AL/OE-TR-1993-0059
NMRI-93-18

PROCEEDINGS OF THE 1992 CONFERENCE ON TOXICOLOGY: APPLICATIONS OF ADVANCES IN TOXICOLOGY TO RISK ASSESSMENT

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TOXICOLOGY DIVISION

JANUARY 1993

FINAL REPORT FOR THE PERIOD 19-21 MAY 1992

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AIR FORCE MATERIEL COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433-6573

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 1993	Final - 19-21 May 1992

4. TITLE AND SUBTITLE
Proceedings of the 1992 Conference on Toxicology: Applications of Advances in Toxicology to Risk Assessment

5. FUNDING NUMBERS
Contract F33615-90-C-0532
PE *62202F*
PR *6302*
TA *630200*
WU *63020002*

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
ManTech Environmental Technology, Inc.
P.O. Box 31009
Dayton, OH 45437-0009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AL/OET
Armstrong Laboratory
Wright-Patterson AFB, OH 45433-6573

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
AL/OE-TR-1993-0059
NMRI-93-18

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The Conference on Applications of Advances in Toxicology to Risk Assessment covered a multitude of topics with a wide range of interest. Topics presented by noted scientists in their fields of expertise included the role of the military in accelerating the phaseout of substances that deplete the ozone layer, policy considerations in alternative methods and approaches to the use of animals in toxicology research, the application of pharmacokinetics and physiologically based pharmacokinetic models in chemical risk assessment, selected mechanisms in carcinogenicity, and advances in the assessment of toxicological end points. A poster session presenting research in risk assessment/toxicology complemented the platform presentations. The Air Force, Army, and Navy supported the Conference that was attended by representatives from the government, industry, and academia.

14. SUBJECT TERMS
See Reverse

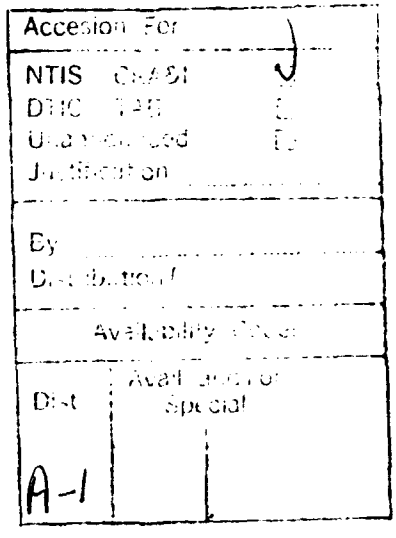
15. NUMBER OF PAGES
286

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

14. Subject Terms

Air Force halon uses
Air Force Regulation 19-15
Airway epithelium
Alternative end points
Alternatives
Animal activists
Animal issues
Animal patents
Animal research
Animal rights
Animal use
Animal welfare
Behavioral toxicology
Biologically based model
Biologically based risk assessment
Cancer risk assessment
Carcinogenesis
Cell cycle
Chemical risk assessment
Chemical spill response
Chromium
Combustion toxicology
Committee on Toxicology
Cytochrome $P_{450}4$ family
Department of Defense
Developmental toxicity
Dichloroacetate
Emergency response
Endotoxin
Environmental assessment
Environmental law
Ethics
Ethyl acrylate
Exposure assessment
Extrapolation
Fluorocarbon alternatives
Growth factors
Halon 1211
HCFC-123
HCFC-124
HFC-125
HFC-134a
HCFC-142b
Health risk assessment
Hepatocarcinogenicity
Hepatocytes
Hydrochlorofluorocarbons
Hydrofluorocarbons
In vitro methods
Installation restoration program
Intermittent exposure
Kinetic model



Liability
Liquid gun propellants
Methods
Military toxicology
Mucous cell metaplasia
Neurotoxicity
Nonmutagenic carcinogens
Ozone
P450
PBPK modeling
Perfluorohexane
Peroxisome proliferators
Pharmacokinetics
Pharmacokinetic models
Physiological modeling
Physiologically based pharmacokinetic modeling
Polycyclic organic matter
Quantitative risk assessment
RA/RM model
Rats
Regulation
Remediation
Risk assessment
Risk management
Screens
Site-specific
Short-term exposure guidelines
Smoke toxicity
TCDD
Test guidelines
Tier-testing
Toxicity testing
Trichloroacetate
Trichloroethylene
Tumor promotion
Uncertainty in cancer risk assessment
UPitt method
Validation

PREFACE

The Conference on "Applications of Advances in Toxicology to Risk Assessment" was held at the Hope Hotel and Conference Center at Wright-Patterson Air Force Base, OH, from 19 to 21 May 1992. The conference was sponsored by the Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory; the Naval Medical Research Institute Detachment (Toxicology); and the Army Biomedical Research and Development Laboratory. The conference was coordinated by ManTech Environmental Technology, Inc., under Department of the Air Force Contract No. F33615-90-C-0532 (Task No. C03). Lt Col James N. McDougal served as Contract Technical Monitor.

Attended by over 150 representatives of government, industry, and academia in the fields of toxicology and risk assessment, the conference featured invited presentations by noted individuals in the field of toxicology plus a poster session on studies relevant to the conference theme. Sessions were held on

- Health Assessment of Halon Replacements
- Alternatives to Animals in Toxicology Research
- Applications of Pharmacokinetics in Risk Assessment
- Selected Mechanisms in Carcinogenicity
- Advances in the Assessment of Toxicological End Points

The papers in this volume span this wide range of topics and hopefully will be of interest to individuals in the fields of toxicology and risk assessment.

We would like to thank the authors for contributing a written document as well as a presentation at the conference. We would also like to thank our colleagues who reviewed the manuscripts, Pam Denton of ManTech Environmental Technology, Inc., for technical editing and Patty Fleenor of ManTech Environmental, for her invaluable assistance in coordinating the review, compiling, and editing processes that resulted in this proceedings. We also would like to thank Lois Doncaster, Jim Stokes, and the members of the ManTech Environmental Toxic Hazards Research Unit for the preparation and coordination of the conference.

The full proceedings of this conference will be published by the journal *Toxicology Letters* as a special issue in May 1993 (Vol. 68).

TABLE OF CONTENTS

(continued)

TABLE OF CONTENTS (Continued)

(continued)

TABLE OF CONTENTS (Continued)

INTRODUCTION

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The Air Force has been involved in the sponsorship of a toxicology conference since 1970. The conference provides an opportunity for issues of interest to the military to be presented and discussed by a wide range of government, industry, and academic scientists who normally would not have the opportunity for this type of interaction. In 1977, the Navy became a sponsor of the conferences, followed by the Army in 1991. All three of the armed services have toxicology personnel stationed at Wright-Patterson Air Force Base, Ohio, as part of a new triservice toxicology effort to promote collaborative studies and eliminate duplication of effort.

The diversity of the topics covered in this proceedings is a reflection of the wide range of interests of the triservice conference planning committee. The 1992 conference attempted to look at as many areas of interest as possible. The committee consisted of the session chairpersons and cochairpersons under the direction of the conference cochairperson and conference coordinator. All three branches of the military were represented on the committee. This committee approach ensures that the conference is a triservice or military toxicology effort.

Military toxicology has many of the same problems and concerns found in the general field of toxicology. However, military toxicology is also faced with unique materials and exposure conditions. The term "environment" to a military toxicologist may mean the cockpit of an aircraft, the inside of a submarine or battle tank, a restoration site on a base or post, a maintenance facility, a flight line or flight deck – and then there is the environment during actual combat. Public concern and legislation have caused the military toxicologist to expand the traditional approaches for determining chemical hazard in all environments and to seek novel ways of applying toxicology to risk assessment.

The first topic presented in this proceedings is of international importance and addresses the role of the military in accelerating the phaseout of substances that deplete the ozone layer. The military uses an extensive volume of halons for fire extinguishants. Efforts are under way by the military to implement toxicology research programs that both complement and augment the scientific work that is being performed by the Environmental Protection Agency and the chemical

industry. The goal is to provide essential information that will be useful for assessing the human health risk of halon replacements.

Another major concern of the military is the use of animals in toxicology research. There is great pressure to find alternatives to current animal models. The pressure is not all external, either, as there are many highly active military programs established to develop *in vitro* screens and assays to limit the use of animals. This topic was considered to be of such importance that a session of the 1992 conference was devoted to animal alternatives and ethical considerations as represented by six manuscripts in this proceedings.

Physiologically based pharmacokinetics (PBPK) is a relatively new area in toxicology and risk assessment but an area that the military has emphasized in its toxicology program. The Air Force, in the mission statement of its Hazard Assessment Branch, states that the toxicologist will work to implement kinetic strategies for use in hazard assessment decision making. Presentations on PBPK models are almost a requirement at each year's conference.

The last two sessions dealt with mechanisms in carcinogenicity and the assessment of toxicological end points. The current knowledge in these areas is changing rapidly and novel approaches are being investigated. These sessions were chosen so that a number of different topics could be presented to describe state-of-the-art techniques and new concepts to all of the committee members and military toxicologists working at Wright-Patterson Air Force Base.

The military will continue to develop new materials and weapons systems to meet the needs of national security. Military toxicology will continue to define the toxic hazards associated with the fuels, chemicals, and structural materials in advanced weapons systems. Methods are being developed to screen chemicals and materials as early as possible in the developmental process. This requires new techniques, tiered approaches, and a thorough understanding of basic mechanisms of action at all levels of interaction in the body. These proceedings represent areas of current interest in military toxicology and are in no way totally inclusive. It is hoped that the continuing forum provided by this conference will prove beneficial in promoting the incorporation of the scientific state-of-the-art into chemical hazard assessments for the protection of military personnel and the public.

WELCOME ADDRESS

Col Erik K. Vermulen
Deputy Director
Occupational and Environmental Health Directorate (AL/OE)
Brooks AFB, TX

On behalf of the Air Force, Wright-Patterson Air Force Base, and the Armstrong Laboratory, I would like to welcome you to the 24th Toxicology Conference. Again this year, the Conference is sponsored by the Armstrong Laboratory, the Naval Medical Research Institute, and the Army Biomedical Research and Development Laboratory. The organizational changes, policy evolution, and technical challenges we face dictate that this conference will never again be the same; I will go into this more later.

For those of you unfamiliar with Wright-Patterson Air Force Base, let me briefly introduce it to you. Daily, 28,000 people come to work here. It is the home to about 100 organizations. Currently it is the Headquarters of the Air Force Logistics Command with responsibility to purchase and repair Air Force systems. A major Air Force Systems Command unit, the Aeronautical Systems Division with its 4950th Test Wing and elements of two major Laboratories, Wright and Armstrong, is here. Additionally, there is the 906th Fighter Group, the Air Force Institute of Technology, the Foreign Aerospace Science and Technology Center, and the 2750th Air Base Wing. These activities make Wright-Patterson one of the most important installations in the Department of Defense (DoD).

All this is to change. Effective 1 July 1992, Air Force Materiel Command comes into existence; the flags of Air Force Logistics Command and Air Force Systems Command will be retired. The new Headquarters will manage the full spectrum of acquisition, support, and disposal of Air Force systems. It will control about half of the Air Force budget and represent 130,000 employees worldwide. But this is only a very small part of the reorganization and downsizing occurring in the DoD.

For years now we have been involved in reorganization and reassignment of responsibilities within the DoD. In particular, the responsibility for biomedical and life science research continues to be assessed, as does the responsibility for environmental research and development.

The three Services have been actively involved in Project Reliance for a couple of years now. Twenty-three different technology disciplines have been harmonized to enhance interservice reliance. Specifically, in the life sciences area, agreements were reached on collocation of disciplines which were further defined by Congressional legislation under Base Closure. This year we are to

implement a portion of the Triservice Toxicology Center here at Wright-Patterson a revisit by the Site Survey team in June will address the actions required to fully implement the legislation by 1996.

This year, we sponsored a preconference seminar on "Managing Hazardous Materials in the DoD." In part, it was to give us a better understanding of the policy changes that impact our customers and improve our vision of future requirements. It is readily apparent that the impact of hazardous materials on life cycle costs is a significant concern to the developers and maintainers of our weapons systems. We have a policy not only to be good citizens with respect to environmental, safety, and health legislation, but also to field the best military systems to ensure secure national interests. The DoD has been aggressive in chlorofluorocarbon replacement, hazardous materials replacement, and pollution prevention. Risk analysis is the principal driver in each of these initiatives.

Some of the significant comments that were expressed in the preconference seminar deserve to be repeated here. In a review of the risk assessment process, it was clearly established that there is a marked difference between science and science policy with respect to how individual materials will be treated. The science policy is recognized as conservative but is being retained because of the significant variance experienced with individual compounds. A consistent message expressed by the Secretariats was that leadership is expected from the Services. In response to public concern, the DoD has implemented a $25 billion restoration effort. However, there is an expectation that enough is enough and that improvements in materials management must occur. The DoD apparently will accept the costs of contractor overhead for prevention programs, but the contractors will carry the total burden for future compliance issues. Through working with aerospace associations, standard language for contracts is being worked to identify and track the use of hazardous materials to essentially create a "sunset" for toxic materials. An infrastructure has been created with a skeleton of policy and tracking mechanisms but now it is a "Commander's" program to implement because "it's the right thing to do." Just as one cannot use the lack of policy as an excuse, the lack of funding as an excuse will be taken away too. In fiscal year 1994 (FY94) an Air Force plan for $125 million was inserted; a requirement for some $80 million was introduced to Congress as a FY92 supplemental requirement. Means to address the FY93 program are under study. The new Air Force Materiel Command has been tasked for developing its systems engineering approach to eliminating hazardous materials and assessing residual risk. The mechanics have been proposed in a recently released draft Military Standard on Systems Engineering (MIL STD 499) which is currently out for comment. In summary, the seminar revealed a demand for Service leadership, an expectation for "green" weapons systems, and a willingness for funding these initiatives to identify and manage environmental, health, and safety risks.

Our agenda for the Conference is to focus on the technical issues and mechanics which promise to enable the DoD to achieve these policies. Product substitutions to achieve the reductions in ozone

depleters impact not only environmental objectives, but also systems performance, health, and safety objectives. The session on Halon replacements describes progress made over the last two years in assessing the human risk of likely replacement candidates. This initiative is a model for addressing future programs of this kind.

With that introduction, please accept our appreciation for your attendance. We have a major challenge in efficiently controlling human risk; your participation, involvement, and guidance is solicited.

SESSION I

HEALTH ASSESSMENT OF HALON REPLACEMENTS

OVERVIEW – AIR FORCE POLICY ON HALONS

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SUMMARY

Halons have been used for decades by the Air Force for a variety of fire protection applications. Their unique combination of effectiveness, low toxicity, ease of use, cleanliness, and low manufacturing cost appear to make them ideal for many situations. Unfortunately, they also deplete the earth's protective ozone layer and, consequently, their production is being phased out globally under the Montreal Protocol. United States legislation implementing the terms of the Protocol required an end to production of ozone depleting chemicals (ODCs) by the year 2000. In November 1991, the Air Force issued a policy requiring an end to ODC purchases by the end of 1997. In February 1992, President Bush announced an even more accelerated phaseout to 1995. The Montreal Protocol is expected to be amended to reflect the more aggressive U.S. phaseout date. This accelerated date increases the urgency of the Air Force's search for ODC alternatives, especially for mission critical uses for which no alternatives have yet been identified. The search is complicated by the fact that the requirements an alternative must meet are unique to their specific application. This paper will provide an overview of the most important Air Force halon uses and review Air Force strategies for ensuring mission continuity until alternatives can be developed.

INTRODUCTION

The Montreal Protocol is the United Nations treaty that establishes international controls on ozone depleting chemical (ODC) production. In September 1987, representatives from 33 nations met in Montreal, Canada under the auspices of the United Nations Environment Program and successfully negotiated the first global phaseout of a family of chemicals that was believed to damage the earth's protective ozone layer. Following the signing of the Protocol, political action to phase out these chemicals in the United States was swift. In December 1988, the Senate ratified the treaty.

Next, the Omnibus Reconciliation Act of 1989 placed punitive taxes on U.S. ODC purchases, which provided strong economic incentive for ODC users to accelerate their search for alternatives. The taxes were not equally applied to all chemicals, but were based on the ozone depletion potential (ODP) of each. For example, halon 1301 has an ODP 10 times greater than CFC-11 (chlorofluorocarbon-11). Using the ODP as a multiplier, in 1994, the tax on halon 1211 will become

$7.95 per pound and for halon 1301 will become $26.50 per pound. Prior to the Montreal Protocol, these halons cost the Government approximately $2.00 per pound, with no tax. The taxes were phased in more slowly for the halons than for the other ODCs, recognizing that more time was needed because of the smaller market compared with CFCs, and the importance of halons to public safety. Table 1 shows the amount and timing of the taxes. It is believed that the high taxes on halons will cause halon markets to collapse and production to stop in advance of the statutory phaseout dates.

TABLE 1. OMNIBUS BUDGET RECONCILIATION ACT OF 1989
Taxes on Ozone-Depleting Chemicals – No Exemptions

	90	91	92	93	94	95	96	97	98	99
CFCs	1.37	1.37	1.67	2.65	2.65	3.10	3.55	4.00	4.45	4.90
Halon 1211	0.25	0.25	0.25	0.25	7.95	9.30	10.65	12.00	13.35	14.70
Halon 1301	0.25	0.25	0.25	0.25	26.50	31.00	35.50	40.00	44.50	49.00

Figures shown are the amount of tax per pound of material purchased for each year the taxes are in effect. They do not include cost of material or any price fluctuation caused by market forces.

Pre-Montreal Protocol government prices for CFCs ranged from about $0.60 per pound to $1.20 per pound. The price for halons ranged from about $1.65 per pound to $2.10 per pound.

On November 15, 1992 President Bush signed the Clean Air Act Amendments of 1990 which incorporated the terms of the Montreal Protocol into U.S. law, but with more frequent interim reductions. Important features of the Clean Air Act to note are its exemption provisions, each of which contains one key phrase, "to the extent that such actions are consistent with the Montreal Protocol." This means that if the Montreal Protocol allows no exemptions, neither does the Clean Air Act. The exemptions in the Clean Air Act, however, include halon production for Aviation Safety, halon production for Fire and Explosion Suppression, and halon and CFC-114 production for National Defense. The Aviation Safety exemption can be granted by the administrators of the Federal Aviation Administration (FAA) and the Environmental Protection Agency (EPA). The Fire and Explosion Suppression exemption can be granted by the administrators of the U.S. Fire Administration and the EPA. The National Defense exemption requires Presidential approval. Currently, there is no production allowed under the Montreal Protocol for National Defense or for any other specific essential uses, because they have not yet been defined.

Before the Clean Air Act Amendments were passed, Congressional concern over the impact of the controls on the military prompted the Defense Authorization Act of 1989 to establish a committee to evaluate military ODC uses, to develop a time schedule for their phaseout within the Department of Defense (DoD), and to identify costs to achieve the phaseout. In response to these events, the DoD issued a Directive requiring the military services to phase out their ODC uses and to

issue service-specific instructions to implement the Directive. The Air Force began issuing specific-use ODC bans in June 1989 based on availability of alternatives for these specific uses. Ultimately, these bans were integrated into Air Force Regulation 19-15 in September 1991, which established comprehensive policy and guidance for dealing with the ODC issue.

The 1987 Montreal Protocol mandated that CFC production be reduced to 50% of its 1986 level by the year 2000 and that halon consumption be frozen at the 1986 level. It is important to note here what the Protocol does and does not control. The Montreal Protocol does not control use of ODCs; it controls consumption. This is defined as a nation's production plus its imports minus its exports.

Based on modest consumption reductions, the original agreement did not represent a significant burden on the Air Force. A 50% CFC reduction was achievable through conservation and recycling programs and modest training and operational procedures changes. A halon freeze at 1986 levels was not significant, considering that 1986 was during the final phase of a major initiative to change the approximately 20,000 flightline fire extinguishers to halon 1211. Because each extinguisher contained 150 lbs of halon, this was one of the Air Force's peak purchasing years.

STRATEGIES FOR MISSION CONTINUITY

At the time the original Protocol was signed, the negotiators recognized two facts that set the stage for rapid and dramatic changes to the terms of the Protocol. First, scientific understanding of the dynamics of ozone layer depletion was less than adequate to accurately balance the economic impact with the environmental necessity for action. Second, the technology to replace ODCs was advancing rapidly, and future availability of acceptable and cost-effective alternatives could not be forecasted with any degree of certainty. As a result, the Parties included a provision in the Protocol requiring periodic reassessment to determine whether the controls were adequate to protect the ozone layer, and to make adjustments based on economic impact, especially to developing countries. This included forming Halon Technical Options Committees to assess the state of alternatives for each major ODC use sectc .

The first assessment of the Montreal Protocol was conducted approximately two years after the original signing. The findings of the first assessment team resulted in significant changes to the terms of the Protocol during the first renegotiation in London during June 1990. The atmospheric scientists advising the Parties determined that the ozone layer was in much worse condition than previously believed, and the Halon Technical Options Committee concluded that alternatives were becoming available for most ODC uses. The proposed 50% reduction in CFC consumption by the year 2000 became a complete phaseout by the year 2000. Halon consumption went from a freeze at 1986 levels to a complete phaseout, placing them on the same schedule as the CFCs.

Although the halons are placed on the same schedule as the CFCs, the Protocol does make a distinction between them. The reasons are that halons are used primarily for fire protection, they contribute substantially to the public safety, and they represent a much smaller market than CFCs. The significance of this last reason is that corporate investment in halon alternatives research is much less attractive than investment in CFC alternatives. This unique "halon situation" prompted the Parties to place a provision in the Protocol to allow for future production for "essential uses." "Essential uses" was deliberately not defined at this time, but will be more precisely defined as circumstances warrant in the future.

Shortly after the 1990 renegotiation was completed, the reassessment process for the 1992 renegotiation was started. News from the Scientific Assessment Panel about the condition of the ozone layer became worse as improved ground and satellite measurement techniques were employed and scientific understanding improved. In addition, technology was providing effective alternatives for most ODC uses. By this time, most halon users appeared resigned to the inevitability of the phaseout, and saw no "silver bullet" replacement on the horizon. As a result, fire protection practices were shifting to alternatives such as water sprinklers, carbon dioxide (CO_2), and better fire protection engineering. Insurance underwriters, long thought to be the prime drivers of requirements for halon systems, began changing their standards for coverage. Rather than insisting on halon systems, the industry began to discourage their use and to recommend sprinklers and better risk management. Unfortunately, there remained a number of small but important halon uses for which there was no acceptable alternative, without compromise to public safety or national defense.

Despite this, the Halon Technical Options Committee concluded during the 1992 reassessment that no additional production was warranted at this time, paving the way for the halon phaseout schedule to overtake the ODC schedule, which was already advancing. The reason was that, unlike other ODCs, most of the halon ever produced still existed in systems and should, theoretically, still be available for reuse. Current thinking within the Committee is that, provided with the right set of incentives, a market for recycled halon will emerge to satisfy these small but essential uses until alternatives become available.

Another force driving acceleration of the phaseout was a dramatic announcement by President Bush on February 11, 1992. He announced that the United States, the largest ODC using and producing nation in the world, would unilaterally accelerate the phaseout of ODC production by five years, from 2000 to 1995. This followed new satellite data from the National Aeronautic and Space Administration showing alarming levels of ozone depletion over the Antarctic and the first ever evidence of depletion mechanisms over largely populated areas of the northern hemisphere.

The rapid decline of the ozone layer, the rapid rate of technological advances to replace ODCs for most uses, the dramatic abandonment of halon as the fire protection strategy of choice for many users, and the agreement on a funding mechanism to help developing countries make the transition to ODC alternatives, culminated in even more dramatic changes during the 1992 reassessment and renegotiation process. Although the renegotiations are not scheduled until November 23, 1992 in Copenhagen, it appears very likely that the halon phaseout date will advance to January 1994; the ODC phaseout date will advance to January 1996; and that carbon tetrachloride, methyl chloroform, and methyl bromide will be added to the list of controlled chemicals. Figure 1 shows how dramatically the phaseout has accelerated in the past two years. The total decrease in consumption allowance also is shown in Figure 1.

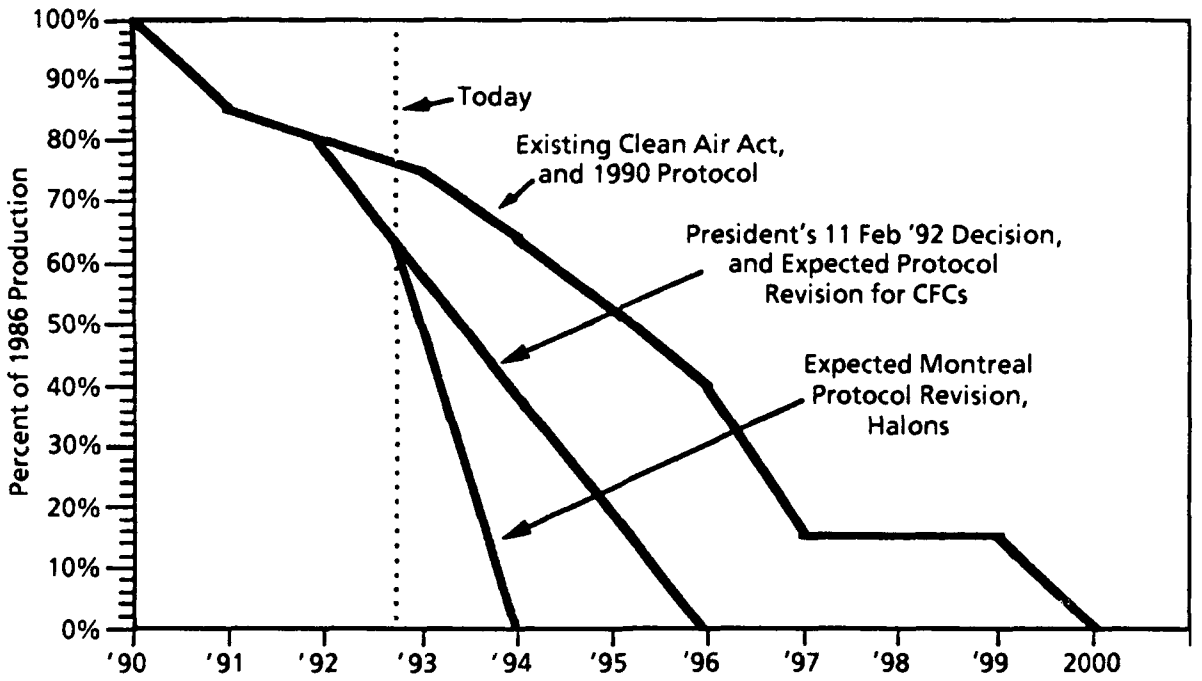

Figure 1. Changes to CFC and Halon Controls.

