indicate an average propellant concentration change of 2.7%. This is well within the expected limits of experimental error. Since the propellant concentration is the only variable in this experiment which affects pH, the high propellant concentrations also maintained a nearly constant pH. The mouth of the flask was left open to the atmosphere but the diffusion of oxygen into the flask by this route was neglected since the experiments were short (most experiments took only two minutes). All experiments were conducted at temperatures between 21 C and 26 C.

TABLE 1. EXPERIMENTAL INITIAL CONDITIONS

Series Number	Cu^{++} mg/liter	Hydrazine mg/liter	MMH mg/liter	UDMH mg/liter
1	1.0	502.6	443.8	393.2
2	0.5	502.6	443.8	393.2
3	0.25	502.6	443.8	393.2
4	0.125	502.6	443.8	393.2
5	0.5	251.3	221.9	196.6
6	0.5	1005.2	887.6	786.4

Eight replicate experiments were performed for each set of conditions in Table 1. The DO data were read from the strip chart recordings starting at the time the DO passed 7.0 ppm. This artificial starting time was used to eliminate propellant mixing transients from the data. Figure 1 shows DO disappearance curves from the hydrazine experiments and illustrates the rate change as a function of copper ion concentration at constant propellant levels. Figure 2 shows the DO disappearance curves with variable propellant concentrations at constant copper ion levels. Similar data resulted from the MMH and UDMH experiments. Each point on these curves represents the average of eight replicate data points.

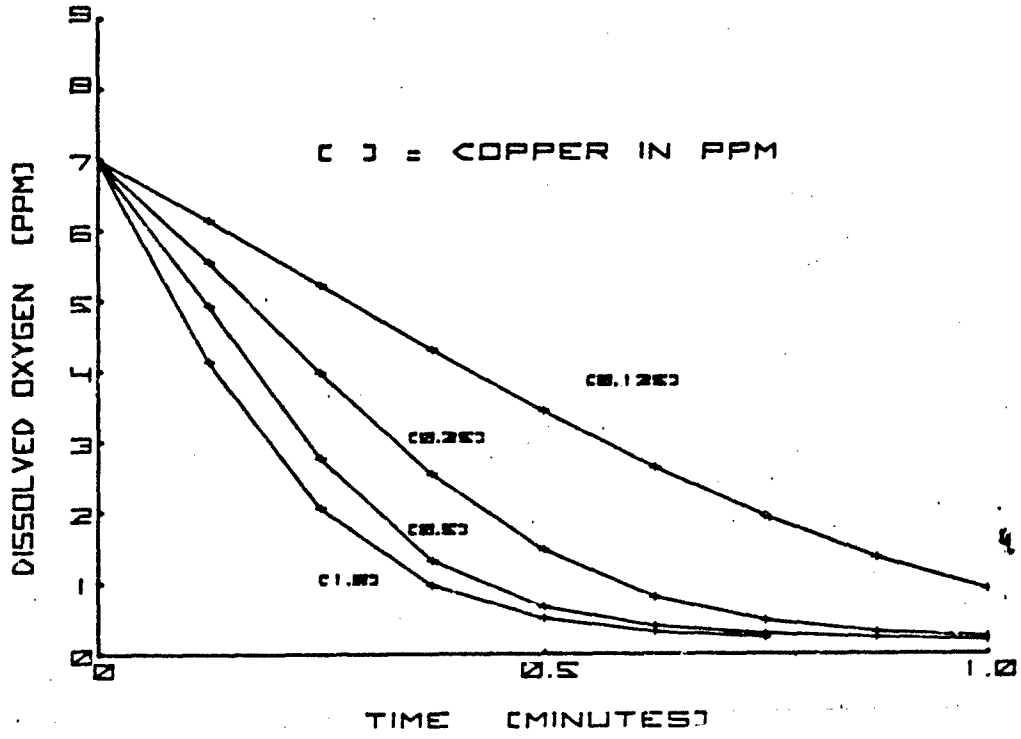

Figure 1. Oxygen utilization by 502.5 ppm hydrazine.

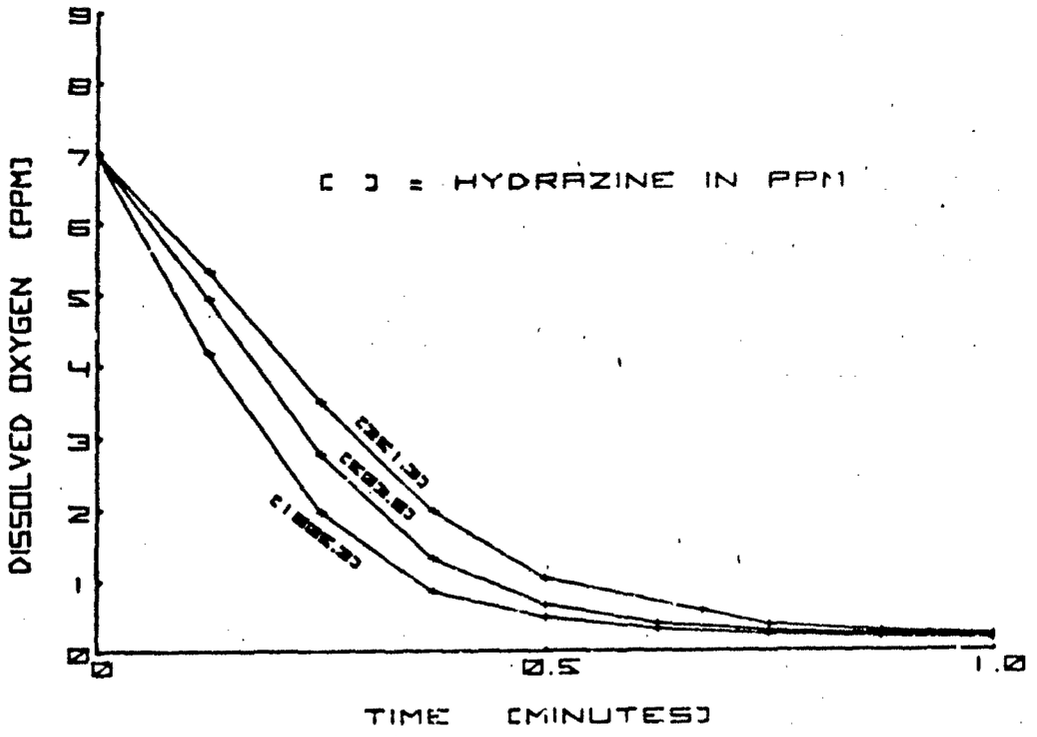

Figure 2. Oxygen utilization by hydrazine with 0.5 ppm copper.

DATA ANALYSIS AND CORRELATION

The average data from each set of eight replicate experiments were fit to the power rate equation shown below.

$$r = k\,(DO)^n = -V\,\frac{d\,(DO)}{dt} \qquad (4)$$

where:

r = rate of DO utilization (mg/min)

k = reaction rate constant (liter/min)

n = reaction order

V = reactor volume (liters)

t = time (minutes)

Equation 4 can be integrated to give the following relationship between DO and time.

$$\frac{(DO)^{1-n}}{1-n} = -\frac{k}{V}(t) + \frac{(DO_o)^{1-n}}{1-n}, \quad n = 1 \tag{5}$$

$$\ln (DO) = -\frac{k}{V}(t) + \ln (DO_o), \quad n = 1 \tag{6}$$

where:

$$DO_o = DO \text{ when } t = 0$$

Using these equations, the experimentally determined reaction order can be defined as that value of n for which a straight line results when the DO data are substituted into the left side of the integrated rate equation and plotted as a function of time. The slope of the line is proportional to the rate constant. A program was written for a Hewlett-Packard Model 9810 calculator system to analyze the experimental data in this manner.

The program was written to load the average data from magnetic tape and then test the fit of the data to the power rate equation for a series of reaction orders between 0.0 and 3.00 using a step size of 0.01. For each value of n, the program used the stored experimental data to calculate a least squares line and a correlation coefficient. The output of the program was the values of n and k/V which gave the largest correlation coefficient (closest to 1.0). The resulting average reaction orders and correlation coefficients for each of the propellants studied are shown in Table 2. To simplify the final rate correlation equations, the reaction order was taken to be 1.0 for hydrazine and MMH and 0.5 for UDMH. Table 3 shows the rate constants for all sets of experimental data using these reaction orders. Since the correlation coefficients in Table 3 are all above 0.9, the fit of the experimental data to the power rate equations using these reaction orders is quite acceptable. The difference in reaction orders between UDMH and the other propellants indicates that different mechanisms may be involved in the degradation of UDMH, but the determination of mechanism is beyond the scope of this report.

TABLE 2. POWER RATE EQUATION DATA ANALYSIS
$d(DO)/dt = - k/V (DO)^n$

Propellant	Average Order - n	Average Rate Constant - k/V (1/min)	Correlation Coefficient
Hydrazine	0.985	4.0245	0.9880
MMH	1.025	3.3296	0.9893
UDMH	0.540	1.6895	0.9967

TABLE 3. POWER RATE EQUATION DATA ANALYSIS
FIXED REACTION ORDER
$d(DO)/dt = - k/V (DO)^n$

Expt. No.	Propellant (mg/liter)	Copper (mg/liter)	Order n	Rate Constant k/V (min^-)	Correlation Coefficient
Hydrazine					
H1	502.6	1.0	1.0	4.8823	0.9871
H2	502.6	0.5	1.0	4.0458	0.9639
H3	502.6	0.25	1.0	3.3922	0.9804
H4	502.6	0.125	1.0	2.1701	0.9494
H5	251.3	0.5	1.0	3.6173	0.9857
H6	1005.2	0.5	1.0	4.3785	0.9316
MMH					
M1	443.8	1.0	1.0	5.1176	0.9351
M2	443.8	0.5	1.0	3.6703	0.9429
M3	443.8	0.25	1.0	2.4444	0.9448
M4	443.8	0.125	1.0	1.1738	0.9352
M5	221.9	0.5	1.0	3.2534	0.9871
M6	887.6	0.5	1.0	3.6782	0.9623
UDMH					
U1	393.2	1.0	0.5	2.4703	0.9941
U2	393.2	0.5	0.5	1.8689	0.9975
U3	393.2	0.25	0.5	1.1725	0.9989
U4	393.2	0.125	0.5	0.5863	0.9749
U5	196.6	0.5	0.5	1.3140	0.9964
U6	786.4	0.5	0.5	2.7627	0.9897

If all of the parameters except DO are considered constant in each set of data, the reaction rate constants are functions of temperature, pH, copper ion concentration, and propellant concentrations. The experimental conditions were selected to allow investigation of the effects of copper ion and propellant concentrations on the rate constants. Figures 3 and 4 are plots of the rate constant values as functions of copper ion and propellant concentration. All of the plots have been forced through the origin since control experiments have shown that there is no degradation when there is no copper ion, and the DO is stable, even with copper ion present, if there is no propellant in the flask. The form of these curves is very suggestive of Michaelis-Menten type curves.

Since the objective of this analysis is to develop rate correlations rather than theoretically significant rate equations which reflect basic mechanisms, the variation of the rate constants was fit to equations of the form

$$\frac{k}{V} = \frac{Vc\ (C)}{Kc + (C)} \quad \frac{Vp\ (P)}{Kp + (P)} \tag{7}$$

where:

(C) = copper ion concentration (mg/liter)

(P) = propellant concentration (mg/liter)

Vc, Vp, Kc, Kp = arbitrary correlation constants

The product form of Equation 7 is based on the same data which required the curves in Figures 3 and 4 to pass through the origin. The correlation constant values which best describe the dependence of the rate constants on copper ion and propellant concentration are shown in Table 4. The final rate equations for the utilization of oxygen in mg/min with the DO, copper ion, and propellant concentrations in mg/liter are:

Hydrazine

$$\frac{r}{V} = \frac{6.79\ (P)\ (C)}{16.07 + 74.4(C) + 0.2160(P) + (P)\ (C)}\ (DO) \tag{8}$$

Methylhydrazine

$$\frac{r}{V} = \frac{13.63\ (P)\ (C)}{52.64 + 44.09(C) + 1.194(P) + (P)\ (C)}\ (DO) \tag{9}$$

Unsymmetrical Dimethylhydrazine

$$\frac{r}{\nabla} = \frac{12.56\ (P)\ (C)}{459.1 + 411.8(C) + 1.1168(P) + (P)\ (C)}\ (DO)^{0.5} \qquad (10)$$

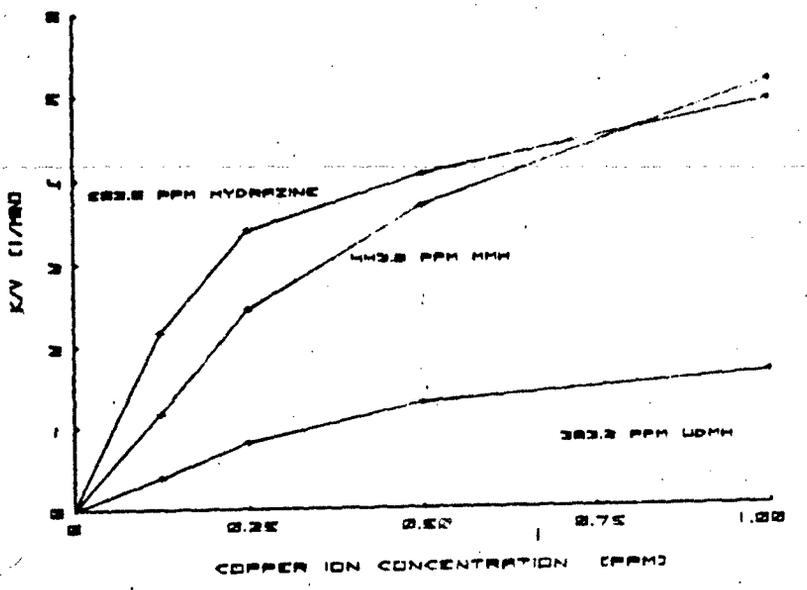

Figure 3. First order rate constants for oxygen utilization by propellants catalyzed by copper ion.