Despite the phaseout acceleration, there remain CFC uses for which alternatives have not been found. Since the halon "bank" will not last indefinitely, research on alternatives continues. However, for some applications there appears to be no short-term solution. One of the prime considerations in the decision on alternatives is safety.

AIR FORCE POLICIES

The DoD CFC Advisory Committee report, produced as a result of a requirement in the Defense Authorization Act for Fiscal Year 1989, provides some useful information to those interested in

conducting research on alternatives to military ODC applications. The report provides specific recommendations for managing DoD ODC dependence and contains an excellent compendium of important military ODC applications. The DoD directive issued after Senate ratification of the Protocol requires the Armed Services to review procedures involving ODC use and to change them where possible; to conserve ODCs where eliminating the use is not possible; and to conduct research and development on alternatives, with an emphasis on halon. This recognizes not only the fact that halons are the most difficult of the ODCs for which to find replacements, but also their important role in military systems.

Air Force Regulation 19-15 was published in September 1991, and prescribes the Air Force strategy – policies and procedures – for managing ODCs. The first policy states that the Air Force will demonstrate leadership by rapidly adopting substitutes as they become available. All offices establishing requirements, including military specifications and standards and technical orders, are responsible for eliminating ODC requirements. This includes all system program offices, depots, and product centers. The regulation makes each of these offices specifically responsible for changing ODC requirements. The policy also stipulates that offices transferring ODC dependence will make those transfers known. The objective of this provision was to gain visibility over our foreign weapons system sales that result in transfer of ODC dependence to other nations.

AIR FORCE USE OF HALONS AND RESEARCH CONSIDERATIONS

Halon is a colorless gas that extinguishes fires through a combination of physical and chemical action. The chemical action accounts for its extraordinary effectiveness. Fires are extinguished in a total flooding application with less than 5% by volume concentration. The maximum human exposure level of halon 1301 is approximately 10%, which gives systems designers a margin of safety sufficient to design and construct a system that extinguishes fires, but not people. Halon leaves no residue, although the decomposition products, hydrogen bromides and hydrogen chlorides, are toxic and corrosive. These are produced in proportion to the amount of halon discharged, the temperature to which the halon is exposed, and the amount of time the halon remains at elevated temperature. Fortunately, the quantities are usually very small. As a gas, halon is drawn into a fire because of the convective currents set up by the fire. This is particularly useful in situations where a fire may start in a space obscured from sight, or in a device with a complex geometry. The halon has the ability to "find" the fire.

Halon research has been frustrated over the years, in part because of the issue of toxicity. The ability to extinguish fires at concentrations compatible with human presence is a unique feature of halon 1301 that has not yet been found in any other material. It is important to note that halon was approved as a total flooding agent for use in occupied spaces in the early 1970s. At that time, our

standards for human exposure were not as stringent, and our understanding of the toxicology was not as sophisticated as it is today. A criterion halon researchers have tried to apply to a halon replacement is that it be no more toxic than halon 1301. A literature search revealed little toxicological data on halon 1301 to use as a basis for comparison, and establishing that data is a costly undertaking. This raises two issues worthy of considering. First, halon 1301 was approved with little toxicological data and little understanding of its long-term effects. Second, if halon 1301 were newly discovered, it is not known if it would be approved for use given today's more stringent acute and chronic exposure standards. This is a formidable challenge to the halon researcher today; although not all halon applications present exposure challenges as critical as in the total flooding situation.

In some military aircraft as well as civilian aircraft, FAA regulations require at least two hand-held halon 1211 portable extinguishers on board all passenger aircraft. In practice, most aircraft use halon 1211 exclusively on board. It is easier logistically to support just one extinguisher type, and it eliminates the possibility of a technician using the wrong type of agent on a particular fire. There is an upper limit on the number of halon 1211 extinguishers allowed on board, which is tied to the toxicity of halon 1211. The upper limit is set by assuming that all extinguishers on board discharge simultaneously, and that no more than 5% by volume concentration of halon 1211 be present in the cabin.

The flight deck space is very small, especially in fighter aircraft. Again, toxicity is a significant consideration in the Air Force's selection criteria for an alternative. It is very important that the pilot is able to continue functioning after the extinguisher has discharged. The Navy is considering CO_2. Carbon dioxide is fairly benign, environmentally. Although it is a greenhouse gas, it is a very minor one. It is also inexpensive and it is easy to produce. Perfluorohexane is a halon alternative being considered for ground applications, and it may be considered for cockpits as well.

Halon 1301 currently is used in a total flooding system in the cargo bays of some C-5 aircraft, in the C-141, and in some C-130s. The C-17, however, does not use a total flooding fire protection system in the cargo bay. This situation leads to a policy question. If the C-17 can function without the use of halon as a total flooding fire protection in the cargo bay, then why cannot similar aircraft? If the risk in terms of flight safety does not warrant such protection in the C-17, then the mission criticality of this halon application to other aircraft types needs reevaluation. Another area in the aircraft that warrants careful assessment are the dry bays. Dry bays are the areas between the inner and outer skin of the aircraft. The reason that dry bays require protection is that they may contain delicate electronics.

Engine nacelles and auxiliary power units account for the largest, most widely used application of halon in aircraft. The engine nacelle is the cowling around the engine, and the majority of

multiengined aircraft employ a halon fire suppressant system to protect this area. If there is an in-flight fire, the pilot is notified and, by pressing a button, can manually discharge the halon into the engine nacelle. Because this area of the aircraft is unoccupied, there is no risk of exposing people to the agent during its use and, therefore, toxicity is not an issue. A similarly configured system is used to protect the auxiliary power units of most military aircraft. Toxicity, however, does become an issue for those who are exposed to the agent during system servicing and while manufacturing the agent. The difficult environment in which the engine nacelle operates, for example, presents additional demands upon a fire suppressant agent. Winds of 600 knots or higher and extremely low temperatures must be factored into a system's design. The complex geometry of the engine nacelle and the flow of fuel under high pressure also complicates the development of an alternative system. Consequently, the nacelle area is one of the more significant applications that the Air Force needs to address, from a mission standpoint.

Halon 1211 is contained in 150 lb cylinders on flight lines, in hangars, and in aircraft shelters. Where aircraft are located on the ground, this agent is considered to be the first line of defense against fire. Halon suits this role very well, primarily because it is a clean agent. That means that in suppressing a small fire, it will not leave a residue and it is unlikely to cause any secondary damage, that is, damage beyond that caused by the fire itself. Cleanliness of a replacement has been a primary objective of most of our halon research and development to date. Researchers at Tyndall Air Force Base, Florida, have done an outstanding job of screening the materials currently available and endeavoring to develop new materials that meet the cleanliness criteria.

Again, toxicity is a concern because maintenance technicians on the flight line are exposed to the fire suppressant agents. Some of the possible alternatives being evaluated include perfluorohexane and some CO_2-based agents. Sodium bicarbonate and other dry powder agents also are very effective fire fighting agents. They are also environmentally benign and are not expensive. Unfortunately, they leave a residue, and some types of powder agents may have undesirable corrosive effects. The Air Force, therefore, needs to take a very thorough look at each of these alternatives, conduct a comprehensive risk assessment of each agent, and reevaluate their assumptions about the need for a clean agent, based on mission requirements.

Until last year, 500 lbs of halon 1211 was carried on every crash/rescue vehicle. Acceleration of the halon phaseout, the halon tax, and the lack of an environmentally acceptable clean alternative led to a policy decision to remove the halon from these vehicles. The rationale behind this policy decision emerged as the Air Force evaluated the reasons for using halon in this particular application. The first line of defense against an aircraft ground fire is the 150-lb wheeled extinguisher. If the fire is not extinguished, the crash/rescue vehicle responds. If the fire is too large to extinguish using 150 lbs of halon 1211, then secondary damage caused by a dirty agent will not be a consideration.

With a fire that large, the primary concerns will be saving lives and preserving as much of the aircraft as possible. Air Force crash/rescue teams now carry dry powder agents, which are more effective than halons outdoors in wind conditions, and aqueous film-forming foam.

Halon hand-held portable extinguishers also have been used extensively in facilities. The purpose of hand-held portables in buildings is to meet life safety codes contained in National Fire Protection Association Code 10. Any extinguisher with the correct type of rating will satisfy this requirement; halon is only one of many choices. Halon is usually chosen to meet this need in computer and communications areas because of its cleanliness. These extinguishers are applied locally, as opposed to a total flooding application, and are used against small fires. Carbon dioxide is an acceptable alternative, because concentrations will not be large enough to affect people.

CONCLUSION

There are a number of halon applications for which the Air Force must find alternatives. Some applications may, as in the case of the crash rescue teams, require not an involved technical solution but a change in policy. In other areas the Air Force may develop "drop-in" replacements. It is more likely that the majority of applications will require replacements that may require modification in the design of the mechanisms they protect. Replacement decisions will be driven by a variety of criteria, including effectiveness, cleanliness, and toxicity. The mandate for the replacement of halon agents means, however, that alternatives must be developed and implemented, and those efforts continue.

HUMAN HEALTH AND ENVIRONMENTAL TOXICITY ISSUES FOR EVALUATION OF HALON REPLACEMENTS

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ABSTRACT

The Clean Air Act Amendments of 1990 require the U.S. Environmental Protection Agency (EPA) to phase out production and use of ozone-depleting chemicals – among them, the fire-suppressants, halons. As part of its rulemaking efforts EPA must evaluate the potential hazards to human health and the environment that could result from exposure to compounds that may substitute for halons.

The EPA bases health hazard assessment on data obtained in studies involving short-term and long-term exposures. The former are used to evaluate potential risks of acute or delayed effects potentially resulting from short exposures at high concentrations, such as might be experienced in episodic emissions in the workplace. Studies with long-term exposure are used to assess potential adverse effects from continued exposure to low ambient concentrations. In addition, reproductive and developmental hazards are evaluated in several animal species.

About ten chlorinated-, brominated-, and/or fluorinated-hydrogen-containing hydrocarbons, to be used alone or in combination, have been proposed as halon substitutes. In addition to health and safety, environmental, efficiency, and marketability, considerations (Table 2) need to be addressed for the selection of proposed halon substitutes.

TABLE 2. FIRE EXTINGUISHING AND EXPLOSION INERTION CONSIDERATIONS

Environmental
Ozone Depletion Potential
Global Warming Potential
Atmospheric Lifetime
Health and Safety
Extinguishing or Inerting Concentration vs. Cardiotoxic No Effect Level/Lowest Effect Level
Chronic Toxicity
Occupational Exposure
Decomposition Products and Oxygen Level in Use

(continued)

TABLE 2. Continued

Efficacy
Extinguishment Concentration
Weight and Storage Equivalent
Other Performance Tests
Market
Availability
Storage Stability
Materials Compatibility
Class of Fire

This presentation will discuss current EPA/Office of Air and Radiation thinking on a decision-tree approach for testing the toxicity of halon substitutes under the Significant New Alternatives Policy program.

The risk to individuals from exposure to halon substitutes is generally from discharges that occur infrequently. Chronic effects are not the usual concern for halon substitutes because when used, these substances are discharged in high concentrations over short periods of time, and thus, are potentially acutely hazardous. Risk from exposure to halon substitutes is accordingly best assessed by analysis of acute toxic effects associated with exposure to these compounds, such as developmental toxicity and cardiotoxicity. In most instances, cardiotoxicity occurs at lower levels than fetotoxicity, and therefore, unless otherwise warranted by the developmental data, the Agency will base the estimates for emergency limits during halon use on the no observable adverse effect level (NOAEL) and lowest observable adverse effect level (LOAEL) reported for epinephrine-sensitized cardiotoxicity in dogs (and in a few instances monkeys). Human heart arrhythmias and sudden death resulting from overexposure to chlorofluorocarbons (CFCs), halons, other halogenated and nonhalogenated hydrocarbons have been documented in workplace settings and in volatile substance abuse (e.g., glue-sniffing).

The determination of the safety of either a flooding or streaming agent substitute is also dependent on a number of related factors. For total flood systems, the magnitude of exposure will depend on the design concentration of the flooding agent (as determined by the substitute's extinguishing concentration plus 20%) and the length of time it takes a person to evacuate the area in which the agent is released. Because total flood systems are designed to achieve a uniform concentration of an agent within a space, the magnitude of exposure is independent of the size of the space, size of fire, or proximity of the person to the fire. In assessing exposure and consequent use restrictions, the agency will compare the total flood concentration to the NOAEL for

cardiotoxicity. The cardiotoxicity LOAEL defines the concentration level at which all personnel must be evacuated from an area. For reentry in an area where the concentration is at or above the cardiotoxicity level use of Self Contained Breathing Apparatus is required.

Exposure to streaming agents can be expected to vary greatly depending on the amount of agent released, the time needed to extinguish a fire, the size of the enclosure in which a fire occurs, the size of the fire, the proximity of a person to the point of discharge of the agent, the rate at which fresh air infiltrates the space, and the air exchange rate near the fire. Assessment of the risk of a streaming agent is more complicated than a flooding agent. Estimations of the above key factors as well as personnel monitoring data may be necessary to complete a risk assessment. The peak exposure will be compared to the NOAEL and LOAEL for cardiotoxicity. The rate at which the peak exposure decays also will be evaluated.

In summary, EPA uses the cardiotoxicity NOAEL, LOAEL, and appropriate exposure measurements or estimates to ensure protection of the user of these agents. Analysis of the risk to humans from these agents will be made together with the fire-fighting groups, such as the National Fire Protection Association. Candidate total flooding and streaming agents that are being considered by EPA are listed in Table 3 and 4, respectively.

The EPA uses short- and long-term exposures to assess environmental effects of chemicals. These include laboratory studies of longevity and reproduction in representative terrestrial and aquatic species. Very few such studies are available for potential halon substitutes.

TABLE 3. FIRE EXTINGUISHING AND EXPLOSION INERTION TOTAL FLOODING AGENTS

Requirements for Occupied Areas

Personnel must be able to evacuate within 30 seconds.

Employees must be alerted to impending system discharge.

All personnel must be evacuated before concentration of the agent reaches the cardiotoxic effect level.

Agent Inertion	Occupied Areas	Unoccupied Areas	Explosion
HBFC-22B1[a]	A	A	A
HCFC-22	A	A	U
HCFC-124	A	A	U
NAF S III	A	A	P

(continued)

TABLE 3. Continued

Agent Inertion	Occupied Areas	Unoccupied Areas	Explosion
HFC-23	P	A	P
HFC-32	P	P	
HFC-125	P	A	A
HFC-134a	A	A	U
HFC-227ea	P	A	A
FC 3-1-10	A[b]	A[b]	A[b]
Inergen	P	P	P
Water	A	A	U
Carbon Dioxide		A	U

[a] Class I substance
[b] Critical uses only; not subject to 30 sec requirement
KEY: A = Acceptable
U = Unacceptable
P = Pending

TABLE 4. FIRE EXTINGUISHING AND EXPLOSION INERTION STREAMING AGENTS

	Consumer Use	Commercial/ Industrial Use	Military Use
HBFC-22B1	A[a]	A[b]	A[b]
NAF P	A[a]	A[b]	A[b]
HCFC-123	P	P	P
Halotron I	P	P	P
HFC-227ea	P	P	P
PFC 614	U	A[c]	A[c]
Dry Chemical	A	A	A
Carbon Dioxide	A	A	A
Water	A	A	A
Foam	A	A	A

[a] Not for Residential Uses
[b] Class I Substance
[c] Critical Uses only, Training prohibited
KEY: A = Acceptable
U = Unacceptable
P = Pending

INDUSTRIAL RESEARCH ON ALTERNATIVE FLUOROCARBONS

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SUMMARY

Fluorocarbons containing chlorine or bromine have been associated with stratospheric ozone depletion and the search for suitable alternatives is progressing at an accelerated pace. The Program for Alternative Fluorocarbon Toxicity Testing (PAFT), an international group representing most of the world's chlorofluorocarbon producers, has been conducting comprehensive toxicological evaluations on several possible replacements for current fire extinguishing agents – 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123), 1-chloro-1,2,2,2-tetrafluoroethane (HCFC-124), pentafluoroethane (HFC-125), and 1-fluoro-2,2,2-trifluoroethane (HFC-134a). Results from short-term experimental studies showing a low order of acute toxicity, as well as results from longer-term toxicity studies on these replacements, will be discussed.

INTRODUCTION

Chlorofluorocarbons (CFCs) have been used as refrigerants, solvents, aerosol propellants, and foam-blowing agents over the past 50 years. Halons (fluorocarbons containing bromine) have been used as fire extinguishing agents over several decades. The main reasons for their wide acceptance in their respective uses were chemical stability, low toxicity, and a lack of environmental reactivity. However, fluorocarbons containing chlorine or bromine have been associated with stratospheric ozone depletion and the search for suitable alternatives is progressing at an accelerated pace. This led in 1987 to the creation of the Alternative Fluorocarbon Toxicity Testing (PAFT), an international group representing most of the CFC producers from Asia, Europe, and the United States. Under this Program, comprehensive toxicological evaluations have been conducted on several CFC/halon replacements and toxicity results on four of these alternatives will be subsequently discussed.

SELECTED PROPERTIES OF CFCS/HALONS AND ALTERNATIVES

In Table 5, selected physical/chemical properties and uses of common, commercial CFCs and halons are given. As mentioned earlier, all of these materials are very low in toxicity but appear to have one serious problem – an environmental stability in the lower atmosphere resulting in their subsequent appearance and breakdown in the upper atmosphere that leads to ozone depletion. Therefore, industry needs to create alternatives that are "less environmentally stable" with a low

ozone depletion potential, a low global warming potential, and low photochemical reactivity. Such chemicals should still possess, however, a high degree of chemical stability in their end uses, be relatively low in toxicity, and also be available at an acceptable cost to users.

TABLE 5. CHLOROFLUOROCARBONS AND HALONS: PROPERTIES AND APPLICATIONS

CFC No.	Chemical Formula	Boiling Point °F	Vapor Pressure psig at ~21 °C	Principal Applications
113	CCl_2FCClF_2	117.6	(5.5 psia)	Solvent, chemical intermediate
11	CCl_3F	74.9	(13.4 psia)	Aerosols, foams, solvent
114	CCl_2CClF_2	38.8	12.9	Aerolsols, foams, refrigerants
12	CCl_2F_2	-21.6	70.2	Aerosols, foams refrigerants
115	$CClF_2CF_3$	-37.7	103	Refrigerants
Halon No.				
1301	CF_3Br	-72.0	199	Fire-extinguishing agent
1211	CF_2ClBr	24.8	25.3	Fire-extinguishing agent
2402	CF_2BrCF_2Br	115.5	(0.5 psia)	Fire-extinguishing agent

Fluorocarbons, in general, have different properties and different applications. Therefore, when one looks for replacements, a good starting point is a comparison of the physical properties of the old versus the new type of fluorocarbon as shown in Table 6. Because boiling point often dictates the type of use for a particular fluorocarbon, the preceding table groups old versus new fluorocarbons accordingly, to provide some suggestion of possible substitution.

Some alternatives that are available now for specific uses include dimethyl ether (DME), chlorodifluoromethane (HCFC-22), 1-chloro-1,1-difluoroethane (HCFC-142b), and 1,1-difluoroethane (HFC-152a). All four can be used as propellants and the latter three for refrigeration and blowing agent uses. None of these four alternatives have been seriously considered for fire extinguishing use (DME, HCFC-142b, and HFC-152a are all flammable, for example). Selected physical and chemical properties for these four commercially available alternatives are shown in Table 7. Also, HCFC-22 and HCFC-142b have very low ozone depletion potentials, whereas DME and HFC-152a have no potential to deplete ozone. All four of these chemicals also have a low order of toxicity on both an acute and chronic basis and pose no hazard to humans from the viewpoint of carcinogenicity, mutagenicity, or teratogenicity when used within their recommended occupational exposure limits.

TABLE 6. CFCs AND ALTERNATIVES

Formula	Halon or CFC No.	Boiling Point	(°F)	HCFC or HFC No.	Formula
CCl_2FCClF_2	113	117.6	133.0	225cb	$CClF_2CF_2CHClF$
CF_2BrCF_2Br	2402	115.5	124.0	225ca	$CF_3CF_2CHCl_2$
CCl_3F	11	74.9	+ 90.0	141B	
CH_3CCl_2F			+ 82.0	123	$CHCl_2CF_3$
$CClF_2CClF_2$	114	38.8	+ 14.0	142B	CH_3CClF_2
CF_2ClBr	1211	+ 24.8	+ 12.0	124	$CHClFCF_3$
Cl_2F_2	12	-21.6	-13.0	152A	CH_3CHF_2
			-13.0	DME	CH_3OCH_3
			-16.0	134A	CH_2FCF_3
$CClF_2CF_3$	115	-37.7	-41.0	22	$CHClF_2$
			-54.0	125	CHF_2CF_3
CF_3Br	1301	-72.0			

TABLE 7. PROPERTIES OF COMMERCIALLY AVAILABLE ALTERNATIVES

	HCFC-22	DME	HFC-152a	HCFC-142b
Formula	$CHClF_2$	CH_3OCH_3	CH_3CHF_2	CH_3CClF_2
BP (°F)	-41	-13	-13	+ 14
VP (psig) 70 °F	121	63	63	29
ODP, vs. CFC-11 = 1	.05	0	0	.06
GWP, vs. CO_2 = 1	.34	negl.	03	.36
VOC	NO	YES	NO	NO
Flammability	NO	YES	YES	YES
Workplace Exposure (ppm by volume)	1000TLV	1000 AEL	1000 AEL	1000 AEL

PROGRAM FOR ALTERNATIVE FLUOROCARBON TOXICITY TESTING

There are eight other fluorocarbon alternatives under development as replacements for CFCs and for possible use as fire-extinguishing agents. These chemicals, all of which contain hydrogen,

making them more degradable in the lower atmosphere, are HCFC-123 (CF_3CHCl_2), HCFC-124 (CF_3CHClF), HFC-125 (CF_3CHF_2), HFC-134a (CF_3CH_2F), HCFC-141b (CH_3CCl_2F), HCFC-225ca ($CF_3CF_2CHCl_2$), HCFC-225cb ($CClF_2CF_2CHClF$), and HFC-32 (CH_2F_2). Worldwide fluorocarbon producers are sponsoring the cooperative PAFT program to expedite the acquisition of toxicology data on these compounds. Five PAFT programs have been established to assess worker safety, risk assessment, and consumer protection issues surrounding the use of CFC alternatives. PAFT 1, begun in 1987, is examining HCFC-123 and HFC-134a; PAFT 2 (HCFC-141b), PAFT 3 (HCFC-124 and HFC-125), and PAFT 4 (HCFC-225 isomers) began in 1988, 1989, and 1990, respectively. The latest PAFT program, PAFT 5 (HFC-32) just started at the end of 1991. The PAFT Program is sponsored by up to 13 of the world's leading CFC producers at a cost of approximately three to five million dollars per compound and an anticipated testing time for each material of 5 years of less. An overview of the toxicity testing schedule established by PAFT for each of its five programs is shown in Table 8. Relative to potential use as fire-extinguishing agents, the short-term toxicity studies are the most important consideration.

TABLE 8. PAFT-PROPOSED TOXICITY TESTING OUTLINE

PHASE I (6 to 8 Months)
- Acute inhalation (LC_{50}, ALC)
- Dermal and eye irritation/sensitization
- Dermal Toxicity (2 species)
- Cardiac sensitization
- Genotoxicity (Ames, lymphocyte, micronucleus)
- Other (acute oral, human patch studies)

PHASE IIa (6 to 8 Months)
- Subacute inhalation (4 weeks)
- Preliminary pharmacokinetics (blood levels, uptake, elimination)
- Teratology probes (2 species)
- Environmental toxicity

PHASE IIb (1 Year)
- Teratology (2 species)

PHASE III (1 Year)
- 90-Day inhalation
- Metabolism – CNS effects

PHASE IV (4 years)
- Chronic inhalation toxicity/carcinogenicity study

For the remainder of this paper, I will concentrate on four of the eight PAFT alternatives that have received some consideration as fire-extinguishing agents. Selected properties of these four alternatives, as well as pertinent environmental indices, are summarized in Table 9. Note particularly that the hydrofluorocarbons (HFC-125 and HFC-134a) have an ozone depletion potential (ODP) of

zero. The status of toxicity test results on each of the preceding four alternatives is subsequently summarized.

TABLE 9. PROPERTIES OF PRINCIPAL ALTERNATIVES FOR HALON REPLACEMENT

	HFC -125	HFC -134a	HCFC -124	HCFC -123
Formula	CF_3CHF_2	CF_3CH_2F	CF_3CHClF	CF_3CHCl_2
Boiling Point degrees Fahrenheit (°F)	-54	-16	+ 12	+ 82
Vapor Pressure (psig) (70 °F)	165	70	33	--
Ozone Depletion Potential (ODP) vs. CFC - 11 = 1	0	0	.02	.02
Global Warming Potential (GWP)	.84	.28	.1	.02
Volatile Organic Compound (VOC)	NO	NO	NO	NO
Flammable	NO	NO	NO	NO
Workplace Exposure (ppm by volume)	1000 AEL	1000 AEL	500 AEL	10 AEL
Toxicity Testing	(----------Underway through PAFT----------)			

PAFT ALTERNATIVES: TOXICITY RESULTS TODATE

HCFC-123 is moderately low in toxicity on an acute inhalation basis with a 4-h LC_{50} in rats of 32,000 ppm (v/v) and a cardiac sensitization threshold in dogs of 20,000 ppm (results from an experimental screening study using a 5-min exposure followed by a large intravenous dose of epinephrine). In addition, HCFC-123 is not an *in vitro* or *in vivo* mutagen and is not a developmental toxin in rats or rabbits. However, in a chronic inhalation study at exposure levels up to 5000 ppm, rats at the end of the 24-month exposure period showed an increased incidence of microscopic, benign tumors of the liver, pancreas, and testes. This type of finding, coupled with an increased survival rate in exposed rats, an altered lipid metabolism, and slight peroxisome proliferation, has led to a large research effort to determine the relevance, if any, of these benign tumors to humans.