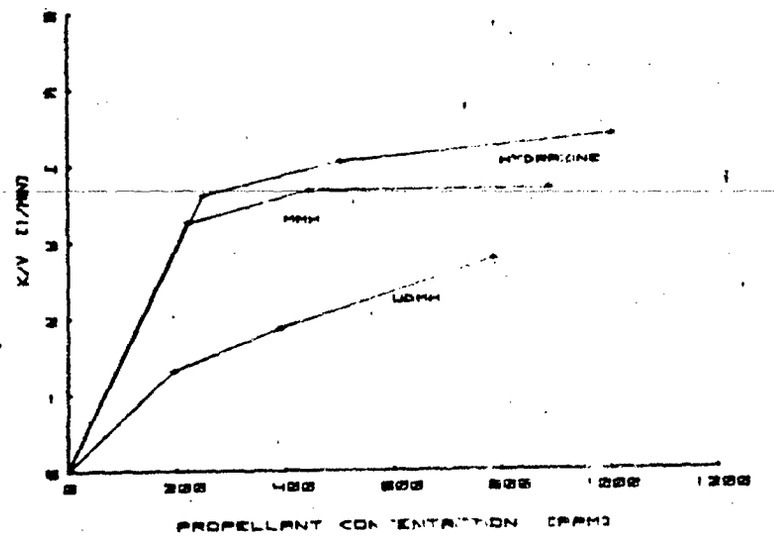

Figure 4. First order rate constants for oxygen utilization by propellants catalyzed by 0.5 ppm copper ion.

TABLE 4. CORRELATION PARAMETERS[1]
(All concentrations in mg/liter)

Propellant	Order n	Kc	Kp	VcVp	Standard[2] Deviation	Percent[3] Error
Hydrazine	1.0	0.2160	74.4	6.7938	0.1081	2.88
MMH	1.0	1.1940	44.09	13.6300	0.2856	8.86
UDMH	0.5	1.1168	411.08	12.5600	0.2181	12.86

[1] Correlation equation

$$k/V = \frac{VpVc\ (P)\ (C)}{KpKc + Kc(P) + Kp(C) + (P)\ (C)}$$

[2] Standard deviation between calculated and observed rate constants.

[3] Percent error = standard deviation as a percentage of the average observed rate constant value.

The percent error shown in Table 4 indicates that these correlations can be expected to give estimated reaction rates to an accuracy of about 10% over the copper ion and propellant concentration ranges studied. If the stoichiometry for the reaction of oxygen with the particular propellant is known, these correlations can be used to find the rate of propellant degradation. For this application, it is more convenient to have the rate equations in terms of molar concentration units. The following equations are for the utilization of oxygen in moles per minute with all concentrations in moles per liter.

Hydrazine

$$\frac{r}{V} = \frac{8.59\times10^{6}\ (P)\ (C)\ (O_2)}{1 + 2.94\times10^{5}(C) + 430(P) + 1.265\times10^{8}(P)\ (C)} \qquad (11)$$

Methylhydrazine

$$\frac{r}{V} = \frac{7.568\times10^{6}\ (P)\ (C)\ (O_2)}{1 + 5.32\times10^{4}(C) + 1043(P) + 5.55\times10^{7}(P)\ (C)} \qquad (12)$$

Unsymmetrical Dimethylhydrazine

$$\frac{r}{V} = \frac{5.83\times10^{6}\ (P)\ (C)\ (O_2)^{0.6}}{1 + 5.7\times10^{4}(C) + 146(P) + 8.3\times10^{6}\ (P)\ (C)} \qquad (13)$$

DISCUSSION

The major objective of this study is the development of decomposition rate correlation equations which can be used in mathematical models to describe the behavior of operational hydrazine-type propellants in environmental waters. Equations have been developed to relate the aqueous degradation rates of hydrazine, MMH, and UDMH to dissolved oxygen, copper ion, and propellant concentration. Temperature and pH are also important variables. The effect of pH has already been described and can be quantitated using ionization constants and measured buffer characteristics of the actual body of water under consideration. The temperature effect has not been studied in this laboratory, but it may follow the Arrhenius equation. We plan to study experimentally the effect of temperature as well as the effect of catalytic ions other than copper. We hope to be able to relate the catalytic effect of the other ions to the effect of copper through effectiveness factors.

Aerozine-50, a 50-50 mixture by weight of hydrazine and UDMH, is the specific hydrazine-type propellant which is used most by the Air Force at this time. The degradation rate equations for hydrazine and UDMH can be used together to describe the degradation of Aerozine-50 in environmental waters if it is assumed that the degradation of one propellant has little, or no, effect on the degradation of the other. Experiments to investigate the validity of this assumption are now in progress.

One environmental effect of the hydrazine-type fuels which was not clearly indicated in our literature survey was vividly demonstrated by the experimental data from this study. The utilization of dissolved oxygen is very rapid in water which has been polluted with hydrazine propellants. Therefore, the environmental threat of these propellants can come from toxicity of the propellants and possibly their degradation products, deoxygenation of the water associated with propellant degradation, or both toxicity and deoxygenation.

CONCLUSION

This study has provided degradation rate correlations for hydrazine, MMH, and UDMH. These correlations may now be used in mathematical ecosystem models to provide computer simulations which will help the Air Force to answer the following types of questions involving the possible environmental impact of hydrazine-type propellants: How long will a body of water be polluted after a spill? How far and how fast will propellant related pollution spread? What is the major pollution hazard related to a specific spill situation, i.e., propellant toxicity, deoxygenation, or both? Will propellants spilled on the ground reach surface waters through underground channels before they have degraded enough to be innocuous and what will be the effect on underground water itself? In addition to answering questions applicable to environmental impact statements, model simulations may be used to plan corrective measures in case of accidental spills. For example, simulation will allow evaluation of the possibility that natural aeration of flowing water, coupled with high natural catalytic ion concentrations, will rapidly detoxify the propellant resulting from a spill while maintaining adequate oxygenation of the water. If this is not the case, simulations will aid the development of techniques such as adding catalysts or providing artificial aeration to rapidly degrade the propellant and maintain acceptable DO levels in the water.

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AMRL-TR-73-125

PAPER NO. 29

EFFECT OF BERYLLIUM ON SOIL MICROORGANISMS

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INTRODUCTION

In an industrial society many simple and complex chemicals enter the soil through purposeful or inadvertent means. In dilute form, most of these are harmless to the inhabitants of the soil and are readily decomposed. When present in concentrated form, however, these compounds may pose a potential threat not only to soil microbes, interrupting many of their activities vital to soil fertility, but also to higher forms of life by transmission through the food chain. Beryllium may be one such substance. Considerable research has been done on the toxic effects of beryllium on higher forms of animal life (Stokinger, 1966) but little is known of the effects of beryllium on soil microorganisms. Since soil microbes occupy a position at one end of the biological food chain, they are capable of transmitting various substances, either in their original form, or more commonly, in a form derived through a series of complex biochemical transformations, to higher forms of life. This may be accomplished by actual ingestion of the soil microbes by larger organisms, i.e., protozoa, by release of concentrated chemicals into the environment upon cell lysis, or by penetration and invasion of plant tissue (Brock, 1966).

Beryllium possesses an impressive list of desirable physical and chemical characteristics which suggest its use in various industrial and military operations. The Air Force has had an interest in beryllium because of its ability to enhance motor performance when included in rocket propellant formulations. This is due mainly to its high heat of reaction and a strong chemical bond formed in the oxide, providing a high specific impulse; because exhaust particles are light in weight, they are exhausted at a higher velocity at a given temperature. The firing of rocket motors containing beryllium, however, may release large quantities of potentially toxic beryllium compounds into the surrounding environment. The effects of a number of different missile propellants on the environment have already been studied (Heck et al., 1962; Heck et al., 1963; Hoover et al., 1964), but there has been little research done on the

effects of beryllium-containing propellants on soil microorganisms. This study was undertaken to assess the effects of beryllium as a soluble salt on soil microorganisms and the eventual fate of this ion in the biological food chain.

MATERIALS AND METHODS

A soluble form of beryllium which could be easily incorporated into a microbiological medium was used in this experiment. Purified beryllium sulfate tetrahydrate was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey. Because of strong hydrolytic properties and a tendency to form chelates with a number of different ligands, the behavior of beryllium salts in solution is quite complicated (Prytz, 1928; Kakihana and Sillen, 1956). Beryllium forms insoluble hydroxides or hydrated complex ions at pH 5 to 8. For this reason 2 g/liter sodium citrate was incorporated into the medium to keep 100 μg/ml beryllium as $BeSO_4 \cdot 4H_2O$ in solution. The growth medium used in this study consisted of 1.0 g dextrose, 1.0 g K_2HPO_4, 0.5 g KNO_3, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.1 g $CaCl_2$ and 0.01 g $FeCl_3$ per liter of distilled water. A concentrated aqueous solution of the beryllium salt was added to the medium to give a final concentration of 100 μg/ml beryllium ion. The pH of the medium was adjusted to 6.8 with sodium hydroxide and the medium divided into 100-ml aliquots in 500-ml Erlenmeyer sidearm flasks. The flasks were autoclaved at 121 C for 15 minutes at 15 psi. Controls without beryllium were prepared in a similar manner. After the medium had cooled, it was inoculated with a mixed culture of soil microorganisms that had been prepared by adding 1 g of soil to 99 ml sterile medium of the same composition as above. The inoculum was serially diluted to approximately 10^4 cells/ml and 1 ml added aseptically to each of three flasks with and without beryllium. The flasks were placed on a reciprocating shaker at 25 C and a growth curve plotted by taking hourly readings of light transmittance through the sidearms of the Erlenmeyer flasks, using a Model 6D Coleman Junior Spectrophotometer at a wavelength setting of 575 nm. Growth curves were compared for cells grown both in the presence and absence of beryllium.

Sixteen bacterial species were isolated in pure culture from the beryllium test broth and tested individually for their ability to remove beryllium from the growth medium. Two-tenth ml of a 24-hour mineral salts culture of each isolate was inoculated into 100 ml of mineral salts medium containing 100 μg/ml beryllium ion in 250-ml Erlenmeyer flasks. The cultures were grown at room temperature on a reciprocating shaker, and at the end of 48 hours all but three of the cultures were growing vigorously. These three cultures were discarded and the others centrifuged individually at 6000 rpm in an Automatic SerVall Superspeed Centrifuge using a SerVall SS-34 head. The supernatant was analyzed for the presence of beryllium according to the procedure of Taylor et al. (Taylor and Arnold, 1971, 1968; Wolf et al., 1972; Kaiser et al.,

1972). This method detects trace (picogram) quantities of beryllium by chelation with trifluoroacetylacetonate, H(tfa), utilizing a gas chromatograph equipped with an electron capture detector. In this procedure two molecules of the fluorine-containing β-diketone combine with a beryllium atom to form a stable, volatile chelate amenable to gas chromatographic detection.

Fifty μl of supernatant from each flask were placed in 100-ml volumetric flasks and diluted to 100 ml with distilled water. Fifty μl of this dilution was placed in a small glass vial made by sealing off one end of a diSPo® pipet. Two-tenths ml of 1% H(tfa) (Pierce Chemical Company, Rockford, Illinois), in nanograde benzene (Mallinckrodt Chemical Works, St. Louis, Missouri) was added to each vial and the vials heat sealed. The vials were shaken for 30 seconds by hand, then placed in an oven and heated for 15 minutes at 121 C. After cooling, the vials were opened and 0.2 ml of a 1:5 solution of aqueous ammonia was added to remove excess H(tfa). The vials were stoppered, shaken for 30 seconds by hand, and centrifuged for 10 minutes at 4500 rpm in a Lourdes Model AAC Centrifuge. One μl of the top benzene layer, containing the chelated beryllium, was injected into a Varian Aerograph Series 2100 Gas Chromatograph equipped with a standard d-c mode electron capture detector, employing a 250-millicurie titanium tritide foil ionizing source. The 6 ft x 2 mm i.d. glass U-tube column was packed with 5% SE-52 silicone gum on 60/80 Gas Chrom Z. Operating parameters were: column temperature 110 C, injector temperature 146 C, detector temperature 178 C, carrier gas (prepurified nitrogen - Matheson, Coleman and Bell) 96.5 cc/min. Standards were prepared by dissolving twice resublimed beryllium trifluoroacetylacetonate, $Be(tfa)_2$, (Monsanto Research Corporation, Dayton, Ohio) in nanograde benzene and diluting to approximately 0.01 μl/ml. The concentration of beryllium in each supernatant sample was calculated on the basis of peak height.

Thirteen bacterial isolates were screened in this way, and the isolate showing maximum uptake of beryllium from the test medium was studied further to determine whether beryllium was merely being absorbed onto the cellular surface or whether the uptake was an assimilative process. To determine this, the test organism was grown in a mineral salts medium for 30 hours, at which time it reached the late logarithmic phase of growth. The contents of the culture flask were then centrifuged at 6000 rpm (SerVall Centrifuge) for 10 minutes and the pellet resuspended in 100 ml of sterile mineral salts medium and washed once. After recentrifuging, the pellet was again suspended in 100 ml of sterile mineral salts medium and one-half the cellular suspension removed and heat killed at 70 C for 10 minutes. Again the cells were centrifuged, and this time the pellets of live and dead cells were each suspended in mineral salts medium containing 100 μg/ml Be as $BeSO_4 \cdot 4H_2O$. The flasks were placed on a reciprocating shaker at room temperature for either 25 minutes in one experiment or 48 hours in another and both supernatant and cells analyzed for the presence of beryllium. In a third experiment live and dead cells obtained as described above were suspended in distilled water containing 100 μg/ml beryllium for 24 hours; only the supernatant was analyzed for beryllium.

To ascertain if beryllium uptake was related to cell number, a growth curve was determined and the supernatant analyzed periodically for beryllium. To 3000 ml of mineral salts medium was added 100 μg/ml beryllium ion; another 3000 ml of mineral salts medium contained no beryllium. The pH of both batches of media was adjusted to 6.8 with NaOH, and each batch divided into 1000 ml quantities in 2000 ml Erlenmeyer flasks. The flasks were autoclaved at 121 C for 15 minutes at 15 psi, and when cool each flask was inoculated with 2.4×10^5 cells of the test organism and placed on a reciprocating shaker at 25 C and sampled every 8 hours for a total of 56 hours as follows: 40 ml were removed aseptically from each flask and centrifuged at 6000 rpm (SerVall Centrifuge) for 10 minutes. A slightly modified procedure was used for preparing the samples for GC analysis than had been used previously (Frame, 1975). One-tenth ml of supernatant was removed from each flask and diluted to 10^{-4}. Five ml of this dilution was placed in a polyethylene bottle to which was added 2.0 ml 0.05M sodium EDTA, 2.0 ml 1:1 acetic acid-sodium acetate buffer, and 5.0 ml 1.0% H(tfa) in benzene. The bottles were capped and shaken vigorously on a reciprocating shaker for 15 minutes. An 0.5 ml portion of the benzene layer was removed and placed in a 1-ml glass-stoppered volumetric vial along with 0.5 ml 0.5M NaOH. The vials were shaken by hand for 30 seconds to extract excess H(tfa) and the tubes centrifuged 2 minutes at 4500 rpm in a Lourdes Centrifuge. A 1-μl sample of the benzene layer was injected into the gas chromatograph. GC parameters were the same as previously stated.

The cellular pellets recovered earlier, after centrifugation of the 40-ml samples from the culture flasks, were washed once with 40 ml of mineral salts medium and the suspension recentrifuged at 6000 rpm (SerVall Centrifuge) for 10 minutes. The cell wash was analyzed for beryllium the same as was the supernatant. To the remaining cell pellet was added 1.0 ml concentrated nitric acid. The cellular digest was poured into a 10-ml Erlenmeyer flask and the mixture heated on a hot plate and boiled gently for 15 minutes. The contents of the flask were washed into a 1-liter volumetric flask, rinsing the Erlenmeyer several times with distilled water, and washing the contents into the liter flask. The flask was stoppered and the contents mixed thoroughly. A 0.025 ml sample of this solution was removed and diluted to 10^{-1}. These dilutions gave a final beryllium concentration consistent with standards, which were in the range of 0.01 μg/ml $Be(tfa)_2$.

Plate counts were also performed by removing a 1.0 ml sample from each 2000-ml sample flask every 8 hours and making serial dilutions, using mineral salts medium as the diluent. One-tenth ml of each dilution was plated in duplicate onto mineral salts agar plates, using the spread plate technique, the plates incubated at 30 C for 72 hours and then counted.

An experiment was also performed to determine whether soil microorganisms could substitute beryllium for magnesium in the growth medium. A mixed culture of soil microorganisms, as well as 8 individual soil isolates,

were inoculated into a mineral salts medium in which 100 μg/ml Be as $BeSO_4 \cdot 4H_2O$ was substituted for magnesium sulfate. Mineral salts medium to which no magnesium or beryllium sulfate had been added was used as the negative control, while the positive control consisted of mineral salts plus magnesium and beryllium. All flasks were prepared in quintuplicate.

In order to determine the tolerance of soil microorganisms to higher concentrations of beryllium, thirteen soil isolates obtained from a mixed culture grown in 100 μg/ml Be were inoculated in mineral salts medium containing 500 and 1000 μg/ml of Be as $BeSO_4 \cdot 4H_2O$. One and two percent sodium citrate were used, respectively, to maintain the beryllium in solution.

RESULTS

Growth curves for a mixed microbial soil population grown both in the presence and absence of 100 μg/ml of beryllium are shown in Figure 1.

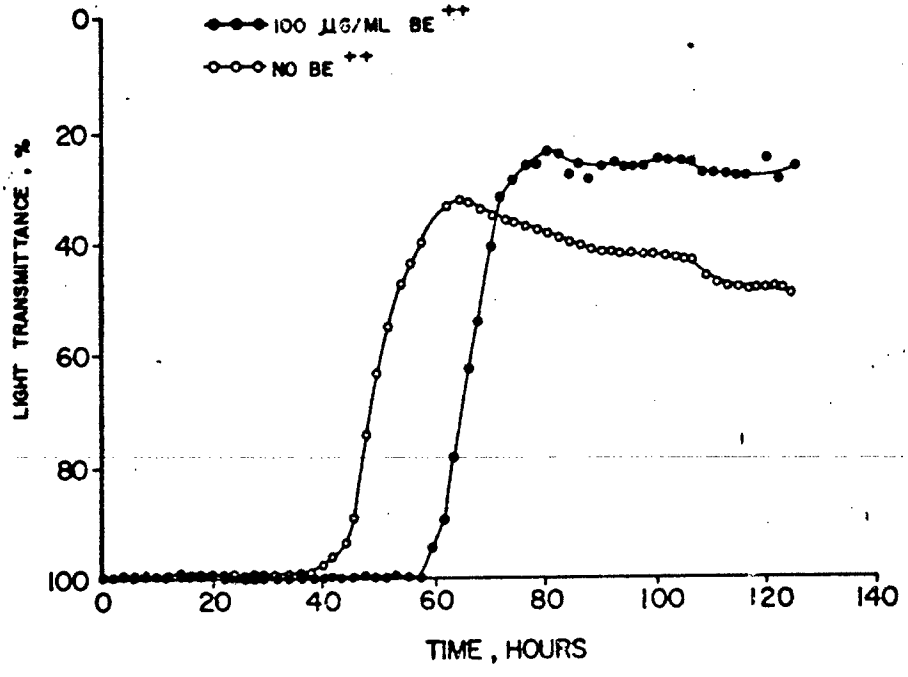

Figure 1. Comparison of growth curves for a mixed microbial soil population grown in the absence and presence of 100 μg/ml beryllium ion. Each point on the curve is the mean value of three replicate cultures.

It can be seen from the curves that the addition of 100 μg/ml beryllium ion to the growth medium resulted in two effects on the growth of the mixed soil cultures: a delay of 20 hours before the onset of the log phase occurred in the beryllium cultures as opposed to the controls, and the attainment of a higher level of growth. The essential identity of slopes of both curves during the log phase indicates that generation times were equal. The lengthened lag phase was not observed in subsequent growth curves of another mixed soil population, probably due to the presence of different organisms in the mixed culture. For all curves determined subsequently for both mixed and pure cultures, however, little or no inhibition of cell growth was observed in beryllium-containing cultures when compared to beryllium-free controls.

The sixteen isolates obtained from the beryllium test medium were examined microscopically and a number of biochemical tests performed to establish tentative identification. The results of these tests are shown in Table 1.

TABLE 1. TESTS FOR IDENTIFICATION OF SIXTEEN SOIL BACTERIA

	Soil Isolate															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Motility	NM	SM	SM	SM	VM	SM	SM	NM	SM	VM	VM	NM	SM	SM	NM	SM
Gram	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-
Methyl Red	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malonate	-	-	-	-	+	-	-	-	+	-	+	-	-	+	+	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Nitrate Redn.	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	-
Oxidase	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-
Urease	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
TSI	A	-	-	-	AG	-	-	-	-	AG	AG	-	-	AG	-	-
Litmus Milk	Al	Alc	Al	Alc	Ac	Al	Al	Al	Al	Ac	R	Al	Al	Ac	Al	Alc
H_2S Prod.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
Adonitol	-	-	-	-	+	-	-	-	-	+	+	-	+	+	-	-
Inositol	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-
Lactose	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-
Dextrose	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-
Sucrose	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-
Coagulase	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-
Mannitol Salt	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

NM = Nonmotile
SM = Slightly motile
VM = Very motile

A = Acid
G = Gas
R = Reduction

Ac = Acid, clear
Al = Alkaline
Alc = Alkaline, clear

All but one of the cultures examined were gram-negative rods. When these isolates were tested individually for their ability to remove beryllium from the test medium over a 48-hour period, uptake was seen to range from 0 to 95%, as determined by gas chromatographic analysis of the supernatant (Table 2).

TABLE 2. REMOVAL OF BERYLLIUM SULFATE BY SIXTEEN SOIL BACTERIA AFTER 48 HOURS INCUBATION FROM A MINERAL SALT MEDIUM CONTAINING 100 μg/ml $BeSO_4 \cdot 4H_2O$

Soil Isolate No.	Beryllium Removed (μg/ml)[1]
1	*
2	14.8
3	91.4
4	58.8
5	45.4
6	-0-
7	84.8
8	90.7
9	*
10	95.2
11	27.4
12	-0-
13	*
14	89.8
15	88.1
16	88.7

* No growth

[1] Values are averages of three determinations.

The culture showing maximum uptake was studied further to determine if beryllium was being assimilated by the bacteria or merely adhering to cellular material by a simple process of adsorption. In the first experiments in which the organism, tentatively identified as a pseudomonad, was suspended either in mineral salts medium containing 100 μg/ml beryllium for 25 minutes or in distilled water containing 100 μg/ml beryllium for 24 hours, neither live or dead cells affected the concentration of the beryllium in the supernatant. However, when both live and heat-killed cells of the test organism were suspended in a mineral salts medium containing 100 μg/ml beryllium ion for 48 hours, the cells removed by centrifugation and the supernatant analyzed for the presence of beryllium, it was found that only 7% of the original concentration of beryllium remained in the supernatant separated from the live cells, whereas the supernatant from the dead cells contained the original concentration of approximately 100 μg/ml beryllium. The live cells were digested with nitric acid and approximately 70% of the original concentration of beryllium was recovered. It is assumed that the difference between the original concentration and that recovered is due to experimental error and adherence of some of the beryllium to the glass surfaces of the culture flasks. No beryllium was found associated with the dead cells.

A growth curve was determined and the supernatant from the pure culture of Pseudomonas sp. sampled intermittently during a 56-hour period; the beryllium concentration in the supernatant was seen to decrease as the cell population increased (Figure 2). The cells were removed from the medium by centrifugation, washed, recentrifuged and the cellular digest analyzed; approximately 81% of the original concentration of beryllium was recovered. The cell wash showed only trace amounts of beryllium, further suggesting that beryllium was not adsorbing to the cells.

This experiment was repeated using a different soil bacterium. The results, shown in Table 3, indicate several significant differences. In contrast to the results depicted in Figure 2, this organism did not exhibit a relationship between cell concentration and the amount of beryllium assimilated but rather removed all the beryllium from the medium only after a critical cell number, approximately 10^9/ml, was reached. Although supposedly identical, the three replicate cultures grew at different rates: culture #1 reached the critical concentration between 144 and 148 hours; culture #2 attained this level between 156 and 160 hours; culture #3 did not grow to this extent during the observation period nor did it affect the beryllium concentration in the medium. Variation of the growth rates in the three replicate cultures was probably due to differences in agitation caused by position on the shake table.

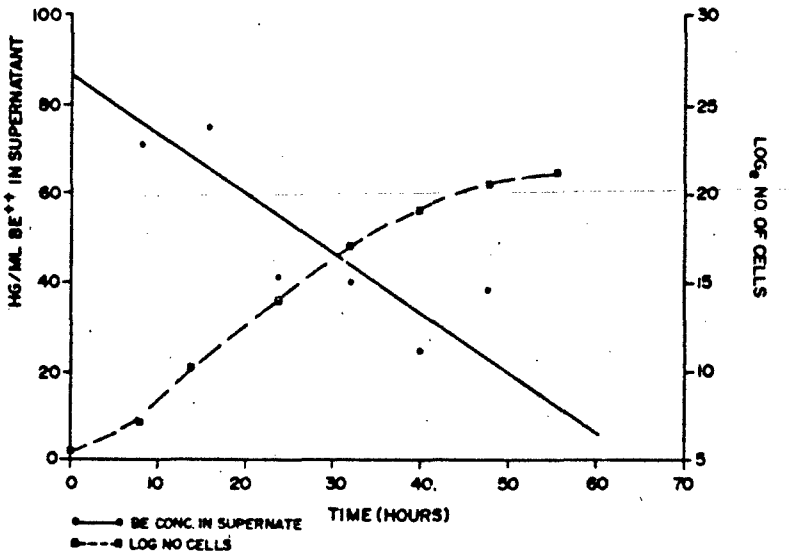

Figure 2. Relationship between cell number and removal of beryllium by a soil pseudomonad. Each cell count and beryllium analysis was done in duplicate on three replicate cultures. The beryllium curve was drawn by least squares analysis.

TABLE 3. RELATIONSHIP OF CELL CONCENTRATION AND ASSIMILATION OF BERYLLIUM BY A SOIL BACTERIUM

	1		2		3	
Time Hrs	Count[1]	Be[2]	Count	Be	Count	Be
0	3.00	79.5	3.70	65.8	-	70.0
6	3.48	76.0	3.00	77.4	3.30	60.0
18	3.61	78.1	3.66	94.2	3.59	89.3
24	3.70	91.4	3.67	86.5	3.48	76.7
42	4.33	87.2	4.45	90.0	4.39	77.4
48	4.73	95.6	4.75	82.3	4.48	57.2
72	5.08	71.2	4.99	54.4	4.83	54.4
84	5.52	98.4	5.36	82.3	5.26	-
96	5.85	76.0	5.73	59.3	5.32	71.6
133	7.74	62.2	7.53	78.1	6.02	ND
137	8.00	74.6	7.60	71.8	6.16	73.9
141	8.51	84.4	7.78	83.0	6.49	79.5
144	9.04	71.8	7.98	86.5	6.35	91.4
148	9.10	0	8.27	81.6	6.68	92.1
152	9.25	0	8.80	80.2	6.57	84.4
156	9.25	0	9.12	82.3	6.62	94.9
160	9.31	0	9.18	0	6.71	83.0
169	9.29	0	9.23	0	6.73	-

[1] Log of cell count/ml

[2] Be concentration in supernatant, µg/ml

The growth of a mixed soil population in which beryllium sulfate was substituted for magnesium sulfate in the growth medium is compared with a culture containing neither beryllium nor magnesium in Figure 3. All cultures began growing within 48 hours, but by the third day the magnesium-deficient culture began to level off. However, the beryllium-containing cultures continued to increase in optical density and by the fourth day showed a 20% increase in optical density compared to the magnesium-deficient cells, indicating that the cells were able to substitute beryllium for magnesium in the growth medium. The growth that was observed in magnesium-deficient cultures was probably due to the presence of trace quantities of magnesium impurities in other chemicals used in the growth medium in the soil inoculum or in the distilled water used in the experiment.