HCFC-124 is very low in toxicity on an acute inhalation basis with a 4-h LC_{50} in rats of 262,500 ppm and a cardiac sensitization potential in dogs of 25,000 ppm. This fluorocarbon is also very low in toxicity on a subchronic basis, is not a mutagen, and is not a developmental toxin. A chronic toxicity/carcinogenicity study in rats exposed by the inhalation route will start later this year.

HFC-125 is also very low in acute inhalation toxicity. It has a 4-h LC_{50} in rats of >800,000 ppm and a cardiac sensitization potential in dogs of 100,000 ppm (threshold). Subchronic toxicity studies in rats and mice, developmental toxicity evaluations in rats and rabbits, and both *in vitro* and *in vivo* mutagenicity testing are currently in progress.

HFC-134a is the last alternative to be discussed. This hydrofluorocarbon is very low in toxicity on an acute inhalation basis with a 4-h LC_{50} in rats of >500,000 ppm and a cardiac sensitization threshold in dogs of 75,000 ppm. On a repeated exposure basis, HFC-134a is also very low in toxicity. It has produced no significant adverse effects to date in rats exposed for a lifetime at ≤ 50,000 ppm, and it is not a mutagen or a developmental toxin. The exposure phase of the lifetime inhalation toxicity study is complete (as of October, 1991) and histopathologic examination of tissues is currently under way.

In addition to the preceding four chemical alternatives, PAFT is also conducting an extensive battery of toxicity studies on HCFC-141b, on two isomers of HCFC-225, and on HFC-32, none of which have been considered as potential fire-extinguishing agents. However, the preceding HCFCs may find some uses as solvents, foam-blowing agents, or possibly refrigerants (HFC-32, for example).

COMMENTS

The Program for Alternative Fluorocarbon Toxicity Testing to date has initiated well over 100 individual toxicity studies on eight possible CFC/halon alternatives in 12 different testing laboratories located throughout Europe, the United States, and Japan. Although the first testing program (PAFT 1 – HCFC-123 and HFC-134a) will not be completed until early 1993, current data are encouraging. Testing results on HCFC-123, HCFC-124, HFC-125 and HFC-134a suggest a low order of toxicity, but higher fire-extinguishing concentrations will probably be required compared to current halon agents. The development of alternative products is difficult and time-consuming because the goal is total product safety, not just worker and user safety considerations.

The Program for Alternative Fluorocarbon Toxicity Testing does intend to publish the results of all toxicity and environmental studies in peer-reviewed scientific journals and to present the information at scientific conferences whenever possible.

AIR FORCE APPROACH TO RISK ASSESSMENT FOR HALON REPLACEMENTS

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SUMMARY

Finding safe, environmentally acceptable, and effective replacements for halon fire extinguishing agents and other chemicals banned by the Montreal Protocol is a formidable task for Air Force research and development organizations. One factor that makes this task a challenge is the uncertainty in relating toxicology studies in laboratory animals to the human situation. This uncertainty from toxicology studies affects the risk assessment process by calling for very conservative decisions. Because of this uncertainty, public pressure and politics also impact the regulatory process. The Air Force approach to assessing health hazards for halon replacements is to provide scientific information that directly applies to the parts of the extrapolation process that are responsible for the most uncertainty. Most regulatory agencies readily incorporate scientific information, when it is available, which can reduce uncertainty. These Air Force studies will be used to provide realistic exposure levels for replacement chemicals which will allow mission accomplishment and provide safety for the worker and the populace.

INTRODUCTION

A variety of the research capabilities of the Air Force Toxicology Division, including toxicity screens, mechanistic research, and a unique risk assessment approach, have been applied in the halon replacement program. Significant teamwork has occurred over the past five or six years between various Air Force agencies, industry, the U.S. Environmental Protection Agency (U.S. EPA), and a wide variety of contractors to address the safety of halon replacements. To complement the industry's traditional toxicology programs, we are making pharmacokinetic and mechanistic contributions. We are working closely with the U.S. EPA on exposure levels for Air Force-specific chemicals to ensure the safe use of these chemicals.

About five years ago, a list of 30 to 40 chemicals was prepared for consideration as candidate replacements for halon 1211. These chemicals were selected based on their physical properties and

the understanding that enough information was available currently, or would be within the next couple of years, to be able to assess the toxicology. This list of candidates was tested by New Mexico Engineering Research Institute (NMERI) under a contract by Air Force Civil Engineering Support Agency (AFCESA), the agency responsible for halon 1211 replacements. These chemicals were tested for their fire-fighting ability, ozone-depletion potential, global-warming potential, and several other parameters. The New Mexico Engineering Research Institute recommended a small number of replacements to AFCESA that went to the fire fighting community to obtain their acceptance. This is an important process because of the concern the general public has about toxicology and hazardous chemicals. Fire fighters and the general public have a tendency to see chemicals as either safe or toxic. So, it takes a great deal of education to get them to understand and put into perspective toxicology information.

Air Force Approach to Risk Assessment

Traditionally, toxicology testing requires a "great leap" from the laboratory studies to the human situation (Figure 2). In toxicology studies, nonhuman species are exposed to known concentrations of chemicals for known periods of time under controlled conditions. It is often necessary to expose the surrogate species to high concentrations for prolonged periods of time in order to find statistically significant toxicity. In contrast, human exposures in the environment or workplace are often at very low concentrations and may be either intermittent or continuous. The two biggest uncertainties in this "great leap" from the laboratory to realistic situations are differences in exposures and physiological or anatomical characteristics of species.

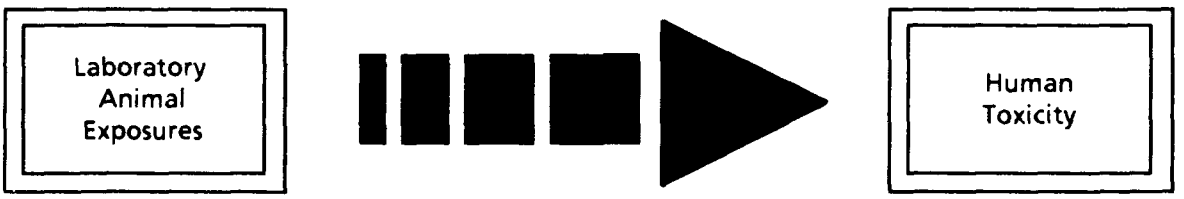

Figure 2. The "Great Leap."

One way to improve the great leap is to take smaller steps. Figure 3 shows intermediate points between the level of contaminant and toxicity. These boxes enclose measurable parameters which form the framework for a more rational extrapolation process between laboratory studies and environmental exposures. Prediction based on a firm understanding of the processes is the key. The contaminant level reflects the concentration of chemical in the air, water, or soil. Internal dose is the

total amount of chemical that enters the animal or human. Tissue concentration is the concentration of chemical that actually reaches the target organ. Area under the curve or peak concentration of either the parent or metabolite may relate best to the toxicological end point of significance. Tissue toxicity defines the biological end point of concern, such as cell death or loss of cellular function over the short or long term.

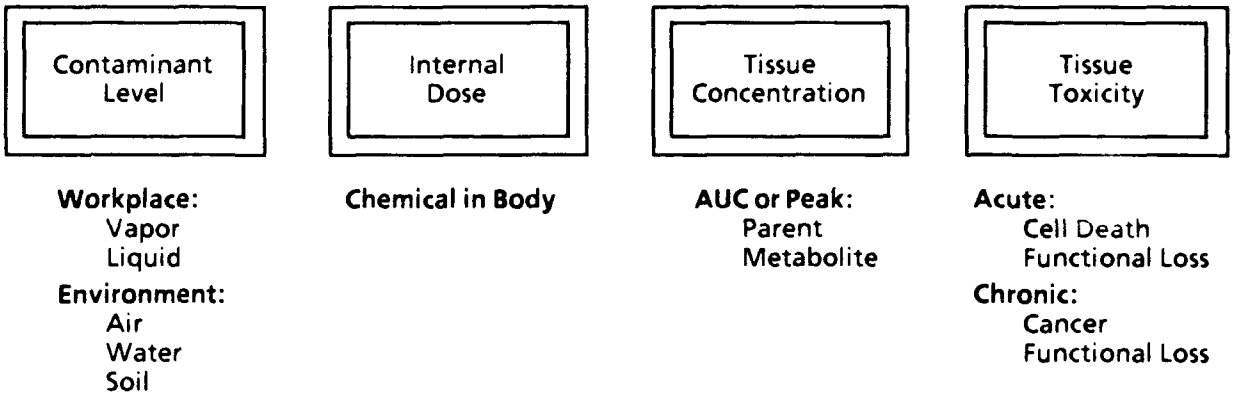

Figure 3. Measurable Parameters.

In Figure 4, those parameters are connected by what could be described as transfer processes. These transfer processes can be measured, understood, and mathematically described. The transfer process between the contaminant level and the amount that ends up in the organism is exposure. The magnitude of this transfer process is affected by any protective equipment, industrial hygiene practices, and personal habits (such as bathing frequency) as well as by the duration and frequency of exposure. Pharmacokinetics is the relationship between exposure-dose and tissue concentration. The concentration that gets to the target organ tissue is affected by absorption, distribution, metabolism, and elimination. Pharmacodynamics is the relationship between tissue concentration and tissue toxicity. Tissue toxicity is affected by species sensitivity, repair processes, and the general health of the organism. Properly validated mathematical descriptions of these transfer processes allow prediction of toxicity for different exposure scenarios. Quantitative adjustments of differences in the transfer processes between laboratory animals and humans allow prediction of human toxicity.

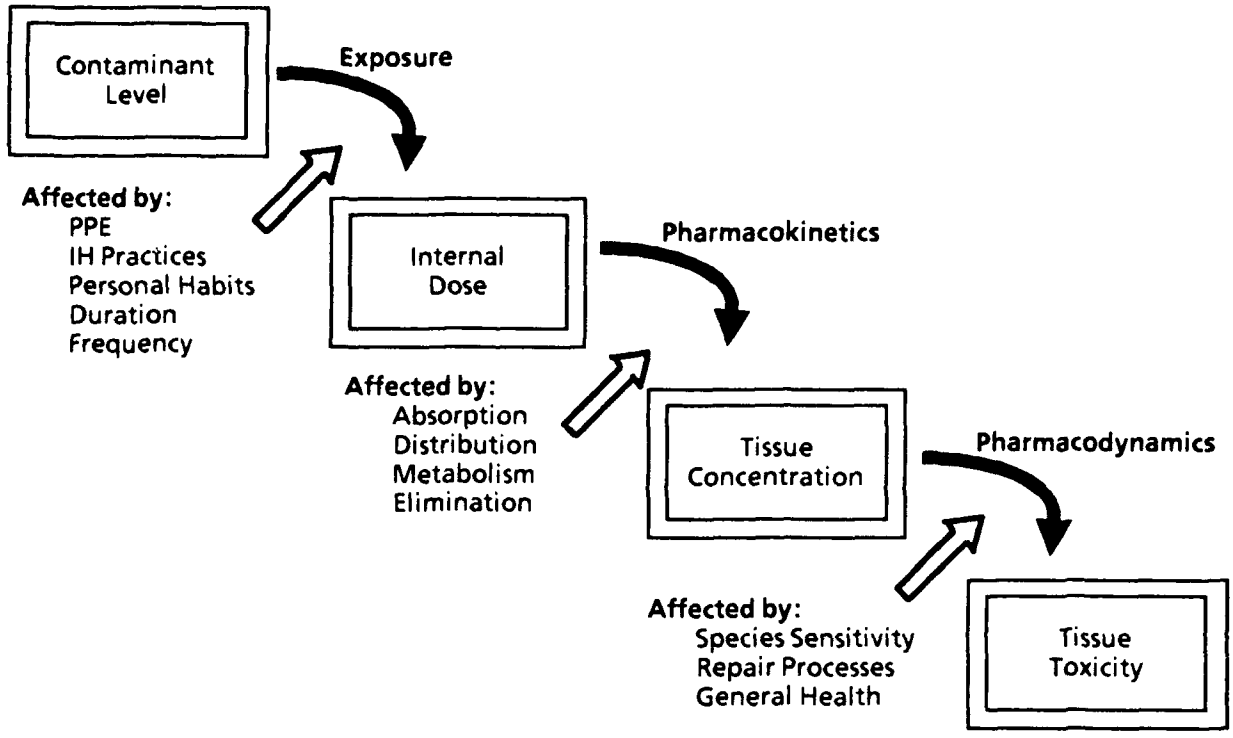

Figure 4. Transfer Processes.

The animal–human differences are present at each of the transfer processes. These processes can be addressed by physiologically based pharmacokinetic (PBPK) and pharmacodynamic modeling. On the left in Figure 5 is a schematic that illustrates a PBPK model for dermal absorption of an organic chemical. This mathematical model is made of physiological compartments (skin, rapidly perfused organs, liver, and such) which are connected by physiological blood flows. Each compartment has a distinct and measurable affinity for the chemical. Some compartments may include metabolism or elimination. The mathematical model requires organ volumes, blood flows, partition coefficients, and metabolic rates. The model keeps track of the mass balance of the chemical in the body and can be used to predict tissue and blood concentrations when properly validated. It also can be used to understand and predict the results of exposures to mixtures. The most important advantage of such a model is the ability to extrapolate to human exposures based on an understanding of the species differences. This approach is gaining favor with the U.S. EPA. When PBPK models are available, the U.S. EPA considers using them for setting standards.

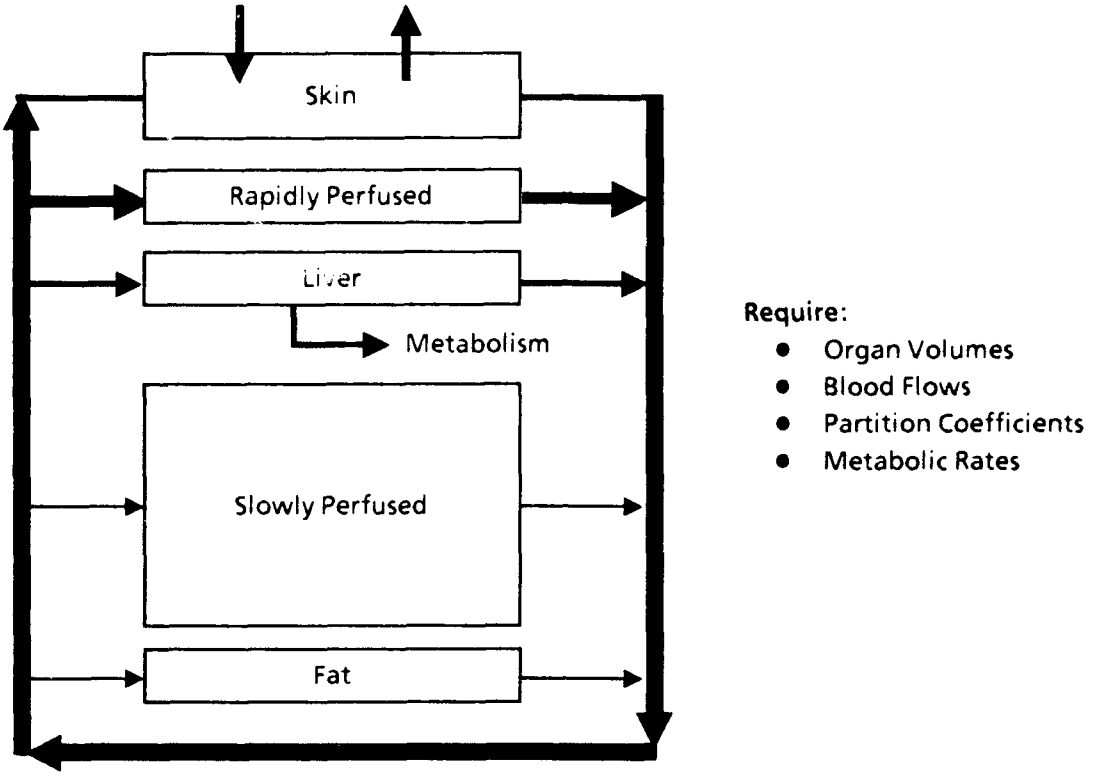

Figure 5. Physiologically Based Pharmacokinetic Model.

The final aspect of our general approach is to specifically address the uncertainty factors and other conservative factors by focusing on providing the scientific information necessary to avoid them. Figure 6 shows the uncertainty factors used by U.S. EPA for chronic noncancer end points linked to the steps of the extrapolation process. Each of these uncertainty factors relates to one or more of the transfer processes previously described. Less-than-lifetime exposures can be addressed by understanding the time-concentration relationship of exposure. Variation in sensitivity in human population can be addressed experimentally by several techniques, including using human cells to understand differences in sensitivity. Lowest observable adverse effect level (LOAEL) versus no observable adverse effect level (NOAEL) can be addressed by understanding the relationship between concentration and determining the level mechanistically. Extrapolating animal data to human data can be addressed with models for pharmacokinetics and pharmacodynamics. Inability of a single study to address all outcomes in humans can be addressed by performing additional toxicological studies of other end points (reproductive, developmental, and neurobehavioral). The approach should be to determine the transfer processes described in Figure 4.

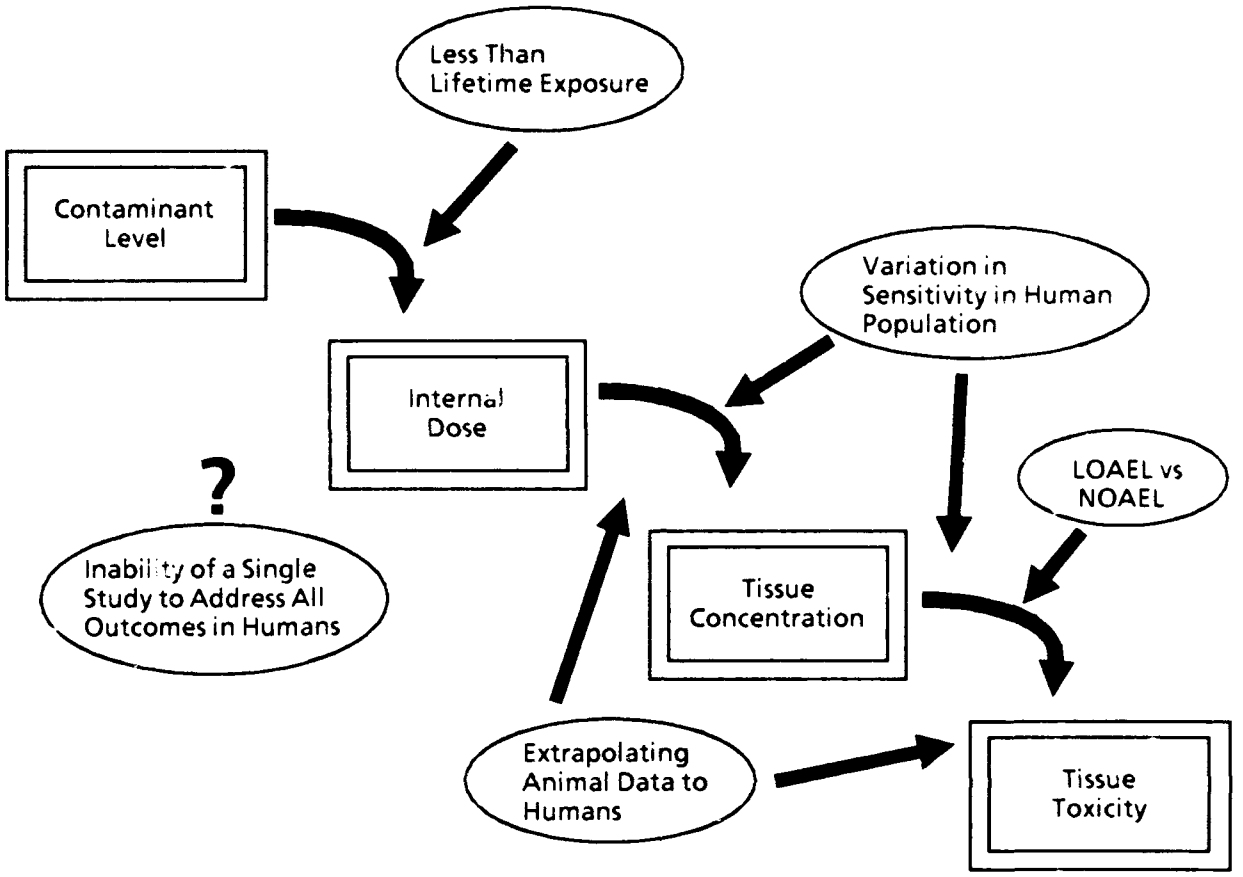

Figure 6. Uncertainty Factors.

At the Toxicology Division, we are practicing a different type of risk assessment that relies more on explicit quantitative methods than on uncertainty factors. Halons are very important to the Air Force because we need safe, clean, fire fighting agents. This method requires a teamwork approach with several different agencies; with Air Force, industry, and the U.S. EPA. This type of program will be applicable to many other chemical replacement projects that will be coming in the future, such as halon 1301, refrigerants, and solvents.

METABOLISM AND PHARMACOKINETICS OF SELECTED HALON REPLACEMENT CANDIDATES

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SUMMARY

Metabolism studies were conducted using Fischer 344 and Sprague-Dawley rats following inhalation exposure to 1.0% (v/v) air atmospheres of 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123), 2-chloro-1,1,1,2-tetrafluoroethane (HCFC-124), 1-chloro-1,1-difluoroethane (HCFC-142b), bromochlorodifluoromethane (Halon 1211), and perfluorohexane (PFH) for 2 h. There were no remarkable differences in results between the two strains of rats. Animals exposed to HCFC-123 or HCFC-124 excreted trifluoroacetic acid in their urine. Urinary fluoride concentrations were increased in rats exposed to HCFC-124, and urinary bromide levels were increased in rats exposed to Halon 1211. Small quantities of volatile metabolites 2-chloro-1,1,1-trifluoroethane (HCFC-133a) and 2-chloro-1,1-difluoroethylene were observed in the livers of rats exposed to HCFC-123. Rats exposed to HCFC-142b excreted chlorodifluoroacetic acid in their urine; no volatile metabolites were detected in tissue samples. For PFH studies, no metabolites were detected in the urine or tissues of exposed animals. These results are consistent with proposed oxidative and reductive pathways of metabolism for these chemicals. Pharmacokinetic studies were carried out in rats exposed by inhalation to 1.0%, 0.1%, or 0.01% of HCFC-123. Following exposure, blood concentrations of HCFC-123 fell sharply, whereas trifluoroacetic acid levels rose for approximately 5h and then declined gradually. Using a physiologically based pharmacokinetic model, saturation of HCFC-123 metabolism was estimated to occur at approximately 0.2% (2000 ppm) HCFC-123.

INTRODUCTION

Important to phasing out the production of ozone-depleting halogenated hydrocarbons is the successful development of functionally similar substitutes. Much emphasis has been placed on the development of hydrofluorocarbons (HFCs), hydrochlorofluorocarbons (HCFCs), or perfluorocarbons as appropriate substitutes. Part of the strategy for developing chemical substitutes is to address the issue of potential human health hazards. An industrial consortium known as the Program for Alternative Fluorocarbon Toxicity Testing (PAFT) is currently involved in conducting a series of toxicity tests on selected HFCs and HCFCs. To complement this effort, the Air Force undertook investigations to examine the metabolism and pharmacokinetic characteristics of selected halocarbons that are candidates for replacing the current flight-line fire extinguishant bromochlorodifluoromethane (Halon 1211). The replacement chemicals chosen for investigation were 1,1-dichloro-2,2,2-

trifluoroethane (HCFC-123), 2-chloro-1,1,1,2-tetrafluoroethane (HCFC-124), 1-chloro-1,1-difluoroethane (HCFC-142b), and perfluorohexane (PFH). This research also included Halon 1211 for the purpose of comparing results.

The primary objectives of the metabolism and pharmacokinetic studies were (1) to identify toxicologically important metabolites, (2) to examine distribution of metabolites and the parent compound, (3) to quantitate the amount of metabolites formed, (4) to determine the time-course of metabolite formation and elimination, and (5) to identify the factors influencing metabolite formation and elimination. For example, enzyme induction and/or decreased levels of oxygen may be important factors affecting metabolite formation and elimination for these test chemicals.

EXPERIMENTAL DESIGN

For the metabolism portion of this study, both Fischer 344 (F-344) and Sprague-Dawley (SD) male rats, 6 to 8 weeks of age, were used. Groups of eight were exposed by nose-only inhalation to 1% (10,000 ppm) air atmosphere of the test chemical for 2 h. Details of the test chemicals have been described [1]. Two control rats were exposed to air only. Following exposure, four test and one control rats were immediately euthanatized via carbon dioxide (CO_2) inhalation, and samples from the following tissues were removed, quick-frozen in liquid nitrogen, and stored at -20 °C for analysis: blood, liver, kidney, skin, muscle, testes, heart, lung, and fat. The remaining four test animals and one control animal were placed into metabolism cages for 24 h and then sacrificed. In this procedure, urine and feces were collected. During the collection, urine was kept at 0 °C, and feces were kept below room temperature.