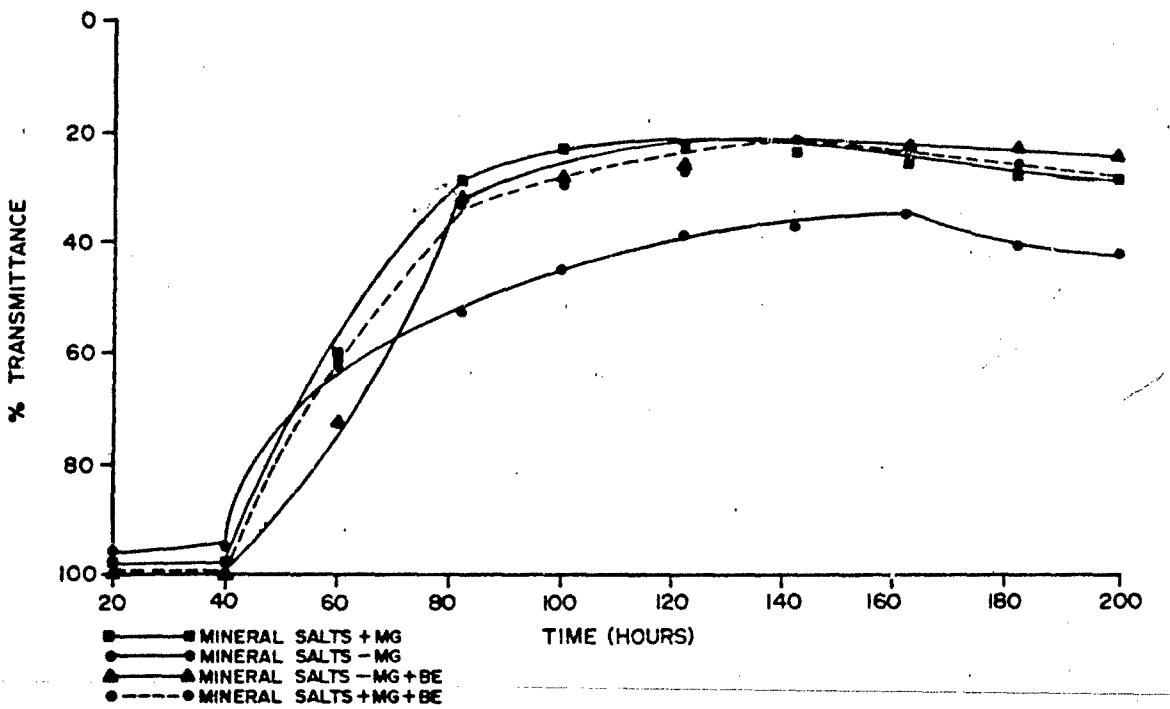

Figure 3. Effect of substituting beryllium for magnesium on a mixed bacterial culture. Each point in the average reading from five replicate cultures.

All 13 soil isolates inoculated in mineral salts medium containing either 500 or 1000 μg/ml beryllium grew well, the lag period and final cell concentration varying with each culture.

DISCUSSION AND CONCLUSIONS

The soil microorganisms isolated in this study were shown to vary widely in their ability to assimilate beryllium. This is not surprising in view of the fact that microorganisms are known to have specific affinities for a wide variety of metal ions. Some of these ions are essential to the nutrition of the microbes, serving as catalysts. Some metals are concentrated inside microbial cells while providing no apparent biological function, while others have been found to increase cellular mass. Zajic and Chiu (1971) found, for example, that whereas strontium metal merely accumulated inside cells of Penicillium, the addition of uranium to the growth medium actually increased pellet size, suggesting a biological function. Tornabene (1973) found that several lead salts in concentrations approaching solubility limits had no apparent effect on cell growth or viability of cultures of Micrococcus luteus and Azotobacter sp. From the data obtained in this experiment, it appears that beryllium can be added to the list of metal ions showing no inhibitory effect on some strains of bacteria in concentrations up to 1000 μg/ml. Manil and Straszewska (1953), however, added 0.05% beryllium sulfate to a medium used to grow yeast cells and observed the impairment of fermentative ability and mitotic cell division; the beryllium salt also appeared to stimulate multiple budding of the yeast cells.

Beryllium effects in higher animals have been attributed in some instances to competition of beryllium with essential cofactors, i.e., magnesium, which catalyze specific biochemical activities. Beryllium is known, for example, to inhibit alkaline phosphatase (Veerkamp and Smits, 1953), amylase (McGeachin, 1960), phosphoglucomutase (Aldridge and Thomas, 1966), deoxythymidine kinase (Mainig and Bressnick, 1969), and (Na^+-K^+)-activated ATPase (Toda et al., 1967).

Hoagland (1952) substituted beryllium for magnesium in a nutrient plant growth medium to determine the effect on plant growth. He observed a magnesium-sparing effect, characterized by an approximate 60% reduction in the magnesium requirement of plants in the presence of beryllium. A similar effect was exhibited in this study; soil microorganisms grown in a magnesium-deficient, beryllium-containing medium attained a greater optical density than cells grown in the absence of both beryllium and magnesium.

Considerable difficulty was encountered early in the experiment in keeping beryllium in solution. Beryllium not only precipitates out as the hydroxide in the same pH range at which most soil microbes grow best, but it also forms insoluble salts with other components of the growth medium. This became readily apparent when phosphate buffer was added to the growth medium, with the formation of beryllium phosphate occurring. When pyrophosphate was substituted for orthophosphate in the growth medium, precipitation did not occur at first, but upon standing several minutes a precipitate began to form. In addition, massive quantities of pyrophosphate were required to keep the beryllium in solution. Tris buffer was also tried

unsuccessfully. When 2 g/liter sodium citrate were added to the growth medium, it was found that this amount would maintain 100 μg/ml in solution at pH 6.8 indefinitely. It was, therefore, selected as the complexing agent of choice. It may be noted that this compound is often added to bacteriological media to make otherwise unavailable ions available by the formation of soluble, diffusible complexes.

Schubert and White (1950) proposed the use of citrates as a possible form of therapy in cases of beryllium poisoning, believing that the citrate might immobilize ionized beryllium and facilitate removal from the body via the kidneys. They found that although urinary excretion increased, survival time decreased. They, therefore, proposed that "the citrates may even enhance the toxicity of Be by increasing local concentrations of ionized Be."

Crowley et al. (1949) and Martin and Liddy (1952) studied isotope turnover in bones using both ionic and citrate-complexed beryllium. They found that both forms of beryllium were bone seekers and tended to accumulate and remain in the bones for long periods of time.

In view of these studies, it is difficult to know with certainty whether the addition of citrate to the growth medium in this experiment tied up beryllium, making it unavailable to exert its toxic effect, or whether citrate merely increased the solubility of Be and thereby its availability to soil microorganisms.

Although a soluble form of beryllium was used in this study, further studies should be performed to determine the ability of soil microorganisms to solubilize beryllium oxide, the form in which beryllium is present in rocket propellant exhausts. Certain microorganisms are known to leach various metals from rock or otherwise insoluble metal compounds (Silverman and Munoz, 1971; Arrieta and Grez, 1971; Nielsen and Beck, 1972; Silverman and Ehrlich, 1964). Since the nature and crystalline structure of a particular mineral are important in determining its susceptibility to microbial degradation, beryllium oxide would be expected to display differences in degradability, because it may be present in several stable forms which differ in refractive index, birefringence and toxicity, depending on the conditions of formation.

Previous studies using rats have already demonstrated a definite gradation in biological response depending on the temperature at which a particular sample of beryllium oxide is calcined during preparation (Spencer et al., 1967, 1968, 1972).

Many of the hazards involved in the handling of beryllium have already been recognized and stringent engineering controls imposed. Therefore, much of the occupational danger of beryllium operations has been eliminated (Donaldson, 1965; Cholak et al., 1962, 1964). However, the potential for

accidental exposure and environmental contamination still exists. Buchauer (1972) recently cited an example of such environmental contamination in her studies of the uptake of environmental pollutants by soil and vegetation near a zinc smelter plant. She found concentrations of 135,000 ppm zinc, 1750 ppm cadmium, and 2000 ppm copper in the soil one kilometer from the plant site; trees within two kilometers of the smelter contained zinc concentrations up to 4500 ppm and cadmium concentrations of 70 ppm.

These studies leave little doubt that environmental contaminants find their way into soil and vegetation and may be greatly concentrated and eventually consumed by animals and man. The data presented here suggest that beryllium may also be just such a contaminant and that appropriate methods of waste disposal of beryllium-containing compounds should be utilized to prevent passage of these compounds through the biological food chain.

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PAPER NO. 30

EFFECT OF HYDRAZINES ON MOUSE SPERM CELLS

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INTRODUCTION

Recent (P. L. 91-190, National Environmental Policy Act, January 1, 1970) and pending (Toxic Substances Control Act of 1973, S.888 and H. R. 5356) United States legislation and the increasing number of ecological disasters (Fishbein, 1973) in the past decades have illustrated a great need to understand and predict both the short and long-term effects of chemicals, radiation, and other stresses on the entire biota, especially man. This is of even greater concern in light of the ever-increasing number of new chemicals to which the human population and the environment are exposed each year. With conventional toxicological techniques it is possible to make good estimates of the short-term effects of these agents (overt toxicity, etc.). Long-term effects, e.g., mutagenicity, carcinogenicity, and teratogenicity, are considerably more difficult to evaluate and have been the recent subjects of extensive research and development in search for sensitive, rapid and inexpensive screening procedures (Hollaender, 1971). Numerous test systems based on the effects of agents on nonmammalian species (bacteria, yeast, drosophila, etc.) have indicated some feasibility as screens, but clearly an in vivo mammalian system would be preferable for extrapolation to man.

A recent report suggests that murine spermatogenesis may be applicable as a rapid and inexpensive in vivo mammalian test system (Bruce, 1973). This system makes use of the high degree of synchrony of murine spermatogenesis (Bruce, 1972; Lam., 1970), the high level of homogeneity of the physical characteristics of the mature spermatozoan head, and the ease of quantitation and the sensitivity to low doses of x-rays (Bruce, 1973).

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With this system the biological impact on the mammal is evaluated by monitoring the percent of mature sperm with abnormal head shapes. Bruce (1973) suggested that abnormally shaped sperm are the consequence of abnormal chromosome complements.

Here we report the results of investigating three rocket fuels, hydrazine (H), monomethylhydrazine (MMH), and unsymmetrical dimethylhydrazine (UDMH) with this test system. The same characteristics, however, that make them very advantageous as fuels, i.e., small molecular weight and high reactivity (Audrieth and Ogg, 1951), also confer on them considerable biological impact. The pharmacology and toxicology have been well described by Clark (1968) and Back (1970). These compounds have been reported to be carcinogens (Mirvish, 1969; Biancifiori, 1971; Evans, 1972; Toth, 1972a + b) and mammalian mutagens (Brown, 1966; Shukla, 1972; Rohrborn, 1972; Epstein, 1972) in a variety of test systems. The biological hazard of these compounds is further indicated by the considerable range of potentially genetic effects shown from studies with bacteriophage (Goldfarb, 1963; Timakov, 1963), bacteria (Lingens, 1964), isolated mammalian DNA (Banks, 1967; Mc.roson, 1969; Hawks, 1972) and whole chromosomes (Hampel, 1968; Gupta, 1970). UDMH and H have been further shown to have considerable embryotoxic and teratogenic activity in mice (Mercier-Parot, 1969).

The purpose of this study was to determine the applicability and sensitivity of murine spermatogenesis as a test system by investigating three chemicals known to be biologicaliy active. This report describes the effects of time and dose on the levels of sperm with abnormal head shapes after subacute exposure to either H, MMH or UDMH. To evaluate the sensitivity of this approach, we compared results with those obtained from more conventional toxicological and pathological techniques.

MATERIALS AND METHODS

Mice

Hybrid male mice, $BC3F_1$/CUM (C57BL♀ x C3Hf♂, Cumberland View Farms, Tennessee) ranging in age from 11 to 18 weeks, were caged in groups of 3 to 5 animals each. All animals were housed in plastic cages in air conditioned rooms with automated light-dark cycles (10:14 hours) while maintained on food and water ad libitum.

Chemicals and Injections

Test animals received a subacute exposure, i.e., five similar daily intraperitoneal injections of various doses (in fractional units of the lethal dose-50) of H, MMH or UDMH (see Experimental Design). Each dose was

prepared in 0.5 ml glass distilled water less than 1/2 hour prior to injection. The control animals received 5 daily injections of 0.5 ml of distilled water and otherwise experienced the same handling as the test animals. The one-tenth LD_{50}'s for H, MMH (Eastman Kodak Company, Rochester, New York), and UDMH (Matheson Coleman and Bell, Norwood, Ohio) were determined from the review by Clark (1968) as being 8, 3, and 12.5 mg/kg body weight, respectively.

Experimental Design

Time dependent response: Three groups of 50 mice each received a subacute insult of H, MMH, or UDMH at a dose of one-tenth the LD_{50} per day. Five animals of each group were sacrificed by cervical dislocation at weekly intervals. The body weights, the ratios of testis to body weight, the histopathology of the testes, and the number and percent of abnormally shaped sperm per cauda epididymis were scored and results compared.

Dose dependent response: Four to six mice received daily injections of UDMH at doses of one-tenth, twenty five-hundredths, four-tenths, fifty-five-hundredths, or seven-tenths the LD_{50}. At 0.8 and 3.0 weeks postexposure 2 or 3 animals at each dose level were sacrificed. Each group of animals was scored for body weights, testis to body weight ratios, testicular histopathology, bone marrow erythrocytic to myeloid cell ratios, hematocrit values, histopathology of five major organs, and the number and percent abnormally shaped sperm per cauda epididymis. The effects of two subacute exposures for MMH (0.25 and 0.4 LD_{50} per day) and 0.25 LD_{50} for H were determined by describing only the level of abnormally shaped sperm in the cauda epididymis 3.5 weeks after the insult.

Examination of Sperm Morphology

Sperm suspensions from cauda epididymides were prepared as previously described by Bruce (1973). Briefly, the cauda epididymides were removed immediately after cervical dislocation, submerged in saline (2 ml per epididymis) and cut into fine pieces. The sperm were dispersed from the tissue fragments by agitation and separated from the tissue by filtering through an 80 μm stainless steel mesh. The percent abnormally shaped sperm was determined by discriminating eosin Y-stained sperm on microscope slides. The average of 1000 to 2000 sperm were analyzed on two separate air-dried smears prepared from a pooled suspension of the sperm from the cauda epididymides of two or three mice. All countings of the sperm were conducted as blind experiments.

Four types of sperm abnormalities were observed in these experiments: amorphous, subnormal, twin-tailed, and folded. The amorphous type of abnormality is recognized by complete loss of the characteristic

sperm shape when compared to normally shaped sperm (Figure 1a). The subnormal sperm has a subnormal hook (Figure 1b) or has an abnormal midpiece attachment site (Figure 1c). In the twin-tailed type of abnormality the head often is of normal shape, but of larger overall size when compared to the normal sperm heads (Figure 1d). There may also be more than two tails at the attachment site. The folded sperm, the fourth type of abnormality (Figure 1e), has a part of the midpiece folded over an improperly developed head. Each sperm head of this type must be subnormal as well as folded under a section of midpiece. The relatively high frequency of the latter type warranted its inclusion as a separate classification. There is always some overlap among these types but generally all abnormals have a main characteristic which qualifies them for one particular type of abnormality. The definition of abnormally shaped sperm has been applied mainly to the head region of the sperm and requires for consideration that each head be attached to a midpiece.

Examination of Other Parameters

Testes weights were obtained by removal of the organs, stripping of the peripheral tissue, and weighing each testis individually to the nearest milligram. The testes were immediately fixed in Zenker-Formol solution, and later sectioned and stained with PAS-hematoxylin (Oakberg, 1955). Tubular morphology was evaluated based on the frequency of completely or partially depleted tubules, as reported for x-rays (Oakberg, 1964), and for observable histopathological lesions. Sperm numbers were determined by hemacytometer counts of the cell suspension prepared from the cauda epididymides. The body weights of 20 animals in each of the three test groups and control group were monitored from immediately after the chemical insult until 5 weeks later at weekly intervals.

The degree of anemia was estimated at sacrifice by the collection of jugular venous blood in a heparinized capillary tube for hematocrit determination. To establish the level of hematopoietic activity, sternal bone marrow smears were fixed, stained, and scored for the myeloid to erythrocytic cell ratio. The histopathologic examination of lung, liver, kidney, heart, and spleen was performed after formalin fixation, sectioning and staining with hematoxylin-eosin.

Statistical Analysis

The statistical analysis was generally limited to means and ranges. The body weight data in the time response experiments was interpreted by two way analysis of variance and the Duncan new multiple range test. The

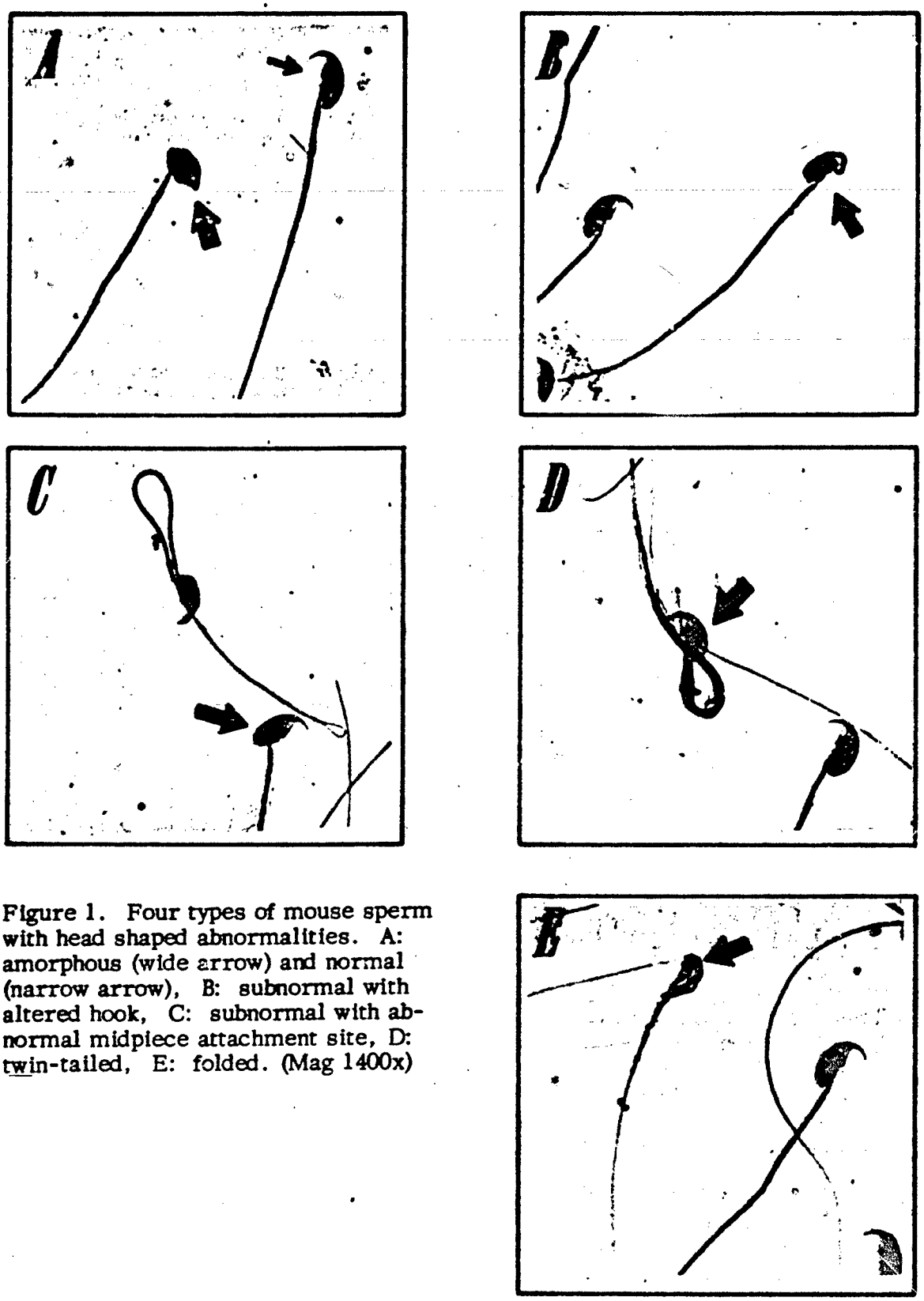

Figure 1. Four types of mouse sperm with head shaped abnormalities. A: amorphous (wide arrow) and normal (narrow arrow), B: subnormal with altered hook, C: subnormal with abnormal midpiece attachment site, D: twin-tailed, E: folded. (Mag 1400x)

comparison of the dose dependence of the cauda epididymal sperm numbers at 0.8 and 3.0 week post injections was analyzed by 2 x 6 factorial analysis (time and dose) with four events (sperm number) at each point (Winer, 1962).

RESULTS

Time Dependent Response Experiments

Effects on Sperm Morphology

The percents abnormally shaped sperm in the cauda epididymides of the chemically insulted animals were compared with results obtained from control animals at weekly intervals (Figure 2). Figure 2a represents the percent abnormally shaped sperm found in control animals. The average value of all 7 weeks of control results (1.8%) is referred to as the "no response level" or NRL. For H (Figure 2c) the percent abnormally shaped sperm increased to a maximum of about twice the NRL at 2 weeks after injection, after which it decreased but did not drop to the NRL after 7 weeks. The response to MMH (Figure 2d) was similar to the H response, with a maximum of more than twice the NRL occurring between 1 and 3 weeks after the end of the chemical insult. At week 7 the effect appears to have dropped to the NRL. After exposure to UDMH (Figure 2b), there was a dramatic increase in the percent abnormally shaped sperm immediately after the insult, reaching 5 times the NRL but rapidly decreasing to less than twice the NRL after 2 weeks, and less than 1.5 times the NRL after 6 weeks.

Effects of Other Parameters

Cauda epididymal sperm number: The range of the weekly sperm numbers was generally larger than the differences between the means of weekly values. The mean sperm numbers did not show any obvious trend during the 7 weeks for any of the test groups, remaining within the normal range of 1.6 to 2.8 x 10^7 sperm per cauda epididymis.

Testicular histology: Based on the frequency of completely or partially depleted tubules and observable histopathological lesions, the test series did not differ from the control series.

Testis to body weight ratio: The range of the ratios for the test series was very similar to the range of the control ratios and did not show obvious tendencies for the 7 weeks.

Body weights: For the 5 weeks immediately after the end of the injections, the mean body weight of each test group was significantly less ($p < .01$) than the control (Figure 3). Both H and UDMH (Figure 3b and 3c) were significantly less than MMH ($p < .05$) (Figure 3d) but did not differ significantly

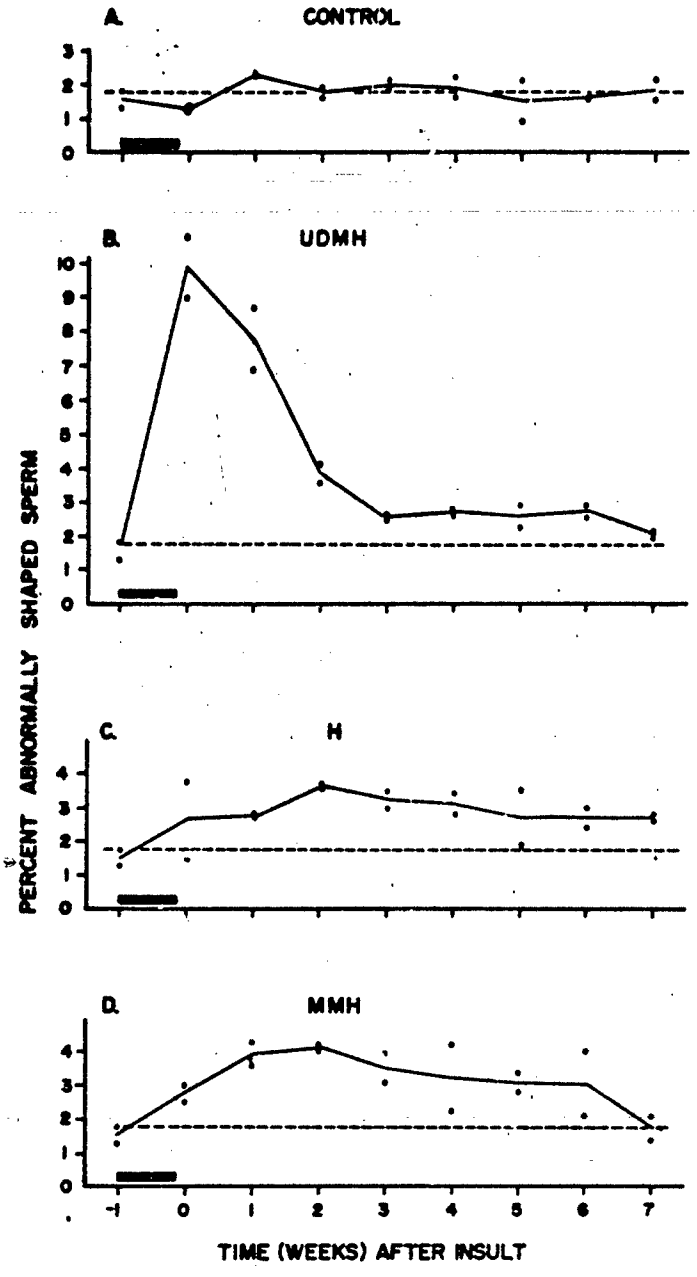

Figure 2. Level of abnormally shaped sperm in the cauda epididymis at various times after exposure to hydrazines. 1000-2000 sperm from 2 to 3 animals were counted for each dark circle. The dashed line represents the NRL (no response level), i.e., the average of the control values in Figure 2a. The length of the bar is the duration of the subacute chemical exposure. The solid line connects the averages of the two weekly scores.