Approximately half-gram samples of blood or tissue were placed into vials for headspace analysis of the volatile metabolites by gas chromatography (GC) with electron capture detection (ECD) or by GC/mass spectrometry (MS) with subambient cryofocusing. Details of the analytical procedures, including instrument conditions, have been described [1]. For urine samples, 2-mL samples were centrifuged, the supernatant decanted, and 100 µL aliquots were combined with TISAB II buffer. Fluoride ion concentration was determined from a standard curve generated with control rat urine. For the analysis of carboxylic acids and bromide, 100 µL samples of urine were derivatized with dimethyl sulfate in headspace vials followed by GC/MS analysis. Because of the low concentrations of bromide in these samples, GC/MS was done using selected ion monitoring. Selected samples of urine, liver, and testes from rats exposed to HCFC-123 or PFH were examined by fluorine-19 nuclear magnetic resonance spectrometry (^{19}F NMR). This technique detects volatile as well as nonvolatile metabolites containing fluorine.

For the pharmacokinetic experiments, the general experimental approach was to cannulate the jugular vein of F-344 rats followed by random placement into two exposure groups. One group

was anesthetized with urethane (1.25g/Kg, ip), tracheotomized, and artificially ventilated with a small animal respirator. 1,1-Dichloro-2,2,2-trifluoroethane exposures ranged from 100 to 10,000 ppm for 2 to 4 h. The other group of rats remained awake and were exposed to HCFC-123 atmospheres of 100 to 25,000 ppm for 2 to 4 h. Chemical analyses were performed on samples of expired air, blood, urine, and tissues. Parameters chosen for physiologically based pharmacokinetic (PBPK) modeling were those of Hoover et al. [2]. Metabolic constants were determined by gas-uptake closed chamber studies. Partition coefficients were determined for the parent compound and metabolites.

RESULTS AND DISCUSSION

Metabolism Studies

HCFC-123

HCFC-123 was detected in all tissues sampled from both F-344 and SD rats sacrificed immediately after exposure (0 h). At 24-h postexposure, small amounts of HCFC-123 were detected in the liver, kidney, muscle, and skin; but most samples did not contain HCFC-123. Of much greater interest was the detection of metabolites. 2-Chloro-1,1,1-trifluoroethane (HCFC-133a) was detected in the liver (Figure 7) and kidney immediately postexposure. Also, in the liver was 2-chloro-1,1-difluoroethylene (CDE) (Figure 8). To distinguish the CDE peak, signal averaging and background subtraction were applied. Mass spectra of HCFC-133a and CDE obtained from liver headspace samples of F-344 rats exposed to HCFC-123 matched the mass spectra of authentic standards of HCFC-133a and CDE, respectively. The most abundant metabolite in the urine was trifluoroacetic acid (TFA). The amount excreted over 24 h was 18 ± 5 μmol. Urinary fluoride values were similar to the control values. Results of ^{19}F NMR spectrometry did not provide additional new information. However, Anders and coworkers [3,4] exposed rats to 1% HCFC-123 and via ^{19}F NMR spectrometry observed the formation of reactive trifluoroacetyl intermediates with liver proteins. These newly formed TFA proteins have been implicated in an allergic-type reaction known as halothane-induced hepatitis [3,5].

Both oxidative and reductive pathways participate in the metabolism of HCFC-123. The reductive pathway begins with reductive dehalogenation to produce a radical intermediate that can either accept a hydrogen atom from a protein or a phospholipid to form HCFC-133a or lose a fluorine to yield CDE. The oxidative pathway catalyzed by cytochrome P450 produces a dichloro geminal halohydrin, which is unstable, and releases hydrochloric acid (HCl) to form trifluoroacetylchloride which is hydrolyzed to TFA. These pathways are similar to those for halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) where TFA, HCFC-133a, and CDE have been detected as metabolites [6,7,8].

The toxicological significance of the metabolites of HCFC-123 has not been determined. Studies by the PAFT consortium [9,10] indicate that the parent compound, HCFC-123, has a low order of toxicity. Toxicity tests with HCFC-133a also indicate a low order of toxicity, but HCFC-133a is tumorigenic as determined in an oral gavage study in rats [9]. The toxicity of CDE has not been evaluated. Halogenated alkenes can react with glutathione causing the formation of conjugates that may be nephrotoxic [11]. Chlorodifluoroethylene (CDFE) inactivates cytochrome P450 which may contribute to an inhibition of chemical metabolism [12]. Because of its similarity in structure to trichloroacetic acid, the potential exists for TFA to produce peroxisome proliferation. Interestingly, rats exposed to HCFC-123 vapor have increased hepatic beta-oxidation [10]. The rare occurrence of hepatotoxicity following halothane anesthesia may be related to either oxidative or reductive metabolites [13].

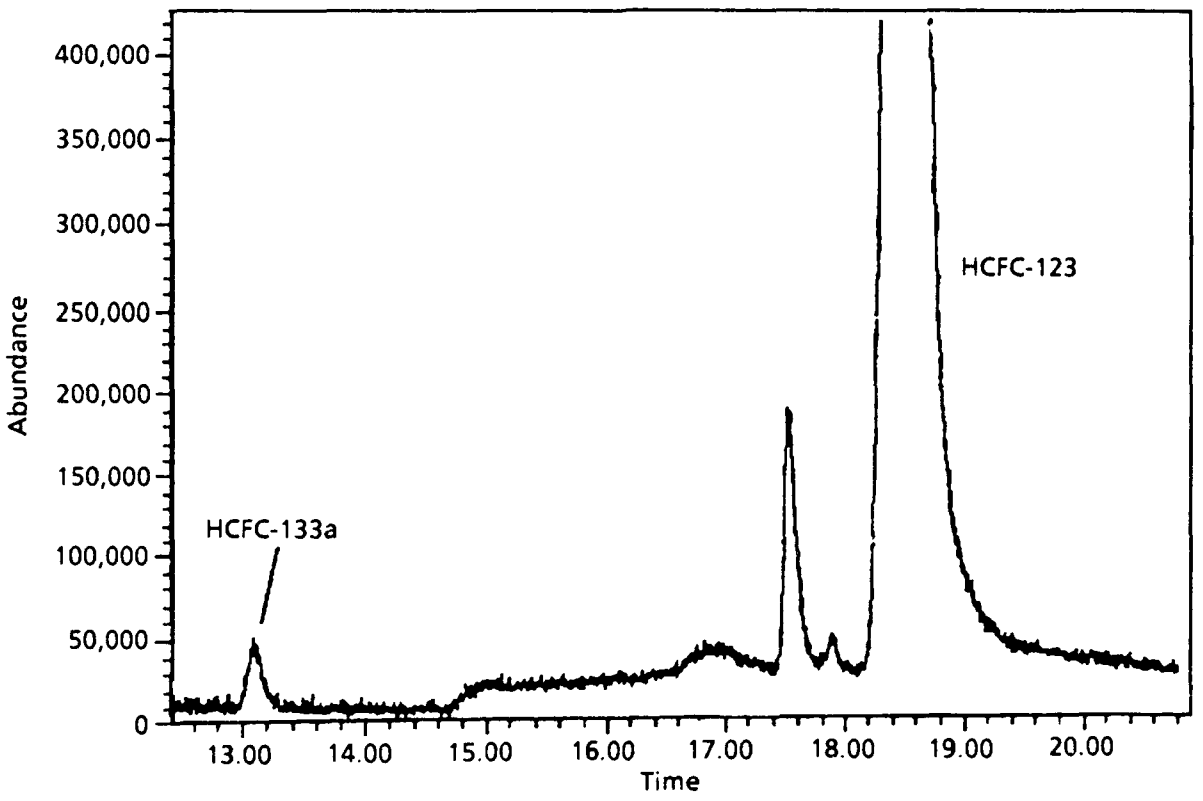

Figure 7. Total-Ion Chromatogram of a Liver Headspace Sample Obtained From an F-344 Rat Exposed to HCFC-123. HCFC-133a has a retention time of 13.2 min; that of HCFC-123 is 18.6 min.

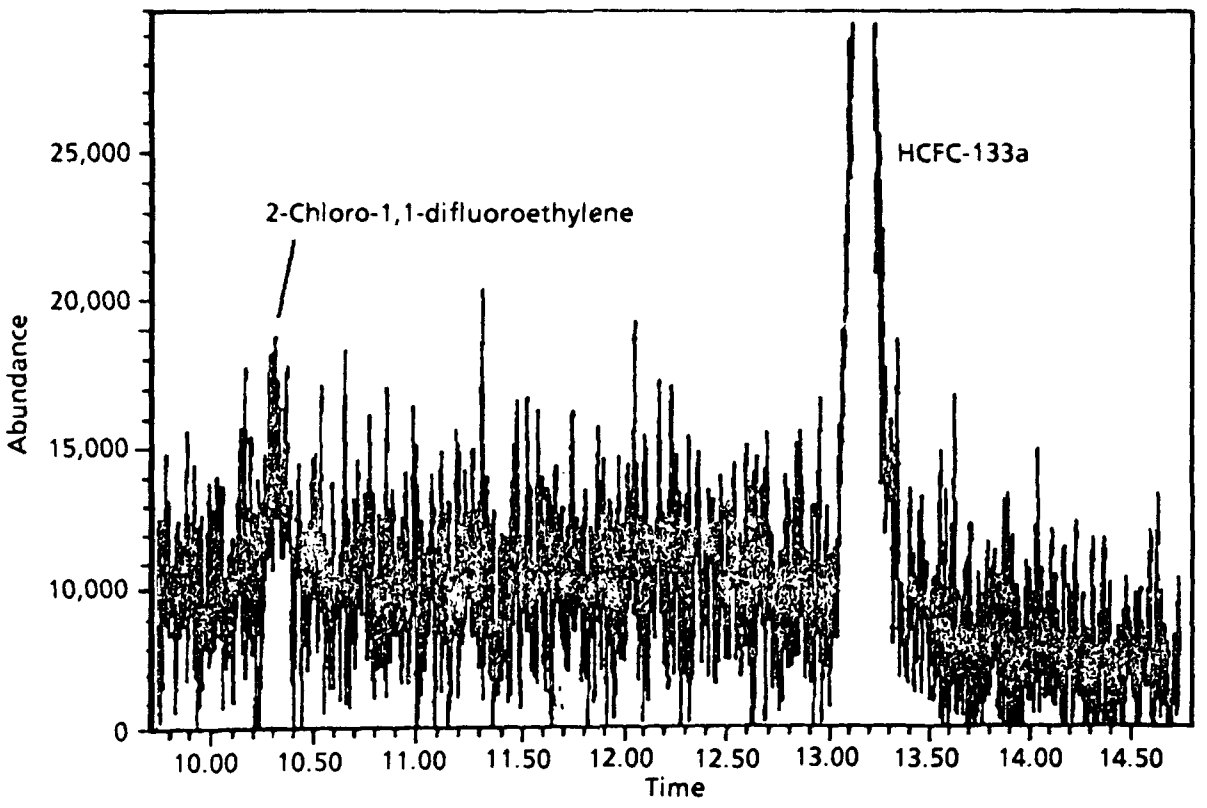

Figure 8. Total-Ion Chromatogram of a Liver Headspace Sample Obtained From an F-344 Rat Exposed to HCFC-123. CDE has a retention time of 10.3 min; that of HCFC-133a is 13.2 min.

HCFC-124

HCFC-124 was detected in all tissues sampled from both F-344 and SD rats sacrificed immediately following exposure (0 h). At 24 h, no HCFC-124 was observed. No volatile metabolites were detected in blood, tissues, or excreta. Trifluoroacetic acid was found in the urine. The amount excreted over 24 h was 2.1 ± 0.3 μmol, which was much lower than the amount excreted in rats exposed to HCFC-123. Urinary fluoride was elevated in exposed rats. Control rats excreted approximately 8 μg over a 24-h interval. HCFC-124 exposed rats excreted 26 ± 5 μg for the same time period. These results are consistent with those of Olson and coworkers [14] who have performed metabolism studies in rats exposed to HCFC-124. For this compound, the presence of urinary fluoride is indicative of oxidative metabolism, not reductive metabolism.

The oxidative metabolism of HCFC-124 is similar to that of HCFC-123. A geminal halohydrin is formed, HCl is released, and trifluoroacetylfluoride is formed. Similar to HCFC-123, this reactive intermediate forms adducts with liver proteins [4]. Hydrolysis of the acylfluoride releases hydrogen fluoride and produces TFA. Although reductive metabolites were not detected in our studies, Olson and coworkers [14] have demonstrated reductive metabolism *in vitro* under low oxygen conditions.

The structural similarity of HCFC-124 to HCFC-123 suggests that the toxicological significance of the metabolites of HCFC-124 would be similar to that of the metabolites of HCFC-123.

HCFC-142b

Similar to HCFC-123 and HCFC-124, HCFC-142b was detected in all samples analyzed from rats sacrificed immediately after exposure (0 h). At 24-h postexposure, no HCFC-142b was found. No volatile metabolites were detected in blood, tissues, or excreta. However, a urinary carboxylic acid metabolite, chlorodifluoroacetic acid (CDFA), was identified (Figure 9). The signal was too small to be able to quantitate the amount of CDFA excreted. Signal averaging and background subtraction were applied to discern the CDFE methyl ester peak. The mass spectrum of the methyl ester of CDFA from a dimethylsulfate-derivatized urine sample from an F-344 rat exposed to HCFC-142b matched the mass spectrum of an authentic standard of CDFA methyl ester.

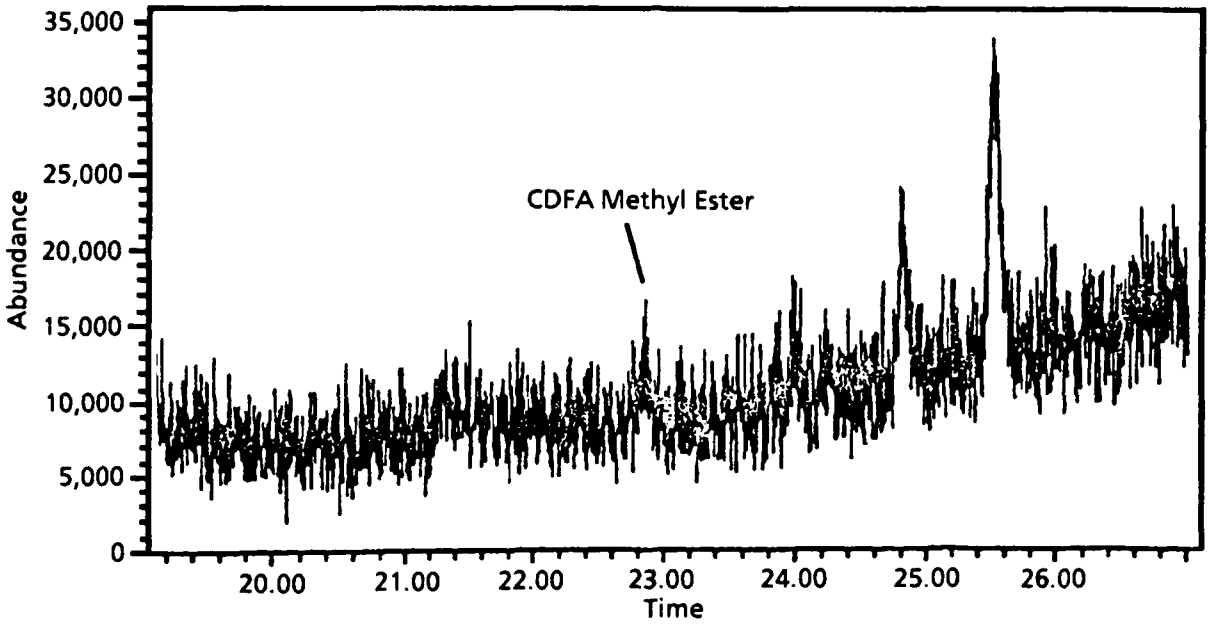

Figure 9. Total-Ion Chromatogram of a Dimethylsulfate-Derivatized Urine Sample Obtained From an F-344 Rat Exposed to HCFC-142b. The methyl ester of CDFA has a retention time of 22.9 min.

The proposed metabolic pathway for HCFC-142b involves oxidation to chlorodifluoroethanol via cytochrome P450 and further metabolism with alcohol and aldehyde dehydrogenases to the metabolite CDFA. Anders and coworkers [15] have investigated the metabolism of other 1,1,1-trihaloethanes, for example, 1,1-dichloro-1-fluoroethane (HCFC-141b). The trihaloacetic acid is the expected metabolite in the urine. However, a glucuronide conjugate was also found. This finding

agreed with results of metabolism studies with trichloroethane [16], which is excreted as a glucuronide conjugate of trichloroethanol, and is excreted to a lesser extent, as trichloroacetic acid.

Although the formation of metabolites of trihaloethanols, such as 2,2,2-trifluoroethanol, are potentially toxic [17], HCFC-142b has been thoroughly evaluated in laboratory animal studies for toxicity [9]. A 2-year inhalation bioassay was conducted in rats at concentrations of 0 (control), 0.1, 1.0, and 2.0% [18]. All end points measured, including tumorigenicity, were negative.

PFH

Perfluorohexane (PFH) was detected in all tissues sampled from both F-344 and SD rats sacrificed immediately postexposure. At 24 h, PFH was detected in the fat only. No metabolites of PFH were detected. The metabolism of PFH, if any, remains to be elucidated. One assumes that the disappearance of PFH following inhalation is entirely due to expiration.

Toxicological investigations with PFH have been limited to acute and subchronic general toxicity evaluations. Results to-date indicate minimal alterations in PFH-exposed rats [19]. A noteworthy consideration for PFH and other perfluoro compounds regarding substitution for ozone-depleting halogenated hydrocarbons is their recalcitrance toward degradation which might, with time, have an environmental impact.

Halon 1211

Halon 1211 was detected in all tissue sampled from both F-344 and SD rats sacrificed immediately after exposure. Halon 1211 was also detected in the liver, skin, muscle, testes, fat, and urine 24 h postexposure. Although no volatile metabolites were present and fluoride concentrations in the urine were similar to control rats, urinary bromide values were increased in exposed rats (Table 10). Bromide excretion rates for SD rats were 64% higher in exposed rats compared to control SD rats. For F-344 rats, a 45% increase was observed in exposed rats.

TABLE 10. URINARY BROMIDE EXCRETION RATE FOLLOWING EXPOSURE TO HALON 1211[a]

Strain of Rat	Group	Bromide, µg/24 h (N)
F-344	Air-only	73 ± 21 [4]
F-344	Halon 1211	106 ± 22 [4]
SD	Air-only	67 ± 13 [4]
SD	Halon 1211	110 ± 24[b] [4]

[a] 1.0% Halon 1211 for 2 h

[b] Statistically significant compared to air-only control ($p<0.05$)

It appears that Halon 1211 undergoes some metabolism. Reductive dehalogenation leads to the release of bromide and the formation of chlorodifluoromethane. Possible subsequent metabolism via cytochrome P450 results in the formation of formylfluoride. Hydrolysis of formylfluoride leads to the production of CO_2 and hydrogen fluoride. The toxicological significance of the formation of these metabolites is unclear. Subchronic inhalation exposure of rats to Halon 1211 concentrations as high as 2.5 to 5.0% did not produce adverse effects [20].

Pharmacokinetic Studies

Rat blood concentrations of HCFC-123 plotted against time during and following a 4-h exposure to 0.01, 0.1, or 1.0% HCFC-123 is shown in Figure 10. Within a few minutes HCFC-123 blood concentrations became stable and were in proportion with exposure concentrations. Following exposure, HCFC-123 blood concentrations decreased fairly rapidly, but small amounts were still present 24 h postexposure. These results are consistent with volatile anesthetics possessing moderate blood-to-air solubility [21]. The blood-to-air partition coefficient for HCFC-123 is 2.4; the fat-to-air partition coefficient is 72.

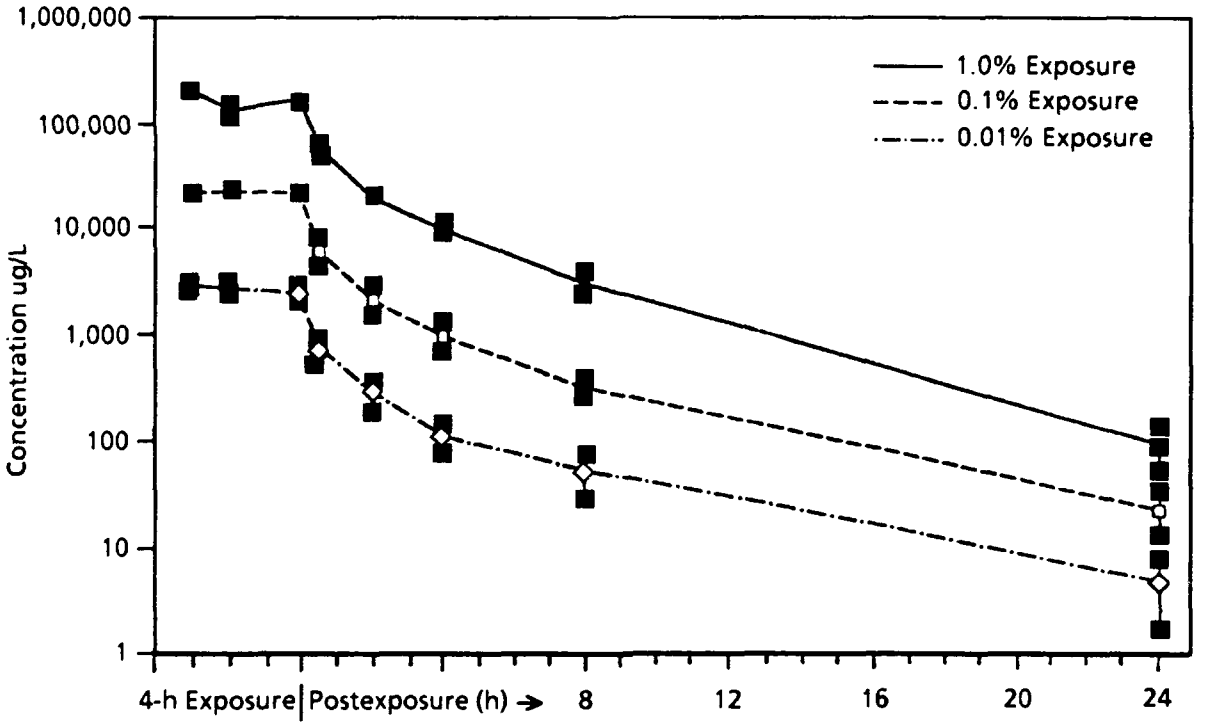

Figure 10. F-344 Rat Blood Concentrations of HCFC-123 During and Following a 4-h Exposure to 0.01, 0.1, or 1.0% HCFC-123.

Figure 11 shows TFA blood concentrations versus time during and following a 4-h exposure to 0.01, 0.1, or 1.0% HCFC-123. Trifluoroacetic acid concentrations slowly rose during exposure and

continued to rise for approximately 5 h postexposure. At 24 h postexposure, TFA blood concentrations were still high, but TFA was slowly being eliminated (this observation was masked due to the log scale of TFA concentration). Elimination of TFA appears to be considerably slower than that of HCFC-123, based on the blood concentration versus time plots. Similar findings have been observed with halothane [22].

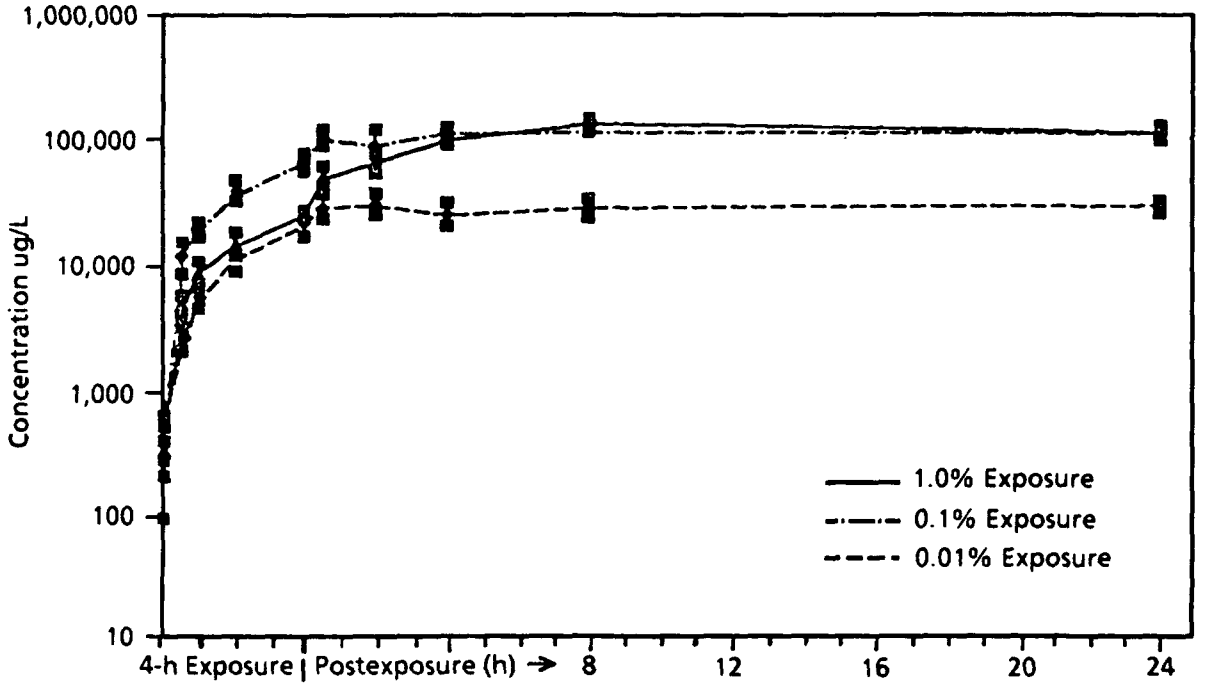

Figure 11. F-344 Rat Blood Concentrations of Trifluoroacetic Acid During and Following a 4-h Exposure to 0.01, 0.1, or 1.0% HCFC-123.