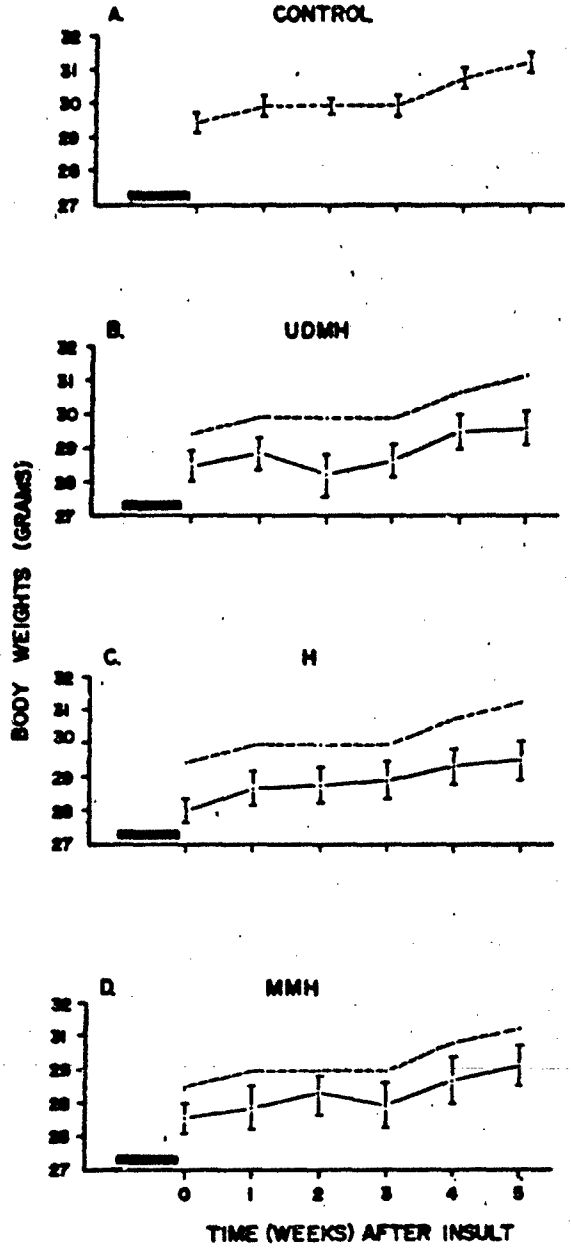

Figure 3. The body weights at various times after exposure to hydrazines. The solid lines connect the means of the body weights of the same twenty animals and are compared to the dashed line representing the control means. The duration of the chemical exposures is shown by the solid bar while the vertical lines represent the means $\pm$ standard errors.

from each other at the same confidence level. There was also a statistically significant differential effect of time among the four groups.

Dose Dependent Response Experiments

Effects on Sperm Morphology

The percent abnormally shaped sperm in the cauda epididymis (Figure 4a) showed a clear response to increasing doses of UDMH when observed 3 weeks after the chemical insults. There was no marked increase over the NRL when observed 0.8 weeks after the insult, apparently in conflict with the results of the time response characteristics shown in Figure 2b. Mass spectrometric analysis of the contents of the UDMH bottles used for the time response study indicated possible contamination of the UDMH with dimethyl nitrosoamine, $(CH_3)_2HNNO$, an oxidation product of UDMH. This possibility is further treated in the discussion. Figure 4b and 4c indicates that the percent abnormally shaped sperm increased with increasing dose for both MMH and H. Freshly distilled and unopened bottles of MMH and H were used for all experiments.

Effects on Other Parameters

During the 5 day injection period, all animals survived the lower doses of UDMH with only 2 out of 6 animals dying within 24 hours after the second and fourth injections of the daily 0.7 LD_{50}, with no further unscheduled deaths occurring.

Sperm number: The sperm number per cauda epididymis was determined at 0.8 and 3 weeks after the graded UDMH insults (Figure 5). Increases in dose had a different effect on the sperm number at 3 weeks than they did at 0.8 weeks ($p < .01$). Inspection of the data indicates that at 3 weeks the higher doses produced lower concentration numbers than at 0.8 weeks, which does not appear to be different than control values. The absolute level of the differences between 0.8 and 3 weeks was not statistically significant at the $p < .05$ level, however.

Testis to body weight ratios and body weights: There was no obvious differential effect of dose level at either time for UDMH. The range of ratios and body weights at various doses was within the range observed for untreated animals, perhaps due to the smaller groups of animals (2-3) used at each dose level.

Testis Histology: There were no obvious differences in the frequencies of partially or completely depleted tubules at either 0.8 or 3 weeks after various UDMH exposures.

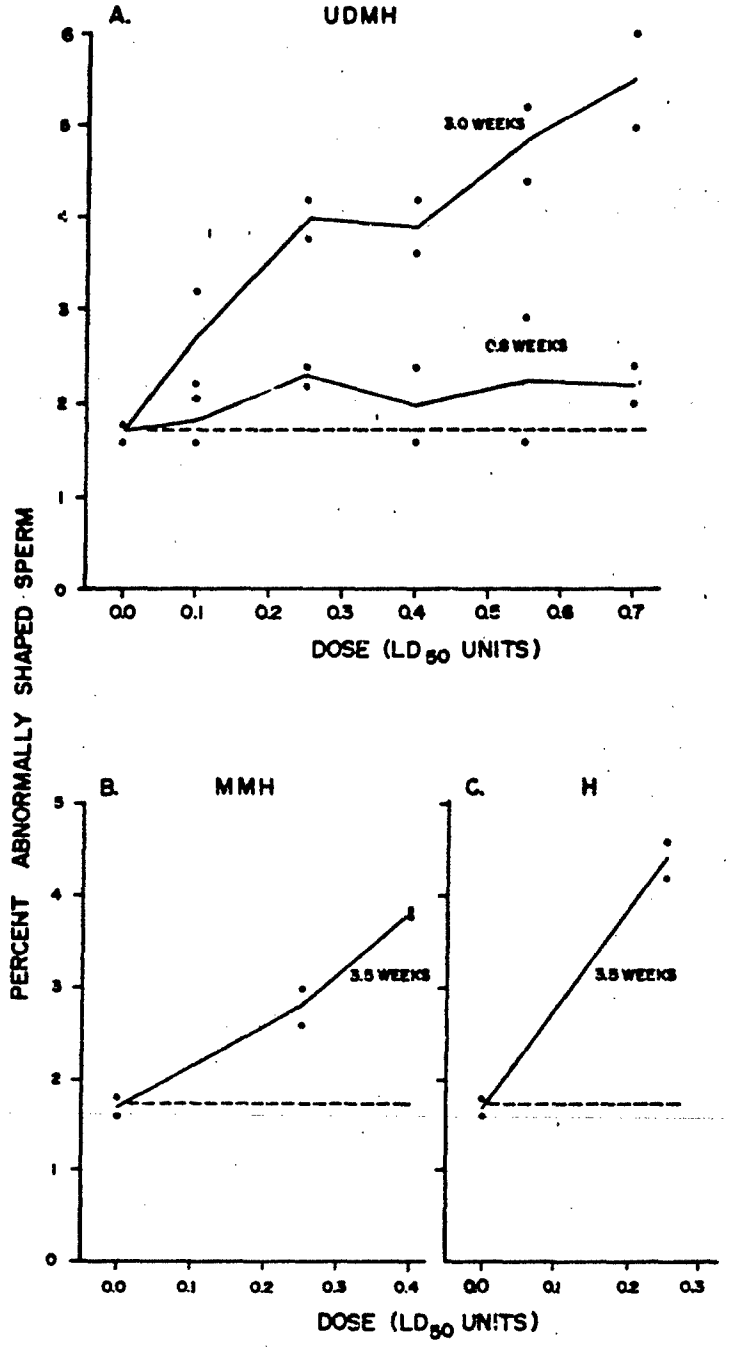

Figure 4. The effects of dosage of hydrazines on the level of abnormally shaped sperm in the cauda epididymis. 2000 sperm from 2 or 3 mice were counted for each pair of circles. Dashed lines represent the NRL.

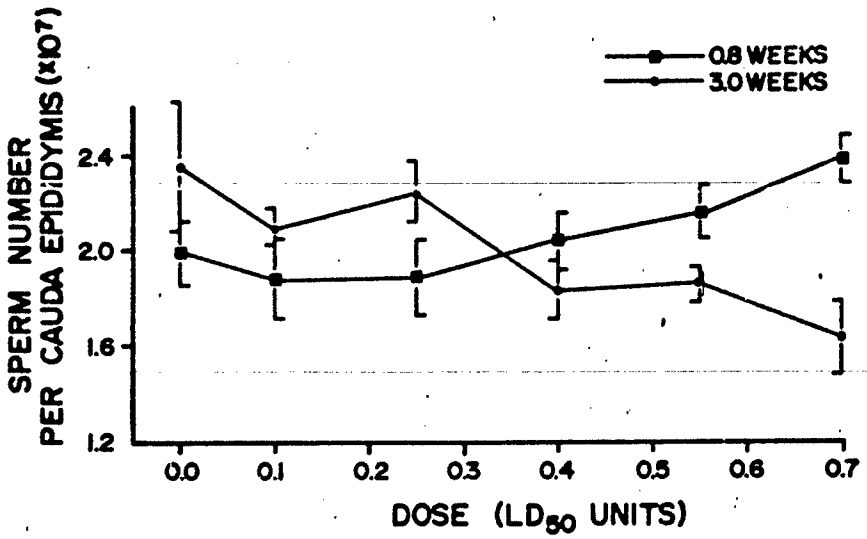

Figure 5. The effects of graded doses of UDMH on the number of sperm per cauda epididymis. The solid lines connect the average number of sperm from 2 or 3 mice. The vertical bars represent the mean ± standard error at 0.8 (flags to right) and 3 weeks (flags to left).

Conventional pathology: The hematocrit values at both 0.8 and 3 weeks were all within the normal range, indicating that obvious anemia was not observed. Comparisons of the myeloid to erythrocytic cell ratios obtained from bone marrow smears for all animals at all dose levels investigated also showed no deviations from the normal values at either time period. At 0.8 weeks, one of the animals in both the 0.25 and 0.4 dose levels showed a possible increase in the number of megakaryocytes in the spleen. One animal in both the 0.4 and 0.7 groups indicated possible cytomegaly in the liver but no other recognizable lesion was found in the remainder of the 14 animals. At 3 weeks one animal in both the 0.4 and 0.55 dose levels showed possible early suggestion of cytomegaly with nodular tendency. There were no other recognizable lesions observed in the remainder of the 14 animals. From the pathologic evaluation we concluded that there were no lesions clearly attributable to the chemical insult.

DISCUSSION

These results showed that the mammalian test system employed by Bruce (1973) for x-ray cytotoxicity in murine spermatogenesis can also be applied for chemicals. Each of three hydrazine fuels was shown to induce a unique and pronounced response in this in vivo system. The overall shapes and the maximum heights of the time dependent response curves varied for

each fuel. All three chemicals induced a maximum level of abnormalities within three weeks after the insult (Figure 2). By 7 weeks, the response to two of the chemicals, MMH and UDMH, appeared to have returned to the NRL (no response level). However, the level of the sperm abnormalities from the hydrazine insulted mice remained at 1.5 times the NRL at 7 weeks even though the response at 2 weeks was the lowest of the 3 chemicals tested. The dose response at 3 weeks after UDMH insult (Figure 4a) agreed very well with the third week time response data. The dose response indications for H and MMH (Figure 4b and 4c) were also in agreement with the time response results (Figure 2c and 2d) at 3.5 weeks after 0.1 LD_{50} exposure. These agreements give support to the ability of this test system to quantitate rather low positive responses with a high level of confidence. A discrepancy in Figures 2b and 3a at 0.8 weeks after UDMH insult raises some question as to the contribution of a contaminant in the UDMH time dependent experiments. The curves illustrated in Figure 2 were obtained by diluting the fuels using polystyrene pipettes (Falcon Plastic, 1 ml disposable) while the dose response curves in Figure 3 were prepared with glass pipettes. Therefore, a plastic related contaminant is suspected for the UDMH. Possible contaminants of UDMH were partially isolated. Mass spectrometric analysis indicated that one contaminant might be dimethyl nitrosoamine, an oxidation product of UDMH, known to have a very broad range of biological impact (Sutton and Harris, 1972). This possibility is currently under further investigation.

The shapes of the time response curves (Figure 2) for the percent abnormally shaped sperm can be interpreted by understanding the timing of sperm production. Murine spermatogenesis is a highly synchronous 31-day process from the late spermatogonia to the mature sperm in the cauda epididymis (Bruce, 1972). In the adult mammal all the many well defined intermediate cell types are always present (Oakberg 1956a + b). The time after the insult that the maximum effect will be observed in the cauda epididymides depends both on which cell type is the most sensitive to the chemical and also whether this sensitivity is manifest in an observable occurrence. The more immature the sensitive cell type, the greater the postexposure interval before the mature form of that cell reaches the epididymis and is observed. Therefore, morphological abnormalities observed within the first week postexposure reflect that the sensitive stage occurred between the last steps of spermiogenesis and the passage of cells through the epididymides. An effect observed from the second to fourth week indicates that the sensitive stage lies between the spermatogonial stage and the formation of the sperm. After the fifth week post insult, the affected spermatogonia should be the main source of any observable effect. In Figure 2 the curves for H and MMH have very similar shapes with the maximum effect at two weeks post insult, suggesting that cell stages near early spermiogenesis were affected. The immediate high response after the UDMH insult suggests that the very late sperm were most affected. All three fuels, but particularly H, appear to have a small effect on the spermatogonial stage.

The comparison and interpretation of the effects of time and dose on the level of abnormally shaped sperm strongly suggest that all three hydrazine fuels are cytotoxic to spermatogenic cells. In view of the particular dangers to the fuel handlers, understanding the time and dose effects of these fuels on sperm production may be helpful in estimating the postexposure time interval during which conception should be avoided. The results of this study would be applicable to either acute or subacute exposures.

The sensitivity of this test system to the hydrazine fuels is strikingly apparent when results were compared to those obtained by more conventional pathological and toxicological techniques. Of the eight parameters monitored in this study (number of sperm per cauda epididymis, percent abnormally shaped caudal sperm, whole body weight, testes to body weight ratio, testis histology, hematocrit values, myeloid to erythrocytic cell ratio in sternal marrow, and the histopathology of five major organs), only three (body weight, sperm number, and percent abnormally shaped cauda epididymal sperm) showed any quantitative relationship to the chemical insult. Presumably with a larger number of mice more of these parameters might have been statistically linked to the insult. As an example, the analysis of variance of the time response data of the whole body weights of 20 mice indicated that after the insult all the test groups weighed significantly less than the control group. However, all other results stem from groups of 2 to 3 mice. Perhaps most indicative of the sensitivity of this assay is the comparison of the dose response characteristics of various parameters for UDMH at 0.8 and 3 weeks post injection. Only the sperm number and the level of sperm with shape abnormalities showed a correlation with increasing dose. The sperm numbers, however, showed considerable variation requiring statistical interpretation. The differential effect of increasing dose on the level of sperm abnormalities at 0.8 and 3 weeks is obvious from just a plot of the data (Figure 4a). From these results, therefore, we can conclude that the level of sperm abnormalities is more sensitive to doses of the hydrazine fuels than any of the other seven parameters studied.

The increased emphasis given to residual effects of toxic exposures demands that sensitive methods be available to observe such effects, at least in experimental models. Murine spermatogenesis as a test system should provide such capabilities suggesting future work to categorize known toxic and mutagenic chemicals in order to establish a rating schedule.

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OPEN FORUM

DR. HODGE (University of California, San Francisco): I'd like to ask a question of Dr. London. Would you be kind enough to show the slide on UDMH dose dependence again? I'm sorry I didn't quite understand what it was that those two solid lines represented. Could we see the slide again?

DR. LONDON (Aerospace Medical Research Laboratory): The slide represents a dose response analysis made of doses ranging between 1/10 and 7/10 of the LD_{50}, and there were two groups of animals. The difference is the bottom line which were numbers taken at 8/10 of a week post insult. The top line is the analysis made at three weeks post insult.

DR. HODGE: Pardon me. I can't read the numbers on the ordinate or the abscissa and I can't even read the legend.

DR. LONDON: I'm sorry that they are not showing up. This is a dose in LD_{50}'s ranging from 1/10 to 7/10 and this is the percent of abnormally shaped sperm, ranging from zero here, up to 6%. This line is three weeks and 0.8 weeks and this is the no response level at 1.8%.

DR. DOST (Oregon State University): I would agree that spermatogenesis is a very effective model. I think something that should be remembered though is that the morphologic stages of spermatogenesis are associated very intimately with successive stages of nucleic acid synthesis and other biochemical events. These associations are highly suggestive of specific interactions which may very well be associated with mutagenic responses, particularly in terms of interaction of intoxicants or products of intoxicants with macromolecules. The second point is that the development of the sperm cell is in many respects a reasonable model of the development of somatic cells and there are some inferences that can quite often be drawn about somatic cell development from toxic effects on sperm cells. This is less a comment on the work itself than the possible extensions of the work to more specific understanding of just what the hydrazines are doing in the biologic system.

DR. LONDON: Perhaps I didn't get the point across clearly. The purpose of this study was to evaluate this technique as a tool for screening and not necessarily to use it to do molecular biology or to do mechanistic studies but to red flag materials when one is faced with a myriad of different compounds to determine if they are mutagenic or carcinogenic. You know the problems associated with exposing animals and then where are you going to keep all these animals. You run out of space and technicians and all kinds of things. The purpose of this study was to perhaps prioritize materials and to select those that, on the basis of this system, seem most likely to

be mutagenic at the lowest concentrations. That was the purpose of the study, not to go any further. My comments on doing the dominant lethal study were to correlate this with a real genetic analysis and to determine if the changes were to occur at the early stages of spermatogenesis where one might expect the target cell to be the stem cell or an early spermatogonium and therefore the effect could be a genetic one and not a cytotoxic one.

MAJOR MC NUTT (Aerospace Medical Research Laboratory): I would like to throw in a word of caution about this type of experiment where there are so many changes of very complex nature between the initial events of spermatogenesis in the evaluation of the final shape of the head. I think there has been quite a bit of study done showing that the epididymis itself affects the morphology of the sperm head and to get a persistent effect, maybe all you have to do is to affect the epididymis to get a very slow cell turnover rate and really not be affecting the primary testis function at all. I think you can't just take the cauda epididymis and count sperm and count abnormal heads and extrapolate back in a very simple way to the very early events in spermatogenesis. I think you would really have to look at a very detailed mechanism. I'm sure Dr. London realizes this, but I think that point should be made for the other members of the conference.

DR. MAC EWEN (University of California, Irvine): Dr. Toth, I tried to make some rough calculations as you went along with your presentation, and as near as I could determine, you are providing UDMH in the drinking water to the animals at about 40 to 50% of the LD_{50} dose on a daily basis. This is quite a massive dose and I can perhaps understand how you might find some of these effects, but is there a threshold for this effect? Have you studied any lower doses than the ones that you presented where you did see some profound changes?

DR. TOTH (Eppley Institute for Research in Cancer): No, you see the purpose was not to find the threshold dose or not to do dose response studies. We just wanted to know in the first stage whether the hydrazines as a class of chemicals were carcinogenic and out of these 20 chemicals how many caused cancer. Obviously, we give the chemicals at the maximum tolerated dose. In the second stage, you see, we are interested more in the problem and we would like to determine the dose response but so far we have not studied it.

DR. MAC EWEN: Did you do any other tests besides what you mentioned, like hematology studies?

DR. TOTH: No, as you may realize, we are not interested in the acute effects. We are only interested in acute effects as far as the dose is concerned. Basically, we are interested in the effect of life span administration of the chemicals.

DR. MAC EWEN: In our work with methylhydrazine and others' work with the hydrazines, we produced anemia at the dose levels you were working with. These animals should be very anemic. I wonder if this might have had some influence on the responses you have seen.

DR. TOTH: I think some of the animals were anemic with certain chemicals, especially those which induce blood vessel tumors, but I would not say that all the compounds produced anemia. But we were not actually interested in this point.

DR. MAC EWEN: I'm referring in particular to the three rocket fuel hydrazines.

DR. TOTH: I don't remember. I can check my record and I can write you a letter.

DR. MAC EWEN: The other thing I wanted to ask is do you have any information from your research or know of others' work that has shown that a single or few isolated exposures to the hydrazines are carcinogenic?

DR. TOTH: First of all, I haven't done such an experiment and I don't know that anyone else has done such things. I don't think so. My guess is that the so-called strong carcinogens like 1, 2-dimethylhydrazine, 1, 1-dimethylhydrazine and possibly others, when given in single doses, are also tumor producers. But I would doubt that compounds like hydrazine itself or monomethylhydrazine would be carcinogenic. However, I have not done such studies.

MAJOR MC CONNELL (Aerospace Medical Research Laboratory): I have a question concerning your vascular lesions in the liver. Was this the only organ you saw those lesions in?

DR. TOTH: You are talking about the angiosarcomas. Those were multiple lesions. I showed a slide on 1, 1-dimethylhydrazine where we have observed them in at least six or seven different organs.

MAJOR MC CONNELL: What organs are those?

DR. TOTH: I have shown slides of the liver, the lungs, the para-renal tissue and sometimes we found the lesions in the vicinity of the uterus, around the paraepididymal tissue, fat tissue, muscle and so forth.

MAJOR MC CONNELL: Of course, all I saw on the screen was the liver.

DR. TOTH: Yes, you see, I had no time during 25 minutes to show all the tumors and all the lesions in the different organs, so I have just concentrated on showing only the lesions mainly in the liver.

MAJOR MC CONNELL: None on the skin?

DR. TOTH: I don't remember now because I have obtained angiosarcomas from 1,2- and 1,1-dimethylhydrazine, ethylhydrazine and also from phenylhydrazine. I don't remember which compound but I do remember that I got a few lesions, angiosarcomas in the spleen but I don't remember with which compound now.

MAJOR MC CONNELL: Are the tumors invasive looking in the other organs?

DR. TOTH: Yes, there is no question that they are malignant tumors.

DR. CROCKER (University of California, Irvine): Further to your discussion just now, Dr. Toth, I'm not aware of other carcinogens producing these vascular tumors which are a fascinating tumor to me. One of the things I am curious about in response to Dr. MacEwen's question as to whether or not anemia was present is whether or not these are initiated by a kind of metaplasia of marrow-like tissue forming blood in the face of the bone marrow depression as may sometimes occur with metaplasias in other organs. Do you know of other types of carcinogens that would do this?

DR. TOTH: Yes, but I don't know of other classes of chemicals that would induce angiosarcomas of the blood vessels in such high incidence. One of the classical experiments I think was done by Dr. Harold in hamsters. I think it was done with diethylnitrosoamine and she obtained a high incidence of malignant blood vessel tumors. Also, as you may remember, you can induce blood vessel tumors, benign and malignant, in various animal species with urethane and a few other compounds but I do not know any of the other chemical carcinogens which would induce something like 90 to 100% incidence of tumors. As to your second point, I have not seen any lesion in the bone marrow because we only took routine sections. Again, I want to say that we had no intention to look at acute effects. We did not kill the animals. We let them live until they died or until they were in the terminal stages and killed them, but we looked for metaplasia.

MAJOR MC NUTT: Dr. Toth, I was perplexed by the alkaline phosphatase data that you gave, you went over it rather rapidly so maybe I missed something, but your control alkaline phosphatase in liver looked negative to me and there should be abundant alkaline phosphatase in the normal liver.

DR. TOTH: I showed the slide with the tumor. It was very strongly positive and in the following slide, I showed you a normal control Swiss liver, and there was one very small area we could call positive but the comparison was striking and obviously there is much more activity in the tumor. I don't know what it means.

MAJOR MC NUTT: The hepatocytes are very well known to have abundant alkaline phosphatase around the bile canaliculis, so that every hepatocyte in the normal liver should have alkaline phosphatase activity.

DR. TOTH: If this is so, we would not be able to show it on the slide with our technique.

MAJOR MC NUTT: I would suggest that something is wrong with the technique.

DR. TOTH: I have shown you the average liver, the normal liver, and then I showed you the tumorous liver.

MAJOR MC NUTT: I guess the point is that I'm not convinced that the tumor tissue that you are showing is really tumor tissue and that the alkaline phosphatase positive cells that you are saying are tumor cells may be damaged hepatocytes.

DR. TOTH: Then why can you not see it in the normal hepatocytes?

MAJOR MC NUTT: I think we would probably have to look at your technique. Many, many people have published good light and electron microscopic evidence for alkaline phosphatase in the normal liver and I think that if your control normal liver doesn't show normal alkaline phosphatase activity ...

DR. TOTH: Now, wait a second! My normal control liver did show some alkaline phosphatase activity but not as much as the tumor tissue. I have shown in the slide that we had activity but not as much as in the tumor tissue. I'm very, very convinced about this, sir.

MAJOR MC NUTT: Maybe I could take a look at your slides afterwards.

DR. TOTH: Yes, and also we took both slides with the same magnification.

MR. VERNOT (University of California, Irvine): This is for Dr. London. Did you also add the extra citrate to your control solutions as you had to add to your beryllium solutions?

DR. LONDON: I believe so. I would have to check back, but I believe so.

MR. VERNOT: If you didn't, this might explain the lag time you saw. It might act as a growth suppressant at that level or something of this sort.

DR. LONDON: Generally, citrate enhances growth by making some of the ions more readily available.

MR. VERNOT: But you can't say that a priori.

DR. LONDON: No, you cannot, but I think the difference here is that we used a rather enormous concentration of citrate as compared to what one normally sees in bacteriological media. I believe that both sets were identical with the exception of the beryllium.

MR. VERNOT: The other question was about the rather rapid disappearance of beryllium. Once you achieved a certain cell population, I wonder if they couldn't have scavaged all the magnesium and then at that point, all the cells grabbed on to the beryllium.

DR. LONDON: That certainly is possible although it happens very rapidly, there was a precipitous fall from one analysis to the next. It was all gone, but that is possible. One would assume that there was enough magnesium in the culture to last for the nominal length of time the culture remains in a viable metabolizing form.

MR. VERNOT: You didn't do supernatant magnesium analysis?

DR. LONDON: No, we did not in that experiment.

DR. THOMAS (Aerospace Medical Research Laboratory): I have one question for Dr. Toth. What happens to hydrazines in the environment? I think Mr. Vernot can elucidate on what happens to monomethylhydrazine in air. What's the half life of it? Do you ever analyze your solutions and see what your animals are really drinking? Is it the original compound you put in there or is it something else?

DR. TOTH: As you may know, in our Institute we have something like 160 people and half of them are organic and biological chemists. Some of the hydrazines that I have been working with have been synthesized by our chemists. Obviously, they are not 100% pure but they are something like 99%. Some of the hydrazines that I am using like methylhydrazine, hydrazine, SDMH and UDMH are commercial preparations. Again, I don't remember exactly but usually I'm consulting with the chemists and I ask

them, "How pure and how stable is this one?" Also, as you may have noticed from my presentation, we gave these hydrazines in the drinking water, in the tap water, and we make fresh solution on Monday, Wednesday and Friday. Periodically, the chemists checked it and they said there was no problem, there was no decomposition.

MR. VERNOT: We've done some work in that area and our indications are that at concentrations not too far removed from the ones that you are speaking of, we get at least a 33% loss in 24 hours with freshly prepared MMH in tap water.

DR. TOTH: Monomethyl, I don't remember. I think it was 0 to 0.1%. I don't remember exactly. Do you also find this with MMH sulfate?

MR. VERNOT: At that concentration it doesn't make any difference what form you put it in, the pH of the water is going to determine what form it is.

DR. TOTH: I did not mention another point. As you know, before we started the studies, we discussed the problems of how we should give the hydrazines to the animals. At that time, there were already a number of publications on the subject. Some people gave some of the hydrazines by subcutaneous injection or intraperitoneal injection. We decided, after looking at the whole class of hydrazines, not just rocket fuel components but the whole class, that we would be much better off giving all the hydrazines uniformly. We did not want to start experiments in which we would give MMH subcutaneously and another orally and so forth. We also realized that this may not be the best method when it comes to decomposition but we had to compromise on how to deliver the hydrazine. Obviously, if you gave MMH by subcutaneous or intraperitoneal injection, decomposition would be minimized or zero. I would be very happy if some of you or other people would do the same experiment with this route of treatment. We don't plan to right now.