A PBPK model was developed for HCFC-123. Metabolic constants used were Vmax = 8.74 mg/h/Kg and Km = 1.0 mg/L [2]. Tissue-to-air partition coefficients were 2.45, 72.72, 3.32, and 2.71 for blood, fat, liver, and muscle, respectively [2]. The rate of metabolism is shown as a function of HCFC-123 exposure concentration (Figure 12). Data points are shown at 100, 1000, and 10,000 ppm which are the concentrations at which animals were actually exposed. Metabolism appears to be saturated at about 2000 ppm. Recent gas-uptake investigations with HCFC-123 using SD rats indicate HCFC-123 uptake was saturated at concentrations greater than about 1000 ppm [23]. Blood concentrations of TFA after a 4-h exposure to HCFC-123 are consistent with saturated metabolism (Figure 11). However, interpretation is complicated by the apparent suppression of metabolism during and shortly after exposure to HCFC-123, as evidenced by the lower blood concentrations of TFA resulting from the 10,000 ppm exposure compared with the 1000 ppm exposure.

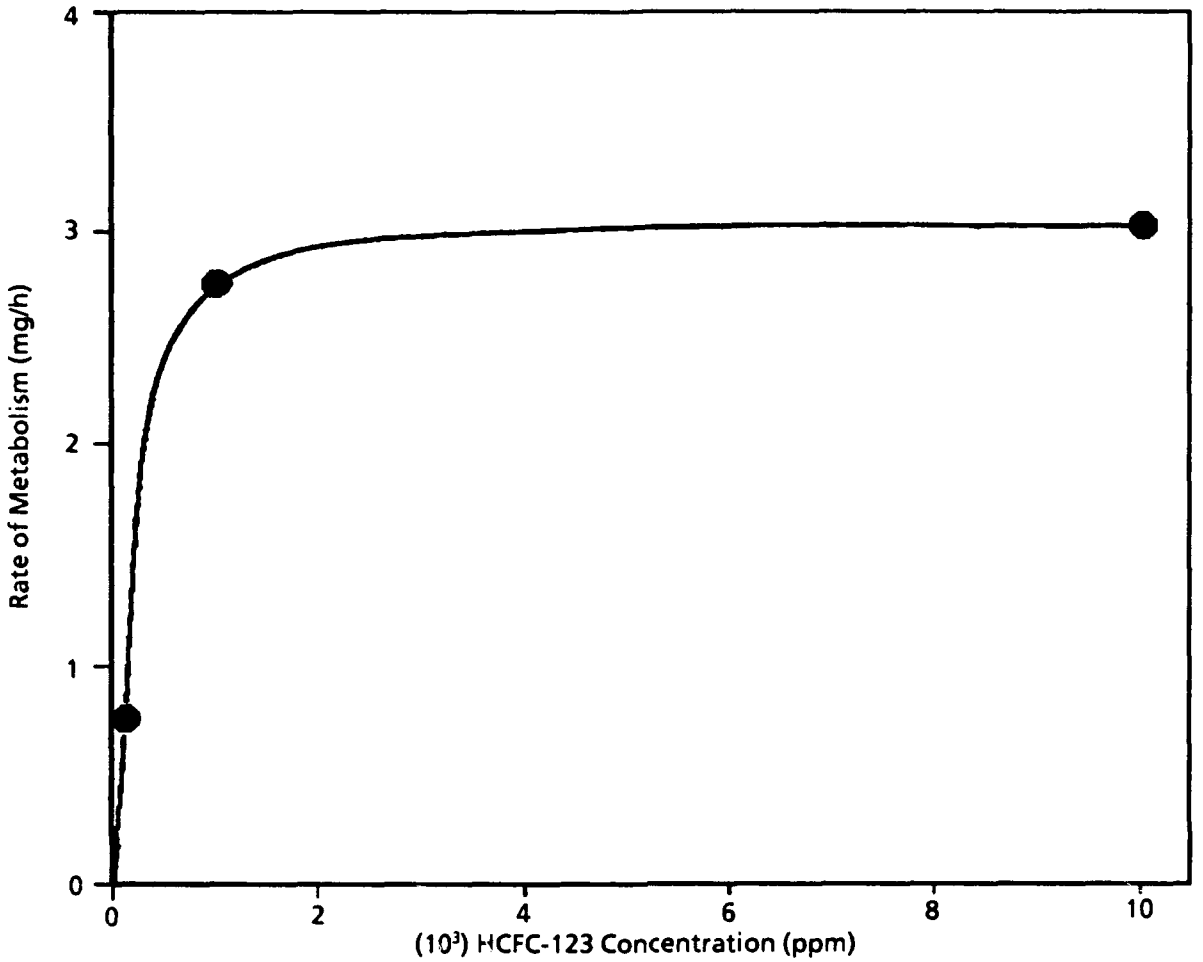

Figure 12. Rate of Metabolism vs. HCFC-123 Concentration. Enclosed circles are actual data points; solid line is simulated following application of PBPK model (see text).

HCFC-133a expired air concentrations during 3 h of exposure to 100, 1000, or 10,000 ppm HCFC-123 were determined (data not shown). If one calculates the proportion of the values to each other and compares the actual ratios with those predicted by the PBPK model, excellent agreement is observed. Thus, the amount of formation and elimination of this reductive metabolite is predictable and is saturable at an exposure concentration of approximately 2000 ppm HCFC-123. The formation of volatile metabolites, such as HCFC-133a or CDE, following halothane exposure has been demonstrated in multiple species [7,8]. HCFC-133a appears to be relatively stable compared to CDE [24] and may be a useful index for additional pharmacokinetic studies with HCFC-123.

This research was conducted under the Department of the Air Force Contract No. F33615-90-C-0532 for the Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory, Wright-Patterson Air Force Base, OH.

REFERENCES

1. **Brashear, W.T., M.M. Ketcha, D.L. Pollard, C.S. Godin, H.F. Leahy, P.P. Lu, E.R. Kinkead, and R.E. Wolfe.** 1992. Metabolite identification of halon replacement compounds. Report No. AL-TR-1992-0078. Wright-Patterson Air Force Base, Dayton, OH.

2. **Hoover, D.K., K.O. Yu, R.K. Black, G.W. Jepson, and J.W. Fisher.** 1992. Kinetic evaluation of Halon 1211, HCFC 123, HCFC 142B, and HCFC 124 using gas uptake methods. *Toxicologist* 12:353.

3. **Harris, J.W., L.R. Pohl, J.L. Martin, and M.W. Anders.** 1991. Tissue acylation by the chlorofluorocarbon substitute 2,2-dichloro-1,1,1-trifluoroethane. *Proc. Natl. Acad. Sci. U.S.A.* 88:1407-1410.

4. **Martin, J.L., J.W. Harris, A.C. LaRosa, M.J. Olson, M.W. Anders, and L.R. Pohl.** 1992. Metabolism *in vivo* of halothane and the chlorofluorocarbon substitutes HCFC-123, HCFC-124, and HCFC-125 to trifluoroacetylated liver protein adducts. *Toxicologist* 12:62.

5. **Owen, A.D. and B.W. Van Der Veen.** 1986. Perspectives in the pathogenesis of halothane-induced hepatitis. *S. Afr. Med. J.* 69:807-810.

6. **Stier, A.** 1964. Trifluoroacetic acid as a metabolite of halothane. *Biochem. Pharmacol.* 13:1544.

7. **Mukai, S., M. Morio, K. Fujii, and C. Hanaki.** 1977. Volatile metabolites of halothane in the rabbit. *Anesthesiology* 47:248-251.

8. **Sharp, J.H., J.R. Trudell, and E.N. Cohen.** 1979. Volatile metabolites and decomposition products of halothane in man. *Anesthesiology* 50:2-8.

9. **EPA, U.S. Environmental Protection Agency.** 1990. Hydrofluorocarbons and hydrochlorofluorocarbons hazard assessment. Document prepared for the Office of Toxic Substances, Health and Environmental Review Division.

10. **Malley, L.A., M.C. Carakostas, J.F. Hansen, H.J. Trochimowicz, and G.M. Rusch.** 1991. Chronic toxicity of hydrochlorofluorocarbon HCFC-123 in rats. *Toxicologist* 11:103.

11. **Anders, M.W., L. Lash, W. Dekant, A.A. Elfarra, and D.R. Dohn.** 1988. Biosynthesis and biotransformation of glutathione S-conjugates to toxic metabolites. *CRC Crit. Rev. Toxicol.* 18:311-341.

12. **Baker, M.T., J.N. Bates, and S.V. Leff.** 1987. Stimulatory effects of halothane and isoflurane on fluoride release and cytochrome P-450 loss caused by metabolism of 2-chloro-1,1-difluoroethene, a halothane metabolite. *Anesth. Analg.* 66:1141-1147.

13. **Brown, B.R. and I.G. Sipes.** 1977. Biotransformation and hepatotoxicity of halothane. *Biochem. Pharmacol.* 26:2091-2094.

14. **Olson, M.J., J.T. Johnson, J.F. O'Gara, and S.E. Surbrook, Jr.** 1991. Metabolism *in vivo* and *in vitro* of the refrigerant substitute 1,1,1,2-tetrafluoro-2- chloroethane. Drug Metabolism and Disposition 19:1004-1011.

15. **Harris, J.W. and M.W. Anders.** 1991. *In vivo* metabolism of the hydrochlorofluorocarbon 1,1-dichloro-1-fluoroethane (HCFC-141b). *Biochem. Pharmacol.* 41:R13-R16.

16. **Hake, C.L., T.B. Waggoner, D.N. Robertson, and V.K. Rowe.** 1960. The metabolism of 1,1,1-trichloroethane by the rat. *Arch. Environ. Health* 1:101-105.

17. **Kaminsky, L.S. and J.M. Fraser.** 1988. Multiple aspects of the toxicity of fluroxene and its metabolite 2,2,2-trifluoroethanol. *CRC Crit. Rev. Toxicol.* 19:87-112.

18. **Seckar, J.A., H.J. Trochimowicz, and G.K. Hogan.** 1986. Toxicological evaluation of hydrochlorofluorocarbon 142b. *Fed. Chem. Toxicol.* 24:237-240.

19. Product Toxicity Summary Sheet on FLUORINERT® Brand Electronic Liquid FC®- 72, 3M Center, September 9, 1990.

20. **Wickramaratne, G.A., D.J. Ti ston, D.L. Kinsey, and J.E. Doe.** 1988. Assessment of the reproductive toxicology of br mochlorodifluoromethane in the rat. *Brit. J. Ind. Med.* 45:755-760.

21. **Goldstein, A., L. Aronow, and S.M. Kalman.** 1974. *Principles of Drug Action: The Basis of Pharmacology*, 2nd ed., pp. 338-353. New York: John Wiley and Sons.

22. Fiserova-Bergerova, V. and R.W. Kawiecki. 1984. Effects of exposure concentrations on distribution of halothane metabolites in the body. *Drug Metab. Dispos.* 12:89-105.

23. **Urban, G., G. Loizou, W. Dekant, and M.W. Anders.** 1992. Pharmacokinetics and metabolism of 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123) in male and female Sprague-Dawley rats: A gas-uptake study. *Toxicologist* 12:217.

24. Maiorino, R.M., I.G. Sipes, A.J. Gandolfi, B.R. Brown, and R.C. Lind. 1981. Factors affecting the formation of chlorotrifluoroethane and chlorodifluoroethylene from halothane. *Anesthesiology* 54:383-389.

ACKNOWLEDGEMENTS

The authors are grateful to several individuals, who contributed to the collection and analysis of data for this investigation. They are R. Godfrey, S. Godin, M. Ketcha, E. Kinkead, H. Leahy, D. Pollard, S. Salins, C. Seckel, and R. Wolfe.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animal* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council, Department of Health and Human Services, National Institutes of Health Publication No. 86-23, 1985, and the Animal Welfare Act of 1966, as amended.

SESSION II

ALTERNATIVES TO ANIMALS IN TOXICOLOGY RESEARCH: POLICY CONSIDERATIONS

ANIMAL ISSUES AND SOCIETY

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SUMMARY

Animal use topics are sensitive issues today. Animal use issues are often presented as black and white or "we" are right and "they" are wrong. This is clearly demonstrated in the available literature from most organizations. Topics presented in this article include: delineation of issues and concerned groups; examples of animal issues in education and agriculture; the terrorist issue; animal issues/sportsman issues; political and legislative impact; and biomedical and toxicological animal use issues. The total context of these issues is not black and white and most of us fall closer to the center of these issues than to the extremes. Dialogue can begin to move our perceived opinions away from the images of black and white and into the many shades of grey.

INTRODUCTION AND PHILOSOPHICAL TENANT

As a veterinarian by profession, trained as a pathologist, and working in a laboratory involved in toxicology research, I believe my work is important and relevant. My neighbor is a young lady of 15 years who thinks I am doing the wrong thing by using animals for research. She is not alone in her belief. I think she does not understand my beliefs and she thinks I do not understand hers. It would appear the individual beliefs on this issue are drawn into a black versus white or right versus wrong polarization, which often seems the case for many social issues.

What leads to the rise of social issues is often the cause of debate. The fact that many social issues led to social change is only debated by the degree of change. Animal use in our society is a current social issue and is very controversial. Although the use of animals in society is as old as history records, the effects of modern communication methods have led many individuals and organizations to assume a position regarding animal issues.

People see animal issues in many different ways. What constitutes an animal-related issue and an individual's position on an issue depends upon a host of variables, some of which might include an individual's geographical location, culture, religion, education, personal beliefs, ethics, and morals. As a veterinarian, I believe those in my profession should assume a leadership role regarding animal use in society. I strongly believe that the majority of opinions on the use of animals for research have much in common.

Presenting issues in a black and white manner is often the strategy of an organization. The objective is to form stereotypes and attach values to them. This process stems from a blend of debate positioning, salesmanship, and self-interests. Groups and organizations present their positions or beliefs as correct, accurate, and honest, yet the positions of the opposition are rarely recognized with equal respect. Organizations are generally composed of loyal individuals who entrust the leadership to defend or promote the concerns that are perceived as being in the group's collective interest or represent the philosophy of the membership.

The transition from individual philosophy to organizational philosophy is the pathway that hinders our ability to see the many shades of grey regarding animal use issues. Much of the hostility associated with animal issues could be reduced by recognizing common objectives despite different philosophies. The recognition of those common objectives and of the many shades of grey can only be accomplished by promoting both dialogue and respect for the right of opinion among all involved.

GROUPS . . . A PERSPECTIVE

The formation of groups to protect domestic animals did not begin in this century. The birth and evolution of the movement has been dramatic. Perhaps it could be traced to beginning in Britain with the Society for the Prevention of Cruelty to Animals (SPCA) in 1824, renamed as the Royal Society for the Prevention of Cruelty to Animal (RSPCA) in 1840 following Queen Victoria's recognition [1]. The number of new organizations founded in the last half of this century has been explosive.

Many cite two publications (*Animal Liberation* [2] by Peter Singer and *The Case for Animal Rights* [3] by Tom Regan), for the inspirational philosophy that has led to the formation of many contemporary animal-related organizations. The flurry of publications related to animal issues has grown proportionally with the formation of groups. Correspondingly, the number of "counter-position" groups has also grown. Today these issues have led to the creation of a very healthy growth-industry that employs thousands of individuals and is funded by merchandise sales, individual donations, and corporate budgets.

Environmental issues are rapidly becoming a dominant focus of public concern. These concerns are having an ever increasing impact on governmental policy, regulatory statutes, and upon those subject to compliance. It is also affecting animal-related organizations. Issues of resource consumption, global warming, and toxic waste are now complemented by the issues of habitat loss, endangered species, and wildlife management. The relative strengths of the environmental movement compared to the animal protection movement is demonstrated by the operating budgets of the top 12 animal-related organization reported by *Animals Agenda* magazine in April 1992 (Table 11) [4]. *Animals Agenda* classified groups as animal advocacy groups, shelter and sanctuary groups, antivivisection groups, and habitat conservation groups.

TABLE 11. TOP 12 MAJOR ANIMAL AND HABITAT PROTECTION GROUPS BASED ON 1990 BUDGET

Organization	1990 Budget
Nature Conservancy	$ 137,734,000
Ducks Unlimited	$ 68,052,939
World Wildlife Federation	$ 51,243,350
Greenpeace USA	$ 39,921,521
Sierra Club	$ 36,062,500
National Audubon Society	$ 33,470,164
North Shore Animal League	$ 22,337,145
American SPCA	$ 19,142,456
Massachusetts SPCA	$ 19,106,328
The Wilderness Society	$ 17,672,779
The Humane Society of the United States	$ 16,485,209
People for the Ethical Treatment of Animals	$ 8,811,252

SPREADING THE WORD FOR CREATING CHANGE AND SUPPORT

Enabling the information to reach the consumer/customer is a complicated art. The ultimate objective is to attain financial support, political support, and philosophical support from those who hear or see the message. The avenues that lead to these objectives extend to educational institutions and mass media.

Animal issues have come to the nation's schools by pamphlets that contain ideas geared for young minds. Organizations promoting change to existing practices were first to initiate major campaigns to reach students. In recent years, many major organizations have recognized the importance of gaining support from those individuals developing opinions for the first time.

Mass media is a term used to represent printed and electronic communication, as opposed to direct verbal communication to an individual or group. It can be effectively utilized by two means. One is free and uncontrolled, using news media forums to convey the message. The other is expensive and controlled, and is very similar to the direct marketing approach.

The free form utilizes press releases, interviews, and events designed for media consumption. The uncontrolled aspect is due to the unknown interpretation or comment attached to effort by journalists or reporters. Gaining the support of a recognized celebrity or publicly respected individual is often used to broaden or strengthen support for the message. The direct marketing approach employs advertisements, mail solicitations, video releases, and other forms of publication. Several organizations involved with animal issues could be considered classic examples or prototypes for study of highly effective media utilization.

The cultivation of public opinion has led to public pressure with political impact. A clear concern of all organizations associated with animal issues is to ensure that the laws and regulations that govern animals and animal use are consistent with the objectives of the membership. This has led to organizations formed specifically to influence legislative opinion, promote legal change, and establish judicial precedent for animal use and/or abuse. Many organizations have attempted to mobilize their members to contact their elected representatives. Recent advances in biomedical technology and research methods, such as genetically altered animals and animal patents, have brought to the surface new issues that are now attracting attention and thought.

ISSUES

Most animal-related issues have been debated from a myriad of viewpoints. A plurality of opinions exists for what constitutes animal abuse and/or animal exploitation. The elimination of needless pain or suffering hinges on what constitutes or is conceptualized as needless. Historically, the major focus of attention has been proper care of domesticated animals, especially horses and dogs. Recent decades have seen concern extend to humane treatment of animals intended for food or fur, wildlife, animals in entertainment, and laboratory animals.

Terminology has been created in an attempt to classify philosophies and organizations as supporters of either animal welfare or animal rights. Although some organizations clearly state that they support animal rights, the majority of organizations choose to claim that they support the concepts of animal welfare. In fact, so many groups have chosen to attach the animal welfare label to their position(s) and image that it is losing the desired public perception for which it was crafted.

"Terrorism" is a word that captures everyone's attention and promotes both a reaction and a counter-reaction. Alvin Toffler in *Future Shock* [5] commented on the rise of terrorism that torments the world today. The motivation of some animal rights activists to strike in an illegally destructive and occasionally fatal manner has fostered fear and hate in many people. Unfortunately, the acts of a few using methods that destroy creditability have cast a new and undesirable shadow on those who seek to reduce or eliminate "animal suffering." The majority of animal-related groups have stated their total rejection of such illegal activities and distance themselves from activities that could be construed as supporting such efforts. Those who serve as spokespersons for these clandestine groups are subject to significant backlash, yet the devotion to their "cause" appears to outweigh the negative public reaction.

The image or idea of a pet/companion animal going to research, for many pet owners, would be like a lost or abandoned child being used for research. This image has affected how shelters and pounds handle unwanted animals. The majority of animals in research are rodents bred for the purpose of laboratory research. Although animal research has lead to numerous scientific

advancements, many seek to promote awareness of alternatives to animals in research. The laws and regulations that govern animal-use research are continuing to evolve. Additionally, researchers are increasingly concerned about animal-use issues.

POSITIONS AND CHANGE

Some people believe that certain organizations, because of their positions on the animal-use issue, threaten existing laws and regulations drafted to govern animal care and use. A common question regarding animals in research asks, "What is the appropriate use of animals in research?" This has led to ideas formulated to reduce the numbers of animals used in research. Similar questions are extending to other animal-use topics now.

We are being exposed to viewpoints to make animal-use issues appear as black and white issues. Which viewpoint is right and which is wrong? What is justified and what is not? What is fact and what is myth? Effectively facing and dealing with these issues requires an open mind, because the polarization of issues into extreme terms prevents the recognition of common concerns. Moreover, accusations often lead first to anger or hurt and then to hostility. Hostility does not promote constructive dialogue. These issues are not going away. I am sure both sides will try to continue to gain public support and awareness. I expect we will continue to see graphic images that reach through to rational minds and touch our deepest emotions.

As a veterinarian, and as a researcher, and as a father, I hope my colleagues and neighbors study these issues not from an emotional basis resulting from the influence of the mass media but rather from a position of careful thought. The total context of these issues is not black and white and most of us fall closer to the center of these issues than to the extremes. In closing, let me share what my young neighbor wrote as she previewed my presentation. I think it is a good example of how dialogue can begin to move our perceived opinions away from the images of black and white and into the many shades of grey. "I think some research is needed in the medical field and the best way to do that is through animals. Animal rights organizations are concentrating too much on killing animals for research. Instead, they should put more effort into banning hunting, promoting vegetarian diets, and stopping the use of animals in relation to beauty products, household items, etc. Also, I think more emphasis should be placed on animals in their natural environment, and there should be more nature reserves where animals can live in a protected place."

RECOGNITION

This publication represents the theme of a presentation made in May of 1992. In preparation, many organizations and groups were contacted and their respective positions and policy statements requested. Those organizations which assisted this effort are:

The American Anti-vivisection Society, Jenkintown, PA

American Cancer Society, Atlanta, GA

American Fund For Alternatives to Animal Research, New York, NY

The American Humane Association, Englewood, CO

American Society For the Prevention of Cruelty to Animals, New York, NY

American Medical Association, Chicago, IL

The American Physiological Society, Bethesda, MD

Americans for Medical Progress, Norwalk, CT

American Veterinary Medical Association, Schaumburg, IL

Animal Industry Foundation, Arlington, VA

Animal Legal Defense Fund, San Rafael, CA

Animal Protection Institute, Sacramento, CA

Animal Rights International, New York, NY

Animal Rights Mobilization, Denver, CO

Animal Welfare Institute, Washington, DC

Applied Research Ethics National Association, Boston, MA

Association of American Medical Colleges, Washington, DC

Association of Veterinarians for Animal Rights, Greenwich, CT

Civitas, Swain, NY

The Doris Day Animal League, Washington, DC

Foundation for Economic Trends, Washington, DC

Friends of Animals, Norwalk, CT

The Fund For Animals, New York, NY

The Humane Society of the United States, Washington, DC

The Humane Farming Association, San Francisco, CA

Incurably Ill for Animal Research

In Defense of Animals, San Rafael, CA

The Johns Hopkins Center for Alternatives to Animal Testing, Baltimore, MD

Medical Research Modernization Committee, New York, NY

Illinois Society for Medical Research, Bridgeview, IL

International Foundation for Ethical Research, Chicago, IL

The International Primate Protection League, Summerville, SC

The International Society for Animal Rights, Inc., Clarks Summit, PA

The National Academy of Science, Washington, DC

National Anti-vivisection Society, Chicago, IL

The National Association for Biomedical Research, Washington, DC

National Cattleman's Association, Englewood, CO

National Institutes of Health

Office of Animal Care and Use, Bethesda, MD

Office For Protection from Research Risks, Bethesda, MD

The National Cancer Institute, Bethesda, MD

National Institute of Environmental Health Science

National Rifle Association

People for the Ethical Treatment of Animals, Washington, DC

Pharmaceutical Manufactures Association, Washington, DC

Physicians Committee for Responsible Medicine

Psychologists for the Ethical Treatment of Animals, New Gloucester, ME

Public Responsibility in Medicine and Research, Boston, MA

Putting People First, Washington, DC

Rocky Mountain Humane Association, Littleton, CO

Scientists Center for Animal Welfare, Bethesda, MD

Society for Animal Protective Legislation, Washington, DC

Student Action Corps for Animals, Washington, DC

Students United Protesting Research on Sentient Subjects, Pasadena, CA

The Texas Society for Biomedical Research, Austin, TX

Tuffs Center for Animals and Public Policy, N. Grafton, MA

United Conservation Alliance, Washington, DC

The Vegetarian Resource Group, Baltimore, MD

Wards, Inc., Washington, DC

The World Wildlife Fund, Washington, DC

The Washington Association for Biomedical Research, Seattle, WA

Washington Humane Society, Washington, DC

Washington Legal Foundation. Washington, DC

The Wildlife Legislative Fund of America, Columbus, OH

REFERENCES

1. **Jasper, J.M. and D. Nelkin.** 1992. The Animal Rights Crusade: The Growth of A Moral Protest, *The Free Press*, p. 57.
2. **Singer, P.** 1975. *Animal Liberation*, The New York Review of Books.
3. **Regan, T.** 1983. *The Case for Animal Rights*, University of California Press.
4. **Who Gets The Money.** 1992. *The Animals Agenda*, Vol XII, No.3, p. 23 (April).
5. **Toffler, A.** 1970. *Future Shock*, Random House.

CURRENT DoD POLICY ON USE OF ANIMALS IN RESEARCH

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SUMMARY

The current Department of Defense (DoD) policy on use of animals in research is contained in the DoDD 3216.1 "The Use of Animals in DoD Programs" and its references. This Directive establishes DoD policy for the procurement, transportation, and care of animals used in research, development, testing and evaluation, clinical investigation, and instructional programs of the Department of Defense. This document lists the responsibilities of the Director of Defense Research and Engineering, and the Heads of the DoD Components. In addition, it defines the specific responsibilities of the Secretary of the Army. This document will be discussed in some detail as the foundation for all research utilizing animals as test subjects throughout the Department of Defense.

It is the policy of the DoD that animals will be used only when it is necessary to our mission, and where it contributes to preserving the health or saving the lives of servicemen and servicewomen. Medical research efforts generally address the unique medical requirements encountered while on field duty whether it be in training, on the battlefield, or in other circumstances involved with fulfillment of a mission. Regulations and rules governing the use of animals in research have been established in strict concert with other government departments and agencies, including the Department of Health and Human Services and the Public Health Service.

Department of Defense Directive 3216.1, dated February 1, 1982, is the most current DoD Directive (DoDD) pertaining to the use of animals in DoD programs. It was implemented on June 1, 1984 by Joint Service Regulations – AR 70-18; SECNAVINST 3900.38B; AFB 169-2; DARPAINST 18; DAINST 3216.1B; and USUHSINST 3203. These documents require compliance with laws and regulations as amended, which means that the most recent changes in the Animal Welfare Act, the *Endangered Species Act*, the *Marine Mammal Protection Act of 1972 (as amended)*, and other laws, regulations, and standards governing the use of animals in research, are the standards used by the DoD to evaluate research proposals and animal programs within the DoD. Not only is the DoDD inclusive of all changes to the laws and regulations (both present and future), but it exceeds the requirements of other Federal regulations. For example, although the Animal Welfare Act exempts many species of animals, including mice and rats (which constitute more than 80% of the animals used in research) this directive applies to "any live nonhuman vertebrate used for research, testing, experimentation, exhibition, or instruction." Furthermore, it applies not only to the Active

Components of the military, but also to Reserve Components engaged in activities involving the use of animals as defined in this regulation. That is to say that it pertains to any nonhuman vertebrate used for research, development, training, and engineering, clinical investigations, diagnostic procedures, instructional programs, or exhibitions (including working, recreational, and ceremonial animals, in displays, demonstrations, or ceremonies).

What then, do these regulations say about the mechanism of review for animal use? They require that all protocols specifying the use of animals be reviewed by a Laboratory Animal Care and Use Committee (LACUC). This review is designed to ensure that the animals will be treated humanely, and that each protocol includes a statement of need for the information being sought, a brief review of pertinent literature on the subject, and a nontechnical description of the general approach and how the animals will be used. Also, a justification for the use of animals rather than some alternative nonmammalian model must be presented. The guidelines for the LACUC are to be found in the *Guide for the Care and Use of Laboratory Animals* (NIH Pub 85- 23, 1985 Revision), the Animal Welfare Act and its implementing rules and regulations (9 CFR Parts 1, 2, and 3), the Endangered Species Act, the Lacey Act, the Marine Mammal Protection Act, and the Department of Health and Human Services guidance on the supply and use of laboratory primates in NIH 80-1520 (the National Primate Plan).

Concurrently, a Scientific Review Committee consisting of members selected on the basis of an expertise in various scientific disciplines and their understanding of scientific design and analysis, must review the protocol with a view toward justification of the species of animal proposed, the numbers of animals required for scientific validity, as well as pain alleviation and to determine if the type of euthanasia, if required, complies with current guidelines set forth by the American Veterinary Medical Association Panel on Euthanasia.

A recommendation for approval, approval with modification, or disapproval is then sent to the institution commander for concurrence. It must be noted that a commander may not approve a protocol in which an "approval with modification" or a "disapproval" has been recommended by either of the aforementioned committees.

Furthermore, all protocols that propose the use of nonhuman primates must, additionally, receive centralized review and approval at Headquarters, U.S. Army Medical Research and Development Command by the Animal Use Review Officer (AURO) to ensure that the protocol contains all required animal use justification and documentation.

The DoD strives to have all laboratory facilities within the DoD fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). At this time, about half of the laboratories using animals in research are so accredited, and there is ongoing effort to bring the other laboratories up to this high standard.

Similarly, protocols submitted by private and state facilities for possible federal funding must, after being approved by their own local LACUC (which must be documented in the proposal), then be submitted to and approved by the command's AURO to ensure justification and documentation completeness. Such grant and contract proposals must also show evidence of compliance with current animal welfare laws and care standards found in the *Guide for the Care and Use of Laboratory Animals*. A signed statement must be included attesting to the fact that the principal investigator understands and accepts full responsibility for the proper care and use of animals in that protocol.

I would like to highlight several questions that are often asked of, or leveled at the DoD. Why does DoD not have oversight laboratory inspections by the U.S. Department of Agriculture (USDA)? The Animal Welfare Act (9CFR Parts 1, 2, and 3), paragraph 2.30 of Subpart C, states: "each research facility other than a federal research facility, shall register with the Secretary ...." Hence, all private and state facilities that receive federal government funding to do research involving animals must be inspected annually by the USDA. This same provision exempts federal laboratories from USDA inspection. It is for this reason, in part, that federal laboratories seek accreditation by the AAALAC. This is an excellent example of the federal laboratories seeking to police themselves even though it is not required.

Another question frequently asked is, "What about the use of animals in chemical, nuclear, and biological research"? Based on the agreement by the representatives of the United States with other participants of the Biological Weapons Convention, April 10, 1972, DoD does not conduct research on, or development of, biological weapons. The Department of Energy, rather than the DoD, is the developer of any nuclear weapons. It is permissible to use animal species to develop chemical weapons, but currently no research is being done in this arena. However, the DoD uses a broad spectrum of animals to develop countermeasures against biological and chemical weapons; not, however, to nuclear weapons. There are no federal, DoD, or Army laws or regulations currently prohibiting the use of any animals for the development of medical countermeasures against biological and chemical weapons. Current work is in the development of vaccines, drugs, and/or other medical therapies and diagnostic tests for the detection of organisms in the environment or the host.

Finally, although significant progress has been made, and continues to be made, both in civilian and federal research facilities, in developing nonmammalian alternatives or adjuncts to animal use for research purposes, it is our firm belief that the continued use of animals remains essential, at this time, for improving the protection of American and allied military personnel from

disease and injury. In the meantime, we will continue to strive to identify, develop, and adopt acceptable adjuncts or alternatives in order to decrease the numbers of animals needed in research.

NOTE: The DoD Instructions listed in this paper can be obtained from the National Center for Disease Research, National Institute of Health, Bethesda, MD 20205. The NIH publication is available, at cost, from the Defense Printing Service, Philadelphia, PA, telephone: 215-697-2179.

FORMULATION OF ETHICAL STANDARDS FOR USE OF ANIMALS IN MEDICAL RESEARCH

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SUMMARY

In the past 200 years, moral theories and attitudes toward both humans and animals have changed considerably. These changes have led to widespread criticism of animal use in some or all research and testing. For the most part, the debate has been marked more by rhetoric than scholarship, but the underlying philosophical theories and their protagonists have been a very important influence on the modern debate over animal use. This paper examines some of the moral philosophy and attempts to present the main arguments concisely and simply and to refute common misconceptions.

INTRODUCTION

When scientists, who are most comfortable dealing with measurable and "objective" phenomena, discuss ethics and moral theories, there is a tendency to view morality as something that is either intuitive and intensely personal or even as simply an emotional response to a difficult conflict. Certainly intuitions, personal backgrounds, and emotions influence our ethical deliberations (as they do our technical discussions), but it would be a mistake to dismiss ethics as an inappropriate topic for scholarly examination. One can, and should, employ logic and scholarly analysis to examine moral theories and, at the very least, to help determine what questions we should be asking. Often, a clear formulation of the essential questions renders the answers fairly obvious.

Apart from the lack of empirical data to help resolve conflicts between theories, moral argument is very similar to scientific argument. In both cases, a lack of coherence or obvious inconsistencies will be evidence of a flawed theory. However, as in science, the existence of flaws does not necessarily mean that a particular moral construct will be abandoned by society. In the case of challenges to fundamental social mores especially, it takes a long time for society to get used to and assimilate new ideas and arguments.

In the past 200 years, moral theories about our fellow humans have changed as modern Western societies have accepted, in theory and law at least, the moral equality of all humans, be they white or black, male or female. Similarly, our attitudes toward animals have changed over the same period although the debate about the proper moral status of animals goes back millenia. For example, the ancient Greeks examined the place of animals and produced four main positions –

Animism, Mechanism, Vitalism, and a fourth, "common sense" position, held by the vast majority of the populace, that animals were placed on earth for human benefit [1]. This last view of animals as resources for humans was not articulated in any detail, much like the situation today where the dominant view of the moral status of animals is based on the largely unexamined notion that humans have been granted dominion over animals. Therefore, it is generally held that we can use animals as we see fit as long as we are not wanton or malevolent about it. This view is now criticized by a wide array of philosophers whose arguments have helped to revitalize the modern animal liberation movement.

For the most part, however, the public debate has been marked by a distressing lack of scholarship and academic debate. The public rhetoric of the protagonists in the animal research controversy usually focuses on the moral turpitude of the other side – antiscience, antihuman, sadistic, and self-glorifying are a few of the adjectives used. On the scientific side, those seeking to maintain the current status have usually responded to critics by increasing the volume rather than by addressing the actual arguments of such critics. Indeed, scientists commonly misinterpret philosopher Singer's arguments [2] and often wrongly assert that he promotes an absolute prohibition of animal research. On the contrary, he argues that animal suffering must be considered as part of the Utilitarian cost-benefit equation in deciding whether certain animal research should be permitted. In the media debate, Singer seems to view most animal research as involving considerable suffering and relatively little potential benefit. Under these circumstances, he would conclude that such animal research should be stopped.

Recently, there have been several considered attempts to reconcile the conflicting arguments about the use of animals in medical research and to begin to develop a reasonable synthesis that reflects a coherent and consistent theory of the moral status of animals. This has proved very difficult because, unlike the argument over research on human subjects, where everybody agrees with the basic proposition that humans should not be used simply as a means to an end, there is no similar base-line consensus on animals.

In the United States, the Hastings Center, a respected bioethics think tank located in Briarcliff, NY, after two years of meeting, has produced a report on the ethics of animal research that identifies the position of what it categorizes as the "troubled middle" [3]. In other words, it recognizes that animal research raises troubling moral questions but does not reject the practice out of hand. In the United Kingdom, a similar body, the Institute for Medical Ethics in London, has produced a book-length report with similar conclusions [4]. Few people are likely to be surprised by the conclusions reached by these two groups. They found that research is important, that animals deserve some

(perhaps increased) moral status in modern society, and that research on animals can be justified given certain conditions.

The Modern Controversy

In the last 20 years, more has been written about the moral status of animals than in the previous 2000, but we seem to be no nearer to resolving some of the central arguments and controversies. For example, what grounds can be used to distinguish humans and animals as different in kind rather than degree? If we choose one criteria like reason or language, then what reasons should we give for excluding chimpanzees while including humans who cannot talk or reason? Whatever criterion is chosen, there are either some animals that appear to meet it or some humans that do not. Moral theories that argue that only those who have duties can have rights [5] would exclude a significant number of humans, who are not deemed capable of having duties, from the category of "rights-holders."

If suffering is to be given a critical place in our moral theory, then how do we decide what animals are capable of suffering and is all suffering equivalent? Are humans, with their considerable capacity for abstract thought capable of more intense mental anguish than nonverbal animals? Are insects or other invertebrates capable of suffering given that most theories of suffering require the presence of significant cognitive abilities?

Despite the difficult questions, we may find that it is much easier to come to a broadly supported consensus on the ethics of animal research than the overblown rhetoric and ad hominem attacks in the media would seem to imply. First, however, it is desirable to examine some of the ethical positions [6] that are evident in the modern debate because the various protagonists, most of whom are not trained in the analytical tools of moral philosophy, often have difficulty articulating their own positions regarding the moral status of animals. As such, it is hardly surprising that they are not aware of all the implications of either their own ethical positions or those of their opponents. The following positions do not represent an exhaustive list but they do provide a broad sweep of the arguments that are evident in the modern debate.

Divinely Granted Dominion. People commonly refer to biblical authority to justify the position that we can use and kill animals as we wish provided we are not careless or malevolent. Some go further and suggest that there are no constraints whatever on our use of animals [7] but the prevailing view is that God-granted dominion falls far short of domination and should be interpreted more as stewardship. One is then faced with questions about the extent of the obligations to animals that are required by this position of stewardship.

The Thomist/Kantian Position. Although Aquinas and Kant did not have much to say on the animal issue, they both argued that we should not abuse animals, not because of any inherent value

that the animals hold, but because animal abusers are more likely to move on to abuse other humans. There is a strong thread of this philosophy apparent in humane education and in anticruelty laws.

Although research tends to support the link between animal abuse and subsequent aggressive behavior towards other humans [8], it is also possible that the tendency of some individuals to abuse humans may be reduced by the opportunity to mistreat an animal. In this case, the counter-intuitive nature of the Thomist-Kantian position is evident. Imagine the public reaction if people were encouraged to engage in cruelty to animals as part of their psychotherapy!

Utilitarianism. Most of the American public probably rely heavily, albeit unknowingly, on Utilitarian arguments to support their moral behavior. Many laws and regulations are based on Utilitarian ideas of maximizing good and minimizing harm. Early Utilitarians, especially the 18th century British philosopher, Jeremy Bentham, identified suffering as a key harm. Bentham then extended his moral orbit to include animal suffering. Indeed, one of his passages is widely quoted in the animal movement's literature.

> "It may come one day to be recognized, that the number of legs, the villosity of the skin, or the termination of the os sacrum, are reasons equally insufficient [as blackness of the skin among humans] for abandoning a sensitive being to the same fate. What else is it that should trace insuperable line? Is it the faculty of reason, or perhaps the faculty of discourse? But a full-grown horse or dog is beyond comparison a more rational as well as more conversable animal, than an infant of a day, or a week, or even a month, old. But suppose the case were otherwise, what would it avail? The question is not Can they reason? nor Can they talk? but, Can they suffer?" [9, emphasis added]

The rationality and linguistic skills of the individual being were not important to Bentham. If the creature could suffer and experience pleasure - that is, if it was sentient – then it would be entitled to have its suffering and pleasure compared and weighed against the similar suffering and pleasure of other sentient creatures, including humans. This does not imply that a chicken and a horse should be treated the same, just that their interests in not experiencing the same type of suffering are equal and should be considered equally.

In the area of animal research, Utilitarian arguments are very common. On the scientific side, people argue that animal research is justified because its benefits to humans and animals outweigh the harms to the laboratory animals. On the animal activist side, philosophers such as Singer [10] use Utilitarian arguments to attack the use of animals in research. One could characterize these two opposing positions as Permissive and Restrictive Utilitarianism. Clearly, the two sides are not arguing about the use of Utilitarian theory itself, but rather about the extent of human benefit and animal suffering. Singer holds that animal suffering in the laboratory is considerable and that most of the benefits are either too limited to warrant such suffering or, with sufficient effort, could be achieved without the use of animals. In addition, Singer places an animal's interests in not suffering on a

virtually equal footing with a human's interests in not suffering. (Singer's arguments have been an important element in the upsurge of support for the animal movement over the past decade.) By contrast, scientists tend to maximize claims of benefit and argue that animal suffering is minimal or nonexistent.

There are several problems with the Utilitarian approach. For example, it is virtually impossible to develop the necessary calculations that permit a measured and rational balancing of harm against good. Frequently one has to attempt to balance very different outcomes. How, for example, does one compare the suffering of a certain number of rats with the increased understanding of a biological phenomenon? In the United Kingdom, where the law governing animal research requires a balancing of costs and benefits, nobody has yet produced a systematic way to compare them [10]. As in the United States, most of the attention is paid to reducing the costs (i.e., animal suffering)

It has also proved difficult to identify what groups of animals experience suffering and distress (do insects suffer?) and whether the suffering of rats is equivalent to that of dogs, or humans? Suffering is usually not defined but the usual implication is that it requires a minimum level of cognitive ability that may not be present in most invertebrates (the octopus being a possible exception). The concept appears to be like obscenity where everybody thinks they can recognize it but nobody can define it for regulatory purposes.

Reverence for Life. Albert Schweitzer argued that our moral concern should be extended beyond just those life forms capable of feeling or sensation (sentience). He held that all life exhibits a "will to live" and that it is "good to maintain and cherish....[and]...evil to destroy and to check" [11]. Nevertheless, his philosophy did not cause him to oppose all animal research, nor was he a vegetarian. His view was that any injury to life must be "necessary" and "unavoidable," but he did not spell out the conditions that make the sacrifice of animals in general, or research animals in particular, "necessary" [12]. Many people make appeals as to the sanctity of life but such appeals are usually reserved for human life. The taking of animal life is usually considered to be acceptable provided little or no suffering is involved and the animal's death is necessary for some human end. The wanton killing of animals is usually not condoned. However, "reverence for life" is a common phrase in the lexicon of animal activists and, in its strong form, it usually implies no killing of animals for human benefit.

Animal Rights. Animal rights is not a new concept. People have talked of the rights of animals for centuries. In the 18th and 19th centuries a number of authors discussed the status of animals using the term "animal rights." These culminated in the 1892 book by Henry Salt, entitled *Animal's Rights* [13], which presented a very modern exposition of the issues. Today, the concept of "animal rights" is a central issue in the clash between opponents and proponents of animal research.

Unfortunately, the term animal rights now tends to cloud and confuse rather than clarify the issues because it has come to be used as a convenient hook on which to hang oneself or one's opponents! There are three contexts in which the term is used - (1) the "common sense," (2) the political, and (3) the philosophical - that are rarely distinguished nor identified in the course of debate and argument.

1. *Common Sense.* Approximately 80% of the public believes that animals have rights. However, about 85% of that same public believes that humans have the right to kill and eat animals [14]. Thus, whatever "rights" the public believes animals have claim to, they do not include the right to life. The concept of animal rights held by the general public probably amounts to no more than a vague and woolly idea that animals have the right to some, rather limited moral consideration.

2. *Political Views.* In the developed world, there is a growing tendency to couch political claims in "rights" language. Thus, we talk of civil rights, women's rights, gay rights, and the like. Therefore, in the political arena, a rights claim carries significant political resonance. It is only to be expected that the animal movement would attempt to appropriate the power of rights language for its own cause. In this sense, the public campaign for animal rights also includes the animal welfare movement although there has been some attempt, both inside and outside the animal protection movement, to distinguish between animal welfare and animal rights organizations.

   Peter Singer who, as a Utilitarian, does not agree with rights language, has ironically accepted his identification with the animal rights movement (although he prefers the term "animal liberation") because he sees it as primarily a political movement with only loose ties to its philosophical roots.

3. *Philosophical Arguments.* In philosophical circles, a right can be defined (simply and simplistically) as nothing more than a claim that cannot be over-ridden by claims to human utility. A rights claim can only be over-ridden by another rights claim. Thus, one has to determine just what is being claimed as a right. The fact that most of the philosophical arguments espousing animal rights have been radical challenges to current human use of animals - usually setting forth a claim that animals cannot be used solely as a means to a human end [15] - does not mean that all animal rights positions need be that radical.

The very strong Animal Rights argument - developed by Regan [15] and others - asserts that we cannot use animals merely as a means to our own ends. A weaker, but plausible Animal Rights argument is one where the assertion is made that animals have the right not to be caused to suffer. In both positions, the term "rights" is used simply to define a claim that cannot be over-ridden merely because it would be useful to do so.

Most rights-based arguments have to identify some characteristic or complex of characteristics that confer moral rights. Regan [15], for example, suggests that animals that have beliefs and desires are the "subjects of a life" and this confers inherent worth that gives them the right not to be killed or used to satisfy human ends. He identifies adult mammals as having this capacity and would give

the benefit of the doubt to birds and perhaps other vertebrates. Thus, Regan's animal rights philosophy tends to require a vegetarian life style and little or no animal use by humans.

Rights-based moral arguments have difficulties dealing with shades of grey. In Regan's philosophy, an animal either is a "subject-of-a-life" or it is not. It cannot half fulfil the requirements. This creates certain problems from an evolutionary perspective although it is conceivable that the capacity to have beliefs and desires is an all-or-none property. Nonetheless, Regan runs into difficulties when he argues (as he does in his book) that a human has a richer life than a dog and is therefore to be favored over a dog when faced with a direct conflict of competing rights [15].

Another Possible Approach

Nearly every articulated argument on the moral status of animals has presented its arguments on the basis of a single morally relevant characteristic. For example, Schweitzer argues that possession of a life is the important characteristic. For Singer, it is sentience. For Regan, it is the possession of beliefs and desires. However, it is very likely that no single characteristic is sufficient to describe a complete ethical theory on animal treatment. The world is not that simple. One has to consider a tapestry of characteristics [16], including the possession of life, the possession of sentience, the possession of beliefs and desires, the possession of self-awareness, and the like [6].

One can develop a two-tiered approach to ethical thinking in which there are proscriptive obligations to animals based on the possession of life, sentience, purposiveness, self-awareness, and personhood that establish baseline levels of moral consideration below which one cannot go. On this relatively complex edifice, one can add a layer of prescriptive obligations that are owed to beings with which one has established certain explicit or implicit contracts. Thus, one is required to treat one's family with greater consideration than a stranger but the stranger is owed certain basic obligations that cannot be voided.

This approach has strong Darwinian overtones in that the ranking of obligations tends to follow evolutionary paths. Thus, sentient vertebrates would be accorded more consideration than living, but not sentient coelenterates and self-aware apes would be accorded more consideration than sentient, but (presumably) not self-aware frogs. The scheme also provides a place for the additional moral obligations incurred by explicit or implicit contracts between humans and animals. Thus, this scheme could explain why we might owe more to the family dog than to a purpose-bred laboratory beagle.

Establishing a Moral Framework

In actual fact, when one looks at the way that we come to decisions about the ethics of animal research, one finds that a variety of ethical approaches are used. For example, we place a high value

on virtue in that we try to identify virtuous people who have high standards of ethical behavior to sit on our animal care and use committees. We also try to identify the values that should guide our decision-making and usually incorporate some mix of the following:

- reduce animal suffering as much as possible.
- reduce the number of animals required as far as possible,
- ensure that the science is properly planned and likely to achieve its goals, and
- ensure that those conducting the research are adequately trained so that they will be able to minimize animal suffering.

Finally, we have also established some rules of behavior that should guide our decision-making. Thus, conducting multiple surgeries on a single animal is not permitted unless it is part of the same protocol.

In other words, our every-day approach to moral conduct incorporates such supposedly disparate traditions in ethics as virtue, value, and deontological rules. Whatever the approach used, it is striking to note that nearly every philosopher who has addressed the question of the moral status of animals in the past 20 years has come to the conclusion that not only are the questions difficult but that society should also seriously consider upgrading the moral status of animals. Only a few have taken a contrary view. Fox produced a book justifying the use of animals in research [17] but then recanted his position shortly after the book was published and now argues that animals have rights that prohibit their use as research tools [18]. Cohen argues that not only can animal research be justified but that it is morally mandated [5]. He is one of the few professional philosophers who have come out in unquestioning support of the traditional position.

In conclusion, the issue of the appropriate moral status of animals is neither an easy nor a trivial question. It involves developing theories for the moral weight to be accorded to such qualities as life, sentience and suffering, self-awareness, and the like. It then requires a more sophisticated understanding of the concepts of sentience so that we can identify which animals might satisfy the requirements. In justifying biomedical research, one is faced with difficult questions about the value of basic knowledge and likely therapeutic benefit. Most of the public and many scientists consider the testing of cosmetics and toiletries on animals as an unjustifiable activity. However, people have been severely injured by unsafe personal care products in the past, so what should we do now about testing?

If we agree that animal use in research involves moral costs that need to be taken seriously (a widespread view despite recent tendencies by defenders of science to avoid "apologizing" for animal use in the media), then how much attention must be paid to the three "R"s of Russell (Replacement,

Reduction, and Refinement) and Burch and the idea of alternatives? In toxicology, a broad public consensus on this issue is developing but there is still much suspicion of the concept of alternatives in the halls of the National Institues of Health, neuroscience, and physiology Nonetheless, much progress in attending to the moral issues related to animal research and testing is evident although there is still a great deal left to do. We will need wisdom, humor, and a good sense of proportion in the decades to come if we are to continue to make progress on these issues.

REFERENCES

1. **Brumbaugh, R.S.** 1978. Of man, animals and morals: A brief history. In: R.K. Morris and M.W. Fox, eds. *On the Fifth Day*, pp. 6-25. Washington, DC: Acropolis Books.

2. **Singer, P.** 1990. Postcommentary: Ethics and animals. *Behav. Brain Sci.* 13:45-49.

3. **Donnelly, S. and K. Nolan.** 1990. Animals, science and ethics. *Hastings Center Report.* Special Suppl. 20(3):1-32.

4. **Smith, J.A. and K.M. Boyd.** 1992. *Lives in the Balance: The Ethics of Using Animals in Biomedical Research.* Oxford: Oxford University Press.

5. **Cohen, C.** 1986. The case for the use of animals in biomedical research. *New Engl. J. Med.* 315:865-870.

6. **Tannenbaum, J. and A.N. Rowan.** 1985. Rethinking the morality of animal research. *Hastings Center Report.* 15(5):32-43.

7. **White, R.J.** 1990. Animal ethics? *Hastings Center Report.* 20(6):43.

8. **Felthouse, A.R. and S.R. Kellert.** 1987. Childhood cruelty to animals and later aggression against people: A review. *Am. J. Psychiatry* 144:710-717.

9. **Bentham, J.** 1962. The Works of Jeremy Bentham. J. Bowring ed. Vol. 1, pp. 142-3. New York: Russell and Russell.

10. **Singer, P.** 1975. Animal Liberation. New York: New York Review of Books/Random House.

11. **Schweitzer, A.** 1929. Civilization and Ethics, trans. C. Champion, Macmillan, New York, pp. 246-47.

12. **Schweitzer, A.** 1950. *The Philosophy of Civilization, trans C. Champion*, p. 318. New York: Macmillan.

13. **Salt, H.S.** 1892. *Animals' Rights.* London. New edition issued by International Society for Animals Rights, Clarks Summit, PA in 1980.

14. *Parents Magazine.* 1989. Parents Poll on Animal Rights, Attractiveness, Television, and Abortion. New York: Kane and Parsons Associates, Sep-Oct.

15. **Regan, T.** 1983. *The Case for Animal Rights.* Berkeley, CA: University of California Press.

16. **Nozick, R.** 1983. About mammals and people. *New York Times Book Review*, p. 11. November 27.

17. **Fox, M.A.** 1986. *The Case for Animal Experimentation: An Evolutionary and Ethical Perspective*. Berkeley, CA: University of California Press.

18. **Fox, M.A.** 1987. Animal experimentation; A philosopher's changing views. *Between the Species* 3(2):55-60, 75, 80, 82.

IN VITRO MODELS FOR TOXICOLOGICAL RESEARCH AND TESTING

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SUMMARY

The objective of this report is to discuss some of the issues involved in utilizing *in vitro* methods in toxicological research and testing. The subject is not new, *in vitro* methods have been used for many years in this context. However, there has been a significant increase in interest in the topic within the scientific community recently as witnessed by the increase in scientific journals dedicated to the topic, symposia held by scientific societies, and commitment of resources to *in vitro* toxicological research activities. Toxicologist should be aware of these developments as the future directions of the science will be influenced significantly by *in vitro* methodology.

INTRODUCTION

Nature of Research and Testing

Before addressing the main topic of this manuscript, it is useful to assure that the distinction between the basic nature of research and testing is clear in the mind of the reader. Research is the discovery of new knowledge. It is accomplished by the application of the scientific method which involves observation, establishment of an hypothesis, and experimental testing of that hypothesis. In many cases in the biomedical sciences, the organism of concern (often human beings) cannot be directly utilized as the experimental system to test the hypothesis. In these cases model systems are employed to test the hypothesis. The particular experimental model chosen is determined by the nature of the hypothesis to be tested. Significant effort is expended by researchers in deciding on the appropriate model system to be utilized in a particular situation. Intact animals are employed when the nature of the problem at hand dictates that they represent the best model to test the proposed hypothesis. At other times non-whole animal models (e.g., *in vitro* systems, computer models) may be better suited. The fundamental point is that in research the best model system is determined by the hypothesis to be tested and cannot be dictated a priori.

In the case of testing, the objective is to determine whether or not some condition exists. Is the woman pregnant? Is the blood contaminated with HIV? Will this chemical cause adverse effects under the expected conditions of use? (referred to as the safety hypothesis). Because the question is always the same, it is feasible to establish standard testing protocols to provide the necessary data sets that will allow regulatory authorities to make appropriate decisions with a certain degree of reliability. In this situation, the model systems to be utilized in the prescribed testing procedures are

determined a priori based on a scientific consensus as to the best available methodology. This is an important distinction between research and testing which has implications for the possibility of replacement of *in vivo* with *in vitro* procedures. If one cannot dictate a priori the model system to be used, as is the case in research, then it is necessary to leave all options for model selection open or risk jeopardizing your research activities to an unknowable extent. On the other hand, in the case of testing, where the model systems are selected a priori, replacement of *in vivo* with *in vitro* models is feasible. It is only necessary to demonstrate that the data set obtained from the replacement models is adequate to make reliable decisions.

Purposes of Toxicological Studies

Toxicological studies are conducted with various objectives in mind. Several of these objectives are listed in Table 12 and range from surveillance activities (hazard identification) to medical treatment. In some cases these studies are conducted in a research mode and in other cases in a testing mode. Often it may be difficult to distinguish between the two. For example, an investigative study of the mechanism of action of a particular chemical may be promoted by a need to understand the mechanism responsible for the adverse effects of a class of chemicals in order to design less toxic chemicals as commercial products. In this situation the study would be classified as research. On the other hand, a similarly designed mechanistic study may be prompted by the need to resolve questions about extrapolating *in vitro* toxicity testing data from animal models to humans for a particular drug. In this case the study would be more appropriately categorized as toxicological testing. Arguing whether this latter situation would be classified as research or supplemental testing is not productive. What is important is that the appropriate model system is used to answer the question. Where issues of fundamental knowledge are concerned both *in vivo* and *in vitro* models can play an important role.

TABLE 12. OBJECTIVES OF TOXICITY STUDIES

1. Identify potential toxic hazards (hazard identification)
2. Determine qualitative or quantitative risk for adverse effects in target populations
   - Establish safe levels of exposure
   - Identify unique populations at risk
3. Priority setting: Select least toxic candidate compound in product development
4. Understand mechanisms of toxic action (product design/risk assessment)
5. Determine symptoms of toxicity and design therapeutic strategies

Applications of *In Vitro* Models in Toxicological Testing

Having made the distinction between research and testing, the majority of this discussion will focus on the role of *in vitro* models in toxicological testing. The utilization of new *in vitro* methods in toxicity testing and safety evaluations can be classified into three major categories based on how the

information provided is to be used in commercial and regulatory decision making. The three categories are screens, adjuncts, and replacements (Table 13) Screens are tests which are used to develop preliminary information. The information provided by the screening test is sufficient to make limited management decisions, usually early in the product development cycle. The term implies that further testing will be conducted to generate definitive toxicological data for regulatory purposes.

TABLE 13. CLASSIFICATION OF *IN VITRO* TOXICITY TESTING ON THE BASIS OF HOW THE INFORMATION PROVIDED IS USED

I.	Screens
	• Product development needs – Toxicity ranking
	• Regulatory needs – Hazard identification/priority setting
II.	Adjuncts
	• Regulatory needs – Tier testing strategy
	• Regulatory/product development needs – Mechanistic studies
III.	Replacements
	• Regulatory/product development needs – Prediction of *in vivo* toxicity (risk assessment)

Adjuncts are test methods that are an integral part of the regulatory evaluation strategy, but in and of themselves are not usually adequate to support a final regulatory safety decision. An example would be an *in vitro* test used as an early phase of a tier testing strategy. In most cases a "negative" test result would not be sufficient to rule that a material was safe, although it would be supporting evidence in a "weight of evidence" argument as to the safety of the material. A strongly "positive" outcome may, in certain circumstances, be sufficient to reach a regulatory decision, such as labelling a material as hazardous. The distinction between screens and adjuncts is somewhat blurred because it is certainly feasible that a given *in vitro* test used in one context as a screen could conceivably be incorporated into a tier testing paradigm as an adjunct. Again, it is a waste of effort to try to split hairs.

Finally, replacements are *in vitro* tests that would completely replace the use of *in vivo* toxicity tests in some prescribed area of toxicity testing. It would be extremely naive to expect that any single *in vitro* test would completely eliminate *in vivo* testing in the general sense. In certain cases it may be possible for a battery of *in vitro* tests to completely replace an *in vivo* test for a particular application, such as ocular irritation testing for product labelling. But, the same battery may not be deemed adequate to substantiate the safety of an ophthalmic drug which would be intentionally placed in the eye repeatedly for therapeutic purposes.

The strategy for the development of *in vitro* models and testing methods will depend to a large extent on the purpose for which they are intended. Furthermore, the criteria used to validate the method will also be influenced by the intended purpose for which the test was developed. The issue of validation will be discussed further below.

SCIENTIFIC BASIS OF CHEMICAL SAFETY EVALUATIONS

The Toxicological Process

One of the main objectives of toxicological testing is to establish the dose-response relationship for a particular material. This relationship is a quantitative expression of what happens to an organism when it is exposed to a test material. Experimentally, *in vivo* toxicity testing consists of exposing groups of test animals to various doses of the test material via an appropriate route and quantitatively observing the response of the animal. The various physical, chemical, and biological processes that connect the exposure of the organism to the ultimate expression of effects in the experimental animal or human being, can be illustrated as in Figure 13. This is a schematic diagram of the *in vivo* toxicological process. It starts with exposure to the material and the subsequent absorption into the biological system. The various processes which determine the delivered dose to the target tissue - absorption, distribution, metabolism, storage, and excretion - are collectively referred to as toxicokinetics.

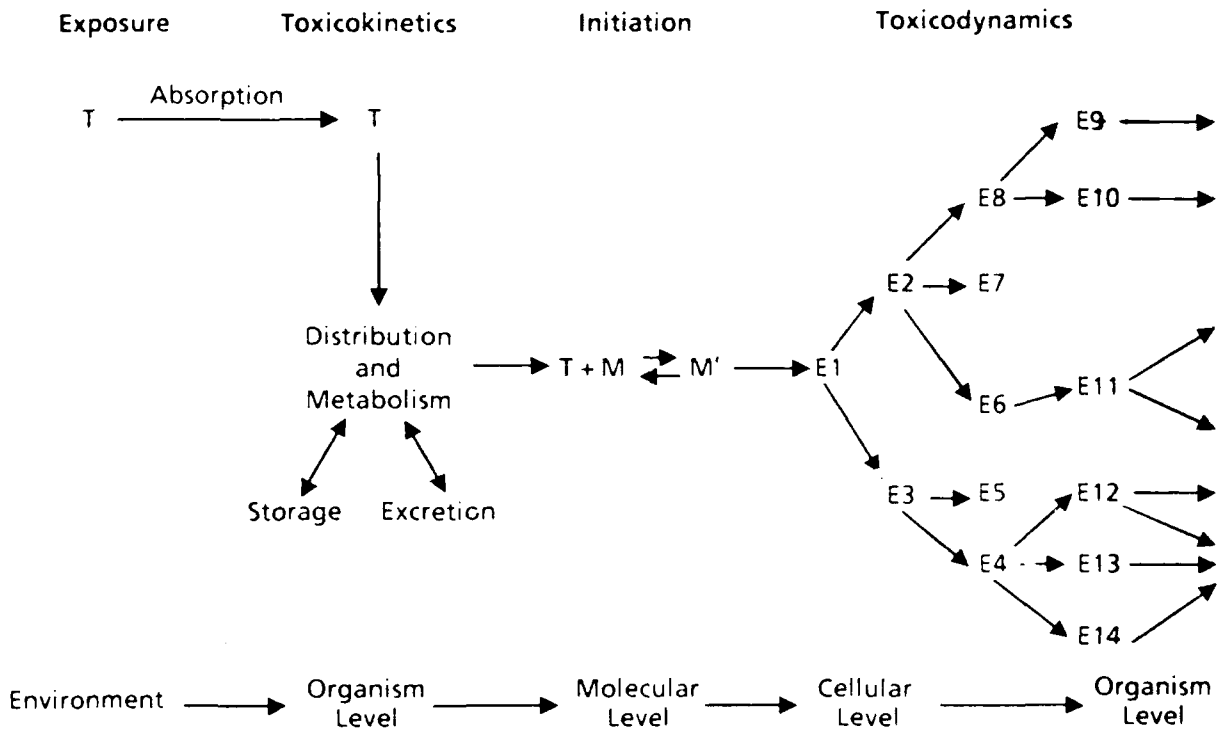

Figure 13. The Toxicological Process *In Vivo*. This schematic diagram illustrates the relationships between exposure to a chemical *in vivo* and the ultimate expression of a toxicological effect in the whole animal.

When the toxicant or an active metabolite reaches the cell, it can react with various molecular targets. Some reactions may be innocuous whereas others may directly alter cellular functions. These first physical-chemical interactions between the toxicant and the molecular targets may be referred to as the initiation of the toxicological response somewhat in analogy with the initiation concept in carcinogenicity. However, in the context of general toxicological phenomena, initiation refers to the molecular interaction between the toxicant and the critical molecular target irrespective of whether this reaction is reversible or irreversible. Initiation may involve a spectrum of reactions ranging from the formation of irreversible adducts between the toxicant and cellular proteins or chromosomal DNA to reversible interactions with enzyme active sites or receptors. The chemical nature of the reaction (e.g., covalent, ionic, hydrophobic) will dictate the degree to which the reaction is reversible under physiological conditions. The cellular system also possesses a wide armamentarium of repair mechanisms which can detect molecular alterations in cellular macromolecules and either repair or replace altered molecular targets. The net balance of the reactions generating molecular alterations and all those reactions correcting damage determine the level of altered molecular targets at any given time. Initially, the magnitude of altered molecular targets provides the driving force for the toxicological response at higher levels of biological organization.

Following initiation, a sequence of events occurs starting at the molecular level and subsequently propagating to higher and higher levels of biological organization. This series of events is referred to as toxicodynamics. Early events occur at the molecular and cellular level. These events can be experimentally observed as: (1) alterations in cellular concentrations of endogenous substrates (e.g., calcium, ATP, glutathione, cyclic-AMP), (2) alterations in the regulation of cellular functions (e.g., macromolecular synthesis, cell replication, cell-cell communication), (3) markers of toxicant-macromolecule interaction (e.g., protein adducts, DNA adducts, inhibition of enzyme activity), or (4) alterations in cellular morphology or structure (e.g., blebbing, calcium deposits in mitochondria, peroxisome proliferation, appearance of autophagosomes). Effects at the cellular level can rapidly propagate to the organ level, system level and eventually to the organism level resulting in clinical toxicosis. Under experimental conditions it is possible to select the level at which we choose to observe these responses. The higher the level of biological organization at which the effects are observed the more complicated the relationship between dose and response because many more processes are involved.

Toxicity Prediction

As mentioned above, one of the objectives of toxicity testing is to obtain the quantitative dose–response relationship. Conceptually, the dose–response relationship is a representation of the probability of a particular toxic response (TR) occurring given a particular exposure to a material (E), represented as $P(TR|E)$. Having segregated the toxicological process into three components –

toxicokinetics, initiation, and toxicodynamics – the overall probability $P(TR:E)$ can be expressed as the product of the three individual probabilities:

$$P(TR:E) = P_K(X:E)*P_I(MT':X)*P_D(TR:MT')$$

where $P_K(X:E)$ is the conditional probability that given the exposure E, the concentration of the toxicant (or its active metabolite) at the molecular target is X, $P_I(MT':X)$ is the conditional probability that the cellular concentration of altered molecular targets will be MT' if the concentration of the toxicant at the molecular target is X, and $P_D(TR:MT')$ is the conditional probability that a particular toxicological response will be observed given the degree of alterations in molecular targets MT'. The first factor is purely kinetic, the second physical-chemical (with molecular repair processes included) and the third factor is biological (biochemical/physiological). Knowledge of these three probability factors would allow for the prediction of the dose–response relationship.

Traditionally, the dose–response relationship for human beings has been predicted on the basis of experimental data derived from animal models. The data derived from these animal studies is extrapolated (high dose to low dose extrapolation, interspecies extrapolation, route-to-route extrapolation) to human beings and certain safety factors are applied to take into consideration uncertainties in the extrapolation process and variability in human sensitivities. Thus, the three independent probability factors discussed above are measured as an integrated product in an animal model and the overall probability in the animal is extrapolated to humans (Figure 14). Refinements in the traditional animal studies have attempted to determine the delivered dose to target tissues, either through direct measurements or through computer modeling (physiologically based pharmacokinetic [PBPK] modeling) in order to extract the kinetic factor [2,3]. Such efforts provide a rational basis for extrapolation of the kinetic component of the toxicological process from animals to human beings [4]. The classical approach has been highly successful in protecting human health and safety as measured by the many toxic materials never allowed to reach the marketplace on the basis of animal testing.

In Vivo Approach to Toxicity Prediction

$P_{ANIMAL}(TR:E) \xrightarrow{\text{Extrapolation}} P_{HUMAN}(TR:E)$

- High Dose-Low Dose Extrapolation
- Route-to-Route Extrapolation
- Species Extrapolation
- Safety Factors

Figure 14. Prediction of Toxicity Based on _In Vivo_ Whole Animal Testing.

The *in vitro* approach to evaluating *in vivo* dose–response relationships again divides the toxicological process into three components, but the division differs slightly from that described above. The three components are toxicokinetics, intrinsic cellular toxicity, and toxicodynamics where the first and last components are defined slightly differently here. The overall probability of an adverse effect in the organism is still the product of the conditional probabilities for each component, thus

$$P(TR:E) = P_K(X:E)^*P_I(CR:X)^*P_D(TR:CR)$$

where $P_K(X:E)$ is the conditional probability that the extracellular concentration of the toxicant or its active metabolite at the susceptible target cell is X for a given exposure E, $P_I(CR:X)$ is the intrinsic cellular toxicity of the test material defined as the conditional probability of an adverse cellular response (CR) when the extracellular concentration is X, and $P_D(TR:CR)$ is the toxicodynamic factor giving the conditional probability that a toxic response will be observed given an adverse cellular response. The major difference between the earlier division of the toxicological process and the division defined for in vitro testing purposes is that the initiation process and the early phases of the toxicodynamic component have been lumped together and called the intrinsic cellular toxicity of the test material. The motivation for this new division is that in vitro experimental systems are well designed to determine the intrinsic cellular toxicity (Figure 15) which is the central focus of the in vivo toxicological process.

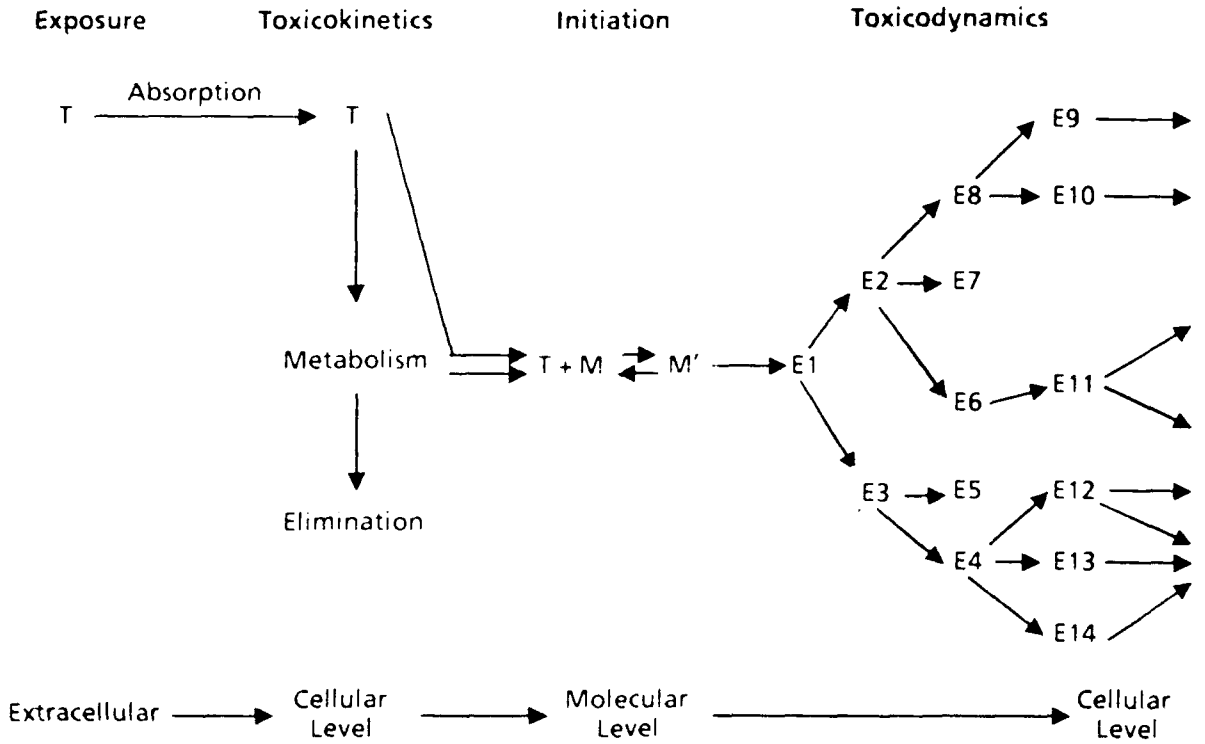

Figure 15. **The Toxicological Process *In Vitro*.** This schematic diagram illustrates the relationships between exposure to a chemical *in vitro* and the ultimate expression of a toxicological effect at the molecular/cellular level.

To fully utilize the *in vitro* approach to toxicity testing it is necessary to also address the issue of estimating the toxicokinetic and toxicodynamic probability factors (Table 14). Many *in vitro* model systems have been developed to investigate metabolism of chemicals (e.g., S9 fractions, isolated hepatocytes) and membrane transport phenomena (e.g., isolated cells, membrane vesicles). Data from these experimental systems can be integrated by physiologically based toxicokinetic models to predict *in vivo* kinetics. These approaches are still in their early stages of development, but the initial successes are encouraging.

TABLE 14. *IN VITRO* APPROACH TO TOXICITY PREDICTION

I. Predictive Toxicokinetics – $P_K(X:E)$

- Models for metabolism

  (1) S9/Microsomes
  (2) Primary cell cultures (e.g., hepatocytes)
  (3) Cell lines

- Models for membrane transport

  (1) Cell models
  (2) Membrane vesicle models
  (3) Isolated perfused organ models

- Physiologically based toxicokinetic modeling

II. *In Vitro* Toxicity Testing – $P_I(CR:X)$

- Concentration-response relationship (intrinsic cellular toxicity)

III. Predictive Toxicodynamics – $P_D(TR:CR)$

- Two-stage cancer models

The problem of predicting the toxicodynamic probabilities is more intractable at this time. The major limitation to progress is the lack of knowledge concerning the sequence of events *in vivo* leading from alterations in cellular functions to overt pathologies. As a consequence, it is difficult to define *in vitro* end points that act as necessary and sufficient markers for the pathogenic process at higher levels of biological organization. This is a new field of investigation. An example of one approach to this problem is the multistage model for carcinogenesis [5]. The development of such models, based on *in vitro* measurements of critical parameters (e.g., cell division rates, mutation rates), will provide the tools necessary to predict *in vivo* dose–response relationships from *in vitro* measurements.

IN VITRO TOXICITY TESTING MODELS

Components of a Toxicity Test

An *in vitro* toxicity test consists of three components: (1) the biological model, (2) the end point measurement, and (3) the test protocol. The biological model consists of the biological system to be utilized (Table 15) and the appropriate culture conditions. The biological model is selected on the basis of the toxicological question to be addressed. Cells of hepatic origin are more relevant to studies of hepatotoxicity than neurotoxicity and vice versa. The greater the ability of the biological model to represent the *in vivo* phenotype of the cell in question, the more confidence will accrue to the toxicity data generated.

TABLE 15. POTENTIAL BIOLOGICAL COMPONENTS FOR *IN VITRO* TESTING SYSTEMS

- Organ Culture
- Tissue Slices
- Primary Cells
  - (1) Suspension cultures
  - (2) Plated cultures
- Subcellular organelles
- Cell Lines
- Plant/Fungi Cultures
- Prokaryotic Cultures
- Macromolecular System

The end point measurement is the analytical determination of the effect of the test chemical on the biological system. The data provided by the end point measurement are used to define the concentration–response relationship in the *in vitro* test system. The better the understanding of the mechanistic basis of the observed end point measurement the more useful the toxicological information provided by the test.

Finally, the test protocol selected will affect the interpretation of the test results. For example, if the biological system is exposed to a test chemical for 24 h and the end point assay is conducted immediately, the data produced would be most relevant to the acute toxicity of the test material. If, on the other hand, the biological system is exposed to the test material for 24 h and then the system is cultured in the absence of the test material for 48 h before the end point assay is conducted, the data provided would be more relevant to issues of recovery from toxicity rather than acute toxicity per se. The choices made for each of these three components of the test will have a significant influence on the information content of the data produced and its application in the safety evaluation process.

Example – Hepatotoxicity Testing

As an example of the application of *in vitro* toxicity testing systems to target organ toxicity evaluation, *in vitro* models for hepatotoxicity testing will be briefly discussed (for a more complete discussion see [6]) Various model systems have been used directly for hepatotoxicity evaluation (Table 16). In addition, hepatocytes play an important role in the metabolic alteration of the parent chemical, either activating or detoxifying it. One example of the metabolic modification of test chemicals is illustrated by the effect of coculturing fibroblast with hepatocytes on the toxicity of a collection of test chemical (Table 17; [7]). In some cases the toxicity of the test chemical is enhanced by the presence of hepatocytes as indicated by a shift of the IC_{50} to lower values In other cases the hepatocytes had no effect or even a protective effect (shift of the IC_{50} to higher values). Thus, the utilization of hepatocytes or S9 fractions in *in vitro* testing systems can prove invaluable in the identification of chemicals which may be activated to more dangerous forms by hepatic metabolic activity

TABLE 16. MODEL SYSTEMS TO STUDY HEPATOTOXICITY

- Isolated perfused liver
- Liver slices
- Primary cultures of isolated hepatocytes
  - (1) Suspension cultures
  - (2) Plated cultures
  - (3) Cocultures with other cell types
- Hepatic cell lines
- Subcellular organelles
  - (1) S9/Microsomes
  - (2) Mitochondria
  - (3) Nuclei
  - (4) Others

TABLE 17. EFFECT OF COCULTURING FIBROBLAST WITH HEPATOCYTES ON THE TOXICITY OF SELECTED CHEMICALS*

Chemical	LD50 (μg/mL) –Hepatocytes	+ Hepatocytes
	Increased Toxicity	
Cyclophosphamide	>500	16
Dimethylnitrosamine	>1000	150
6-Aminochrysine	>200	11
	Decreased Toxicity	
4-Nitroquinoline *N*-Oxide	0.63	3.50
	No Effect	
Carbon Tetrachloride	2000	2000

* Data taken from [7]

Multi-End Point Batteries and Intrinsic Cellular Toxicity

As suggested above, one of the advantages of *in vitro* systems is their ability to serve as models for the central events in the *in vivo* toxicological process. A critical evaluation of the intrinsic cellular toxicity of test material in a cellular system can provide useful information for toxicological considerations. The ranking of toxicity potential, as evaluated by the EC_{50} for a single end point measurement, can provide essential information concerning the relative toxicity of a new product using benchmark toxicants as a measurement scale. Multiple end point batteries increase the power of the evaluation by providing a spectrum of information relating to various cellular functions. These multiple data can be used to diagnose the mechanistic nature of the toxicological effects of the chemical [8]. Some biological functions which can be evaluated in cellular systems are listed in Table 18. The relative positions of the concentration–response relationships for various end points which evaluate the status of these processes can be used to determine the most sensitive markers of toxicity which in turn can suggest a mechanism of action for the test material. An additional factor in the evaluation is the evolution of the responses in time. This added dimension can be employed to further discriminate between potential mechanisms by defining the order in which responses occur.

TABLE 18. POSSIBLE CELLULAR FUNCTIONS THAT CAN BE EXPLOITED TO EVALUATE INTRINSIC CELLULAR TOXICITY

(1)	Maintenance of gross integrity of cellular membrane
(2)	Regulation of physiologically important ions (e.g., K, NA, CA)
(3)	Regulation of biochemically important substrates (e.g., amino acids, sugars, glutathione)
(4)	Regulation of cellular energetics
(5)	Regulation of macromolecular synthesis (e.g., proteins, DNA, RNA)
(6)	Maintenance of cytostructural components
(7)	Regulation of cell cycle
(8)	Maintenance of DNA integrity
(9)	Maintenance of cell–cell communication
(10)	Regulation of secretory (exocytotic) processes
(11)	Regulation of essential biochemical pathways (e.g., lipid metabolism, urea cycle, arachidonic acid metabolism)
(12)	Regulation of signal transduction

VALIDATION

Definition

Validation is the process by which the credibility of a particular test method is established for a particular purpose [9]. Validation of toxicity tests is a different process than validation of an *in vitro* model for toxicological research. In research, validation of an *in vitro* model is ultimately established by confirmation that the information provided by the *in vitro* model correctly describes the process of interest in the intact animal. In research, the *in vitro* model does not stand alone, but serves as a tool to test hypotheses in a continuing quest to understand reality *in vivo*. On the other hand, an *in vitro* toxicity test is a component of a testing strategy which is designed to test the safety hypothesis. It is validated for a subset of the universe of all chemicals to be tested and once validated it is applied to new unknown materials to evaluate their toxicity. Presumably, when validated the test system can be used without the need to confirm results *in vivo*. The reason this works is that the hypothesis to be tested is always the same in the testing situation.

Test validation consists of two components: reliability and relevance. These two attributes are analogous to the traditional concepts of precision and accuracy. Reliability (precision) can be determined in a straightforward manner. Does the test system give reproducible results between laboratories and in the same laboratory over time? The theoretical design of experimental studies to establish reliability can be readily established, however, the practical problems that arise in such studies can be formidable [1]. The establishment of the relevance (accuracy) of test results to risk assessment and chemical safety evaluations is a more difficult issue.

In the traditional approaches to evaluating relevance (accuracy) of a method, it is essential to compare the output of the method to a "gold standard." For example, if the method is designed to measure length, then the accuracy of the method is established by applying the procedure to a standard reference length. The absolute standard is rarely used for methods validation and is kept safely in a stable environment. Validation is usually carried out using a secondary standard which is sufficiently accurate itself to serve the purpose of the validation study. The key to the establishment of accuracy is the ability to directly compare the output of the method to the known value of the standard. If the length measuring method we are using as an example gave its output in yards and the standard reference is meters, there would be no way to validate the method for accuracy unless the conversion factor between meters and yards was known with sufficient accuracy. Thus, direct comparisons between the method and the standard, in equivalent units, is the established procedure used to evaluate accuracy of the method.

The analogy with the validation of *in vitro* toxicity tests is useful. In toxicity testing a major objective is to establish, quantitatively, the safe level of human exposure which is measured as the concentration of the chemical in the air we breathe, the water we drink, or the food we eat (another way to put it is that we wish to know the risk of adverse effects at a given level of exposure (dose–response relationship) to decide whether the benefits of such an exposure outweigh the risk-drug safety evaluations). To establish the relevance of a method for this purpose the method must provide an output which can be compared to the *in vivo* gold standard in equivalent units. For example, if a new *in vitro* method were developed for the purpose of predicting the acutely lethal toxicity of a chemical to human beings then the LD_{50} in humans would be a potential gold standard for validation of the method. The question arises: is this the appropriate gold standard? In general, the *in vitro* assay will yield a concentration-response relationship that can be parameterized by computing an EC_{50}. The units of the EC_{50} are in concentration (μM, μg/mL, etc.) whereas the LD_{50} is in units of dosage (mg/kg). Obviously, these results cannot be compared directly.

Another factor that complicates the validation of *in vitro* methods is the problem of which database constitutes the gold standard. Obviously, *in vivo* human toxicity data would be the optimum database for validation, however the data available are limited [1]. In analogy with the

example of the validation of the length measuring method discussed above, a secondary standard can be used for validation purposes. In this case, the secondary standard is the *in vivo* animal toxicity database. Whether this data base is adequate for validation purposes is still an open question. Rationally, one would expect that *in vitro* toxicity test systems based on animal cells as the biological component should be validated against *in vivo* animal toxicity data and test systems based on human cells validated against *in vivo* human toxicity data. Otherwise, the validation process will be confounded by species-specific responses which the *in vitro* test should not be expected to take into account.

Correlative Versus Mechanistic Approaches

There are several approaches to quantitatively evaluating the validity of *in vitro* methods. Two approaches which will be considered here are the correlative approach and the mechanistic approach. The correlative approach involves a direct comparison of the *in vitro* end point measure and the *in vivo* data (e.g., a comparison of the EC_{50} of the test method with the LD_{50} data for a set of standard test chemicals). Assuming the LD_{50}s are known with absolute certainty (which is seldom the case), the observed EC_{50}s can be compared using a correlation relationship. The mathematical relationship chosen for this computation will affect the outcome. For example, a linear correlation relationship may be chosen and a correlation coefficient determined. However, the data may be better fit with a non-linear correlation relationship in the sense that a better correlation coefficient may result. The question is which relationship is correct and the answer is not easy to determine.

The underlying issue is whether the conversion factor, which relates the concentration units of the *in vitro* test to the dosage units of the *in vivo* standard, is a linear factor or not. (The mechanistic nature of this relationship will be discussed further below). This is usually not known a priori and therefore must be empirically determined. In theory, the true order of the conversion factor can be elucidated by studying a large collection of reference chemicals and determining mathematically the best fit. The more chemicals included in the study the greater the likelihood of correctly determining the order of the relationship. Unfortunately, the success of this approach depends on the assumption that all of the reference chemicals fall into a mechanistically similar toxicological category (i.e., produce their adverse effects by a similar mechanism). If the chemicals fall into many different categories of toxic mechanisms, then it would not be expected that they would all exhibit the same EC_{50} to LD_{50} conversion factor. By employing a mixed collection of reference chemicals (mixed in the mechanistic sense), the result will be a significant scatter in the data, thus hiding the true order of the conversion factor. The real barrier to success of this approach is that toxicology as a science has not satisfactorily established the mechanistic classification of *in vivo* toxicity of a sufficient number of chemicals to allow for a rational selection of test chemicals for correlative validation to progress.

A second form of correlative validation is to determine the ranking of chemical and compare *in vitro* to *in vivo* data using rank correlations (or a concordance evaluation [10,11]). This approach has the advantage of not requiring a specific choice of the mathematical order of the conversion factor, but does require for success that the correct "mapping" of the ranking of the *in vitro* to the *in vivo* data has no crossovers (i.e., the two data sets do in fact have the same inherent ranking of the reference chemicals). This assumption has the same limitation as discussed above. If all the reference chemicals in a validation collection are mechanistically similar, then there is a rational basis to expect that the inherent ranking is the same *in vitro* and *in vivo*. However, if chemicals of different mechanistic categories are included in the collection, then crossovers would be expected and the evaluation fails because the essential criterion for success cannot be fulfilled.

Even if it were possible to obtain collections of reference chemicals with similar mechanisms of toxicity and empirically determine correlative predictor relationships for each class separately, the ultimate limitation of the correlative approach for predictive purposes would be the determination of the mechanistic classification of a new test chemical. Without an independent determination of the mechanistic category, it would be impossible to predict the LD_{50} of the new chemical because different values would result depending on which predictor relationship was selected. Thus, correlative relationships have their value in suggesting possible toxicity, which may be sufficient for certain decisions in product development (screening tests), but are not adequate for definitive toxicological decision making where the inherent uncertainties are not quantifiable.

The second approach to validation to be discussed here is mechanistically based. A mechanistic explanation of a phenomenon is an explanation which describes the processes underlying the phenomenon in terms of events at a lower level of organization. In the case of toxicological phenomena, a description of lethality in terms of target organ toxicity would be a mechanistic description of lethality at the tissue pathology level. A mechanistic description of the tissue or organ responses would entail a description of molecular or cellular events which contribute to tissue pathology. An even more basic description would be to describe the molecular initiation reactions (reactions of the toxicant with molecular targets) at the quantum mechanical level. These are all mechanistic descriptions at different levels of biological organization. What we label as mechanistic is entirely determined by the level of biological organization at which we observe a given phenomenon. The Toxicological Process *in vivo* (Figure 13) is a framework within which we can orient this discussion. It starts at the level where the organism interacts with its environment (exposure), works its way down through the kinetic side to the molecular level where the initiation reactions occur and then it works its way back up the organizational hierarchy to the organism and population level where mortality, morbidity and behavioral effects are observed.

As was pointed out previously, *in vitro* systems are best suited as models to describe the central events of the Toxicological Process – initiation and early molecular and cellular effects. Thus, a mechanistic validation of an *in vitro* toxicity testing system should compare the responses in the *in vitro* test to the appropriate *in vivo* correlates. As an example, if an *in vitro* toxicity test based on human hepatocytes was developed to evaluate the potential of new chemicals to cause hepatotoxicity in humans, the appropriate gold standard for validation would be a set of concentration–response curves which describe the relationship between the peak plasma concentration of the chemical in human beings after a single exposure to the chemical versus an early marker for hepatotoxicity, such as a serum enzyme marker, for a collection of reference chemicals. If the mechanistic hypothesis is correct, then the *in vitro* concentration–response curves would resemble, qualitatively and quantitatively, the *in vivo* curves, assuming that the end point measurement of the *in vitro* test is an adequate marker for the mechanism of action of the reference chemical set. Again the limitation of the validation process is the ability to select a set of reference chemicals that have defined mechanisms of toxicity.

The advantage of the mechanistic approach is that factors that confound the overall dose–response relationship, toxicokinetic and toxicodynamic factors, are isolated from the validation process and the performance of the test is evaluated against the appropriate standard which reflects the mechanistic basis of test system. If we truly want *in vitro* methods to form the basis of a chemical safety evaluation program, then it will be necessary to develop *in vitro* tools to add back into the equation the kinetic and dynamic factors which modulate intrinsic cellular toxicity *in vivo*.

Domain of Validation/Domain of Validity

Before leaving the subject of validation, one additional issue should be explored and that is the range of applicability of a validated test. A test is validated for a limited set of reference chemicals. Usually the chemicals selected are chosen on the basis of the availability of *in vivo* toxicity data and often chemical structural considerations are neglected unless as a secondary consideration. The set of chemicals tested in the validation study defines the domain of validation of the method within the universe of all possible chemical structures. The actual domain of validity (i.e., the total class of all chemicals that would be correctly evaluated by the test) is presumably much larger. The selection of chemicals for validation of tests should attempt to optimize, on a structural basis, the domain of validation in order to form the basis for extrapolation of the method to its maximum range of applicability. These issues have not been adequately explored in the context of validation.

The extrapolation of a method to chemical classes outside the domain of validation of the test requires different approaches depending on whether a correlative or mechanistic validation was applied. Correlatively validated tests have no basis for extrapolation to chemical classes outside the

domain of validation because there is no basis for assuming the predictor equation will apply to the new class of chemicals. It is possible to interpolate a correlatively validated method to new chemicals that fall within the defined class of reference chemicals used to establish the predictor relationship. However, for a correlatively validated method to be fully reliable, even for interpolation, an operational procedure must be described to certify that a new chemical to be evaluated does in fact fall into the same mechanistic class as the reference chemicals. Without some assurance that a new chemical falls within the domain of validation then the uncertainty in the predicted result is an unknown quantity which would be unacceptable for many decision-making purposes, including regulatory decisions.

On the other hand, if the test method is validated in the mechanistic sense (i.e., there is a reasonable level of confidence that a chemical which is positive for a particular mechanistically defined toxicological end point can be expected to exhibit the same response *in vivo*), then it will presumably be a marker for that effect independent of the chemical class of the test material. Thus, a mechanistically validated test will have a domain of validity which is identical to the universe of all chemicals (i.e., out of all chemicals in the universe, the test will identify those chemicals which act through the mechanistically defined end point). As with all model systems, there will always be some exceptions that are attributable to artifacts in the test system, but in principle the method will have universal applicability. This is a major advantage of mechanistic validation.

All in all, validation of new *in vitro* methods for toxicity testing is still a difficult barrier to surmount. The main reason is that *in vivo* toxicity is still poorly understood. In pregnancy testing, there is a single biological condition to be ascertained and definitive biomarkers have been identified which can be determined *in vitro* and are necessary and sufficient conditions in the logical sense for the biological state to exist. Thus, pregnancy testing is an example of an ideal situation for the replacement of an *in vivo* test with an *in vitro* test. In the case of toxicity testing, there are multiple pathological conditions that can develop, definitive biomarkers (i.e., necessary and sufficient conditions for the development of each of the potential pathological conditions) have not been identified by *in vivo* toxicological research, and the mechanistic bases of the measurements in many of the *in vitro* test systems are not fully understood. In the light of these limitations in the scientific basis of *in vitro* toxicity testing, it is no wonder that validation of the new methodology has been such a perplexing problem. For these reasons, *in vitro* toxicity testing methods are still in their embryonic stage of development. Much of the current research is observational and empirical, but then this is always the pattern of evolution of a new scientific endeavor.

Before leaving the subject, it should be pointed out that if validation of *in vivo* testing methods were required today with the same level of mechanistic documentation as is being demanded of *in vitro* methods, the scientific community would be hard pressed to establish their validity on the

basis of quantitative criteria. This is not to say that the *in vivo* methods have not been validated. They have, by a historical data base which shows that *in vivo* methods have successfully protected human health and welfare on many occasions. No model system or testing strategy is perfect and everyone is aware of well publicized failures, but few people outside of the research and development community appreciate the many times that dangerous chemicals have been correctly identified and eliminated from further development, much less commercial marketing, on the basis of *in vivo* toxicity testing. This should not be overlooked.

PARADIGM FOR TOXICOLOGICAL EVALUATION

Short Term Situation

In the near future *in vitro* toxicity tests will play an increasingly important role in chemical safety evaluation. *In vitro* tests will be used as screens by corporate research and development groups to provide useful data for management decisions concerning product development priorities. *In vitro* toxicity tests will be incorporated into regulatory accepted tier testing strategies. A hypothetical example of such a strategy is given in Figure 16. Although this strategy still relies on *in vivo* toxicity testing, *in vitro* tests are incorporated into the scheme at various points to maximize the toxicological knowledge available before *in vivo* testing occurs and to identify extremely toxic materials at an early stage in the process thus eliminating *in vivo* testing in these cases.

Toxicological Testing Paradigm

- Definition of Testing Objectives (Information Needs)
- Evaluation of Physical-Chemical Properties (pH, pK, etc.)
- Evaluation of Structure – Activity Relationships
- Screening Testing (Single Cell Type/Single End Point)
- *In Vitro* Evaluation of Intrinsic Cellular Toxicity
- *In Vitro* Target Organ Toxicity Testing
- *In Vitro* Hazard Identification (e.g., Phototoxicity Testing)
- Selective Animal Testing
- *In Vitro* Mechanistic Studies
- Ciinical Trials/Human Volunteer Studies
- Risk Analysis and Risk Management

Figure 16. A Proposal for an Integrated Toxicological Testing Paradigm.

Long Term Situation

The longer perspective depends on the continued development of the basic science of toxicology to provide the necessary knowledge-base to support a completely *in vitro* safety

evaluation process. *In vitro* toxicity testing, *in vitro* toxicokinetic evaluations coupled with physiologically based toxicokinetic modeling and quantitative structure–activity relationships will form the basis for such an approach. Whether *in vivo* testing will be completely eliminated will remain to be seen; however, we should not loose sight of the primary objective of the chemical safety evaluation process and that is to protect the health and welfare of people, animals and the environment. If *in vivo* toxicity testing is required to accomplish this goal then we must recognize this need and, at the same time, make every effort to establish a scientifically sound toxicity testing paradigm that utilizes the best available testing technologies.

ACKNOWLEDGEMENTS

This research is supported by the National Institutes of Health, National Center for Research Resources grant number P40RR05236-04. The author would like to thank Ms. Dynita Parker for secretarial assistance in the preparation of this manuscript.

REFERENCES

1. **Balls, M., B. Blaauboer, D. Brusick, J. Frazier, D. Lamb, M. Pemberton, C. Reinhardt, M. Roberfroid, H. Rosenkranz, B. Schmid, H. Spielmann, A.L. Stammati, and E. Walum.** 1990. Report and Recommendations of the CAAT/ERGATT Workshop on the Validation of toxicity Test Procedures. ATLA 18.

2. **Anderson, M.E.** 1989. Physiological modeling of tissue dosimetry. *CIIT Activities* 9:1-8.

3. **Bischoff, K.B.** 1987. Physiologically based pharmacokinetic modeling. *Pharmacokinetics in Risk Assessment, Drinking Water Health* 8:36-61.

4. **Reitz, R.H., J.N. McDougal, M.W. Himmelstein, R.J. Nolan, and A.M. Schumann.** 1988. Physiologically based pharmacokinetic modeling with methylchloroform: Implications for interspecies, high dose/low dose, and dose route extrapolations. *Toxico. Appl. Pharmacol.* 95:185-199.

5. **Moolgakar, S.H. and G. Luebeck.** 1990. Two-event model for carcinogenesis: Biological, mathematical, and statistical considerations. *Risk Anal.* 10:323-341.

6. **Rauckman, E.J. and G.M. Padilla.** 1978. *The Isolated Hepatocyte: Use in Toxicology and Xenobiotic Biotransformations.* Academic Press.

7. **Wiebkin, P., J.R. Fry, and J.W. Bridges.** 1978. Metabolism-mediated cytotoxicity of chemical carcinogens and non-carcinogens. *Biochem. Pharmacol.* 2:1849.

8. **Frazier, J.M.** 1990. Multiple end point measurements to evaluate the intrinsic cellular toxicity of chemicals. *J. Molecular Cell. Toxicol.* 3:349-357.

9. **Frazier, J.M.** 1990. Scientific Criteria for Validation of *In Vitro* Toxicity Tests, OECD Environment Monographs, No. 36.

10. **Feder, P.I., R.A. Lordo, L.C. DiPasquale, D. Bagley, M. Chudkowski, J. Demetrulias, K.L. Hintze, K.D. Marenus, W.J.W. Pape, M.T. Roddy, R. Schnetzinger, P.M. Silber, J.J. Teal, S.L. Weise, and S.D. Gettings.** 1991. The CTFA Evaluation of Alternatives Program: An evaluation of potential *in vitro* alternatives to the Draize Primary Eye Irritation Test (Phase I) Hydroalcoholic formulations; (Part 1) Statistical methods. *In Vitro Toxicol.* 4:231-246.

11. **Gettings, S.D., D.M. Bagley, M. Chudkowski, J.L. Demetrulias, L.C. DiPasquale, C.L. Galli, R. Gay, K.L. Hintz, J. Janus, K.D. Marenus, M.J. Muscatiello, W.J.W. Pape, K.J. Renskers, M.T. Roddy, and R. Schnetzinger.** 1992. The CTFA Evaluation of Alternatives Program: An evaluation of potential *in vitro* alternatives to the Draize Primary Eye Irritation Test. (Phase I) Hydroalcoholic formulations; (Part 2) Data analysis and biological significance. *In Vitro Toxicol.* 4:247-288.

IN VITRO METHODOLOGIES FOR ENHANCED TOXICITY TESTING

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SUMMARY

This report will give a general overview of some of the *in vitro* methodologies used in toxicity testing. The use of computer-based structure–activity relationships and cell culture testing systems can provided valuable toxicological data for hazard and risk assessments. *In vitro* systems allow for a more rapid identification of toxic compounds and can be utilized to study mechanisms of toxicity at the cellular and subcellular level. The data derived from these types of studies can be used to improve the predictability of animal models for chemical or drug toxicity. This report focused on primary hepatocytes as an *in vitro* model for cytotoxicity and metabolic studies.

INTRODUCTION

Increased pressures from groups concerned with animal welfare has resulted in the need for alternatives to whole animal testing. However, substitution of *in vitro* methods for *in vivo* techniques must not result in increased health risks to the public. With this in mind, researchers have taken a "3Rs" approach to toxicity testing. The 3Rs are reduction (the use of the least number of animals in toxicologic research that results in meaningful data), refinement (the improvement of whole animal toxicologic research in such a way as to reduce or eliminate pain and discomfort), and replacement (the use of alternative toxicologic testing methods that do not involve the intact animal). The status of this type of approach has been recently reviewed and animal use has been reduced by employing *in vitro* methods [1]. The process of hazard assessment may be envisioned as a series of steps that begins with structure- activity correlation and proceeds through physical and chemical analysis, short-term tests, screening procedures, animal studies, and finally human studies and risk assessment [2]. Based on this scheme of hazard assessment, *in vitro* methods can be incorporated as short-term tests and screens. Furthermore, *in vitro* systems can be utilized to give insight into mechanisms of chemical toxicity at the cellular level thereby allowing possible intervention with therapeutic or antidotal treatment.

The development of liver perfusion procedures to isolate viable rat hepatocytes [3,4] has led to their use in a wide range of toxicity and metabolism studies. Primary hepatocytes that maintain their *in vivo* characteristics make them ideal candidates for these types of studies. Therefore, it is not surprising to find that they have been the predominant cell type used in *in vitro* toxicology studies.

Because the liver was the major target organ of many of the novel compounds screened in our laboratory, examples of *in vitro/in vivo* comparisons were presented using only the primary hepatocyte model for hazard assessment. However, it should be noted that accurate risk assessment will require information from batteries of *in vitro* assays performed on a number of different cell types from a number of different species.

ADVANTAGES AND DISADVANTAGES

The main objectives in toxicity testing are to identify hazardous material and to provide data estimating the quantitative exposure-response relationship for hazardous material in animals and ultimately in humans. The first of these objectives can be addressed by using *in vitro* screening systems, whereas the second can be addressed by using cell systems in mechanistic toxicity studies. There are a number of advantages to using these *in vitro* systems as alternatives to whole animals.

The obvious advantage in using *in vitro* testing methods over *in vivo* methods is a reduction in animal numbers and cost. Animal numbers are reduced because the cells from a single animal can be used to conduct a complete experiment or set of experiments. Cost savings are realized as a result of the reduced animal requirements as well as the reduced maintenance costs in housing and feeding of animals. Another advantage of *in vitro* systems is that the cells used in experiments are of a uniform population, being derived from a single animal. This results in reduced experimental variability. *In vitro* systems also have the added advantage of being able to assess toxicity of compounds of limited quantity because doses are typically in the range of micrograms, or less, per milliliter instead of milligrams per kilogram body weight. Another advantage is that an *in vitro* system can be utilized to rapidly identify metabolites of test agents. Using whole cells, or microsomes, metabolites of chemicals can be identified in a matter of hours instead of days using whole animals. Lastly, the use of *in vitro* systems will ultimately lead to better designed *in vivo* experiments. These systems can be used to identify chemicals or drugs worthy of further *in vivo* study and to give an indication of an appropriate dose range to use in an animal study.

Although there are a number of advantages to using *in vitro* toxicity testing systems, there are also some limitations. Because cells are usually derived from the digestion of a particular organ, these isolated cells have been removed from their normal heterogeneous cellular milieu. The loss of organ architecture and cell orientation may result in cells responding differently to chemical or drug exposure than those observed *in vivo*. In addition, the digestion of a particular organ may result in alteration in cell membrane lipids and receptors of the isolated cell type, which can lead to erroneous conclusions in toxicity studies using these cells. Furthermore, these cells have been removed from their physiologic relationships with the intact animals circulatory system. Therefore, factors such as chemical or drug absorption, distribution, and elimination can not be assessed *in vitro*.

Another limitation of *in vitro* systems is that cellular functions may become unstable over time in culture. This is of critical importance when conducting biotransformation studies with primary hepatocytes. It is well established that cytochrome P450 (P450) content will decrease with time in culture [5]. Factors such as species and culture environment will determine the degree of P450 change [6,7,8,9]. However, cell function and longevity can be preserved using the appropriate culture medium and biomatrix substrate (collagen, laminin, fibronectin, etc.) for cell attachment. Although some of the *in vitro* limitations can be controlled, others can not. One example is in the area of neurotoxicity. *In vitro* systems are not, as yet, capable of assessing behavioral effects such as pain, irritation, or coordination.

IN VITRO STUDIES OF METABOLIC MECHANISMS OF TOXICITY

In vitro studies of cellular metabolic mechanisms of toxicity give insight into the pathway(s) involved in chemically induced toxicity. *In vitro* systems are useful for dissecting cellular biochemical pathways of toxicity because the target cell can be studied directly without interference of other cell types or organ systems. Therefore, measurements of effects on specific cell functions, such as membrane integrity, mitochondrial membrane integrity and function, cellular detoxification systems, metabolic capacity, and DNA damage, can be made directly. In addition, critical measurements of enzyme kinetics, that are often difficult or impossible to determine *in vivo*, can be made due to a more direct dose-response relationship. This data can be used in pharmacokinetic risk assessment models to generate more accurate predictions of risk. Furthermore, by correlating *in vitro* toxicity studies with *in vivo* toxicity studies, predictions concerning the toxicity in other species that possess similar biochemical pathways can be made.

Although microsomes can be used as an *in vitro* system to assess metabolism of chemicals or drugs, they may not accurately reflect the *in vivo* or intact cell situation [10]. It has been shown that the metabolism profile generated from microsomes may not match that generated from whole animals or whole cells [11]. This difference in metabolite profile results because microsome preparations lack cytosolic enzymes and cofactors that may be involved in the metabolism of certain compounds.

IN VITRO ASSAYS

Cytotoxicity assays are designed to detect alterations in cellular structure