DR. THOMAS: I would like Dr. Back to describe some of the historical findings using hydrazines in the buffered and unbuffered states.

DR. BACK (Aerospace Medical Research Laboratory): I think it is pretty obvious that UDMH is catalyzed almost immediately by copper and so is hydrazine so that when you put them in tap water something happens. Within a matter of a few minutes, at almost all dose levels, you don't have the compound any more. You are not exposing your animals to hydrazine or to UDMH. They are rapidly decomposing to something else. If you look at the literature values for SDMH LD_{50}'s which have been published over the years, you will notice that there are two widely diverse toxicity levels quoted for the compound and there's a pretty good reason for it. Much of the work had been done in buffered solutions. As soon as you change the pH and the

buffering capacity of the solution, you automatically change the whole physical chemical characteristics of the compound. One LD_{50} for SDMH is approximately 900 mg/kg. The other one is something like 30 mg/kg. You see the point of the matter is that when you blithely say that I exposed animals to UDMH at 0.001% in their drinking water for their life span, you are not really exposing them to UDMH. You are exposing them to something else which is a breakdown product of that compound.

DR. TOTH: First of all, we do not do LD_{50}'s because for a long-term experiment, they are almost useless. Would you like to comment on that?

DR. BACK: LD_{50}'s, that's not my point. The LD_{50} has nothing to do with it. My point is that the compound is different. What you have to do when you do a feeding experiment with UDMH is to feed the animal the compound. That means that every day you should change your water, not every fourth day.

DR. TOTH: You just said that was impossible.

DR. BACK: I didn't say it was impossible. It can be done, but you have to buffer the water at the right pH so that at least 95% of it is still the compound you are talking about.

DR. TOTH: Do you think humans are exposed to this buffered solution you are talking about?

DR. THOMAS: No, man does not drink water containing hydrazine.

DR. TOTH: Well, I accept your reasons about the solutions, but I don't think that you should have the idea that we wanted to do this experiment because we wanted to prove that rocket propellants are carcinogenic. As a matter of fact, I learned later when I was in the process of this experiment that these compounds are used as rocket fuels. Our interest was not to prove that these compounds are not safe. It was a different reason and I have told you this. I am going to check out the 1,1-dimethylhydrazine in tap water when I go back and I will write you about it.

DR. BACK: My only point is that if one says that a compound is the most potent carcinogenic agent around as you have said that 1,1-dimethylhydrazine is. You have said that quite a few times now.

DR. TOTH: I've said it and I'll say it because in addition to our work as I have mentioned, Dr. Roe in England injected 1,1-dimethylhydrazine and I assume that he injected 1,1-dimethylhydrazine and not the breakdown products.

DR. BACK: You can't necessarily assume that.

DR. TOTH: But he did get lung tumor induction with the 1, 1-dimethylhydrazine, not by orally drinking water, and I assume that he used a fresh solution but when you inject, you cannot claim what I have claimed about lifespan effects. Second, Dr. Druchi, who also does very, very careful work, came to the conclusion at the end of his rat study that 1, 1-dimethylhydrazine is carcinogenic since it induced liver cell carcinoma. I assume that Dr. Druchi also injected the chemicals. I think it was intraperitoneal injection.

DR. BACK: In going over the literature, very often you can't find out how the chemical agents were prepared and given to the animals. When we exposed animals for 6 months to hydrazine vapors, we didn't see a single lesion that looked like a tumor anywhere. We never had, nor am I aware of any work that has ever shown it by the inhalation route. Now, this doesn't mean that in massive, massive doses you might not get a tumor sometime but I would still say let's make sure that we are exposing the animal to the unchanged compound and not some byproduct.

DR. TOTH: What are you talking about, massive doses? I don't know what you're talking about. What do you mean massive doses? Do you call 0.001% in drinking water a massive dose?

DR. THOMAS: Did you calculate total dose given to the animals during their lifetime?

DR. TOTH: Yes. I don't remember what it was but you can find it in my paper.

DR. THOMAS: I am surprised that at that dose level you didn't induce convulsions in your animals.

DR. TOTH: We did!

DR. THOMAS: You did! You didn't report that finding in your paper.

DR. TOTH: I don't remember. I mean I'm not interested in those effects. As you know, we cannot do epidemiology and we cannot do everything in one study. I'm an experimental pathologist. I just wanted to find out, as I have told you, whether this compound at the maximum tolerated dose given orally for life induces tumors or not. I can ask hundreds of other questions. I am not trying to say that we have an answer to all of those.

DR. THOMAS: I don't want to make this look like a debate. My point is that I'm in a position where I'd be aware of any workman's compensation

litigation or claims from the corporation who has been manufacturing this product for the past 15 years, and there have been none. The ultimate point I am trying to make is what I tried to make yesterday and the day before yesterday, that for a yes or no answer, maybe we are putting ourselves in a narrow corner. Is something carcinogenic? Yes, you can get a yes or no answer, but is it realistic?

DR. TOTH: From my studies and other studies, I think I can say safely that the hydrazines as a class, not every single hydrazine but probably 75 or 80%, are carcinogenic.

DR. THOMAS: But are they a toxic hazard?

DR. TOTH: That's your job to find out.

DR. CROCKER: At a moment when there seems to be a genuine national concern for the hazards that may result in the use of these agents, we are encountering the absolute statement of carcinogenicity based on long-term chronic feeding at something near half the lethal dose or at least at the maximum tolerated dose. This is contrasted with the hazards that may result from episodic individual short-term exposures in the industrial process or let's say manufacture or shipping and the issue gets to be really the very difficult one that is being widely faced, namely, is there a threshold limit of tolerability for an agent that has been shown by one rather rigorous test to be carcinogenic when the needs to use that agent have overriding benefits. The risk may have to be undertaken in which case one has to estimate how big a risk one can permit. The value of your work, Dr. Toth, would be very much increased if in the future you would look at the long-term effect of a single high dose. Is that possible? In other words, can you undertake such experiments or do you plan to?

DR. TOTH: Yes, it's possible if we want to spend another 10 or 15 years of our lives. As you know, to do these studies, we use a hundred animals of each species. We conduct a very careful histology study and the studies are very, very time consuming. I have been doing this experiment now almost for 6 years and to finish the other 11 or 12 compounds will take probably another 5 years, so that's 11 years. If you want me to go ahead with at least a few of the compounds, that may undergo degradation, and it may take another 5 or 6 years. I don't know whether I want to spend 15 or 16 years of my life with this problem. I am more interested in working on the inhibition problem and trying to reverse the toxic and the tumorigenic effects of these chemicals. In this case, I will have to inject the chemicals subcutaneous or intraperitoneal because we cannot do the long-term inhibition experiment using drinking water, so probably my answer is no rather than yes.

DR. CROCKER: Not to press you necessarily to undertake such experiments but rather to recognize that this is an important question, it would seem that it would be a prime object for that kind of determination that would resolve whether or not single exposures are ultimately carcinogenic although chronic long-term feeding may cause cancer. In short, we don't want another abandonment of a valuable compound on the strength of an all or none statement when the ultimate statement is based on doses not likely to be encountered by several orders of magnitude in any ordinary industrial episode.

DR. TOTH: To tell you honestly, I really don't worry too much about these three hydrazines that you people worry about here which are used in the rocket fuel. I worry more about those hydrazines which are used as drugs. I have shown in my slides only four which are known or are still used as drugs, but there are many, many more which I did not mention today which I am planning to work with in the future and which are used today for the treatment of various problems. So I think if I am going to do more work with the reactive single injection or repeated injections, I would probably like to start with those compounds which are injected into human beings. But first, I want to know how many of those are carcinogenic by repeated injections and I would say several dozen hydrazines are used as drugs today.

MR. WANDS: I would like to tell you, Dr. Toth, that I am very pleased that you are interested in the drug situation. I do feel that the rocket propellants are very important, particularly to this group, in their responsiveness to national needs. I think in terms of the overall picture that the hydrazines may well be a relatively minor part of public problems. In light of your interest in drugs, would you care to comment on the use of hydrazine sulfate as a cancer chemotherapeutic agent?

DR. TOTH: Well, as you know, it is well known in cancer research that many compounds which were shown to be tumor producers were picked up originally because they were used in chemotherapy. So these are always very powerful toxic substances. We can probably generalize, but it is not 100% true, that most of the chemicals which are very toxic may also produce cancers. So apparently there is a parallel situation going on. These are powerful chemotherapeutic agents. We should not make a generalized rule, however, when you come to the point that people, especially at a young age, are treated with compounds for various reasons which are carcinogenic. We should press to have these agents removed from the market. However, if the same agent is given to a person who is sixty years old, I would not worry that the person could get cancer 35 years later.

MR. WANDS: Would you also then say that not only is the difference between this use of hydrazine sulfate a function of the age of the person receiving it but is it also a function of dose? I don't know what doses are used in this form of chemotherapy. I haven't been able to check that out yet.

DR. TOTH: Well, there is no question that it's a function of dose also. I know that you toxicologists always come back to the question of dose and this is a well known problem, but you should realize that when we treat a mouse or a hamster or a rat for 2 years, we exaggerate the dosage and give a much higher dose than the toxicologist would. I am talking about repeated exposure, you see, because you obviously cannot treat a mouse or a hamster for four years and then observe for another 30 years, so we exaggerate the dose in order to get a maximum effect.

DR. LEWIS (National Institute of Occupational Safety and Health): I'm a little puzzled by the question of the instability of some of the hydrazines in the aqueous solutions because of the high humidity of the respiratory tract. I'm not too sure in certain cases of the kinetics of these compounds, how rapidly they break down. Is it perhaps not the case that you may be studying similar things inhaled and degraded in the humidity of the respiratory tract and the residence times of some of these elements within the lungs as compared to your drinking solutions. I direct that either to Dr. Back or anyone who would care to answer, but empirically I would think this is a relationship that should be considered and that rebreathing experiments or something like that could be used to investigate this problem. Do you have knowledge of some of the degradation products?

MR. VERNOT (University of California, Irvine): We really didn't investigate that very deeply. The only thing that we have noted is that during times of relative humidity, the concentration of water vapor in the air very strongly affects the rate of degradation of monomethylhydrazine. We found that when we were introducing MMH in the chamber in our acute experiments at high concentrations we had to use dry air because MMH is unstable in moist air. This is at several hundred parts per million.

DR. MAC EWEN: Is the reaction rate in air considerably slower than that in water with MMH?

MR. VERNOT: It depends on a whole lot of factors such as concentration, temperature, and relative humidity. Making a comparison between the time of degradation in air and time of degradation in water is almost impossible.

MR. HAUN (University of California, Irvine): At approximately 50% relative humidity, you lose half of the MMH concentration you put into a chamber, but knowing that, you can achieve the desired concentration. One can lower the humidity or simply put in more MMH. You can also increase airflow rates to reduce the time available for MMH degradation so that it reaches the lung unchanged.

DR. LEWIS: I would like someone to comment on the fact when it's in the human body and exposed to 95% relative humidity, the MMH is not all breathed in and out of the lung. There's an equilibrium. Some of the air has a longer residence time. And then you also have a wet surface in the respiratory tract. Are you or aren't you studying similar compounds with inhalation exposures and in the drinking water study?

DR. BACK: We have labelled MMH and UDMH with carbon 14, and we do know that it's metabolized by the body. However, most of the UDMH is gone in 24 hours. That is, most of the nascent compound is. The labelled carbon comes out of the body in a number of ways. One of the ways for MMH is as methane which comes out in the breath and you can measure that. The radiorespirometry work that has been conducted at Oregon State over the years has shown that also. Early after exposure to the compound, you get roughly 50% going around in the bloodstream as MMH or UDMH. The other 50% is something else. We don't know what it is. But it's something else. There are also at least 3 good size slugs of something coming out in the urine, some of which is unchanged compound. Some MMH or UDMH is excreted in the urine almost immediately. So we do know it's changed, but not all of it's changed, and I might add that there is plenty of evidence that it certainly doesn't go to a nitroso compound. We should see hepatotoxic effects. The nitroso compounds are the most hepatotoxic agents we know of. And if they were formed, we should have seen massive liver damage with UDMH. Nobody's ever seen any liver damage with UDMH. So I think this is evidence that we are not breaking down in the body.

MR. VERNOT: We made some analyses of the oxidation products of MMH. A good part of it goes to methane which accounts, on a molar basis, for about 25% of the lost MMH. Eighty percent of the lost MMH can be accounted for as gaseous nitrogen. So most of the MMH seems to break down into gaseous products that are completely innocuous from a toxicological standpoint. That is in the air. We've also found such materials as formaldehyde, which is a very important breakdown product. Theoretically, the formaldehyde and MMH could condense to form all kinds of materials of increasing complexity. Now the only evidence we have for biological reaction in the body is the work that Mr. Harry Leahy did in blood. He found that the same amount of methane is formed by the oxidation of MMH in blood as was formed in air. These experiments were conducted in vivo.

MR. DI PASQUALE (University of California, Irvine): Perhaps I could make one comment to try to answer your question. Captain Gormley mentioned earlier this morning that the presence of copper ion causes a considerable change in the rate of oxidation of MMH. For your analogy to be considered completely true for comparing MMH in drinking water with inhaled MMH, you would also have to make the assumption that ionic content would be comparable. We are also talking about a considerable number

of different ions in drinking water that wouldn't be found in water vapor within the respiratory system. Nitrates for one. Imagination could tell you what those could lead to with the amines.

DR. MAC EWEN: It is now time to bring this meeting to a close. I would like to express my appreciation for the expert technical assistance provided by the attractive young ladies at the desk and by the staff providing the audio-visual support. I'd also like to thank all of the speakers and participants on behalf of the University of California and the Air Force for having made this an excellent conference and we look forward to seeing you next year. Thank you.

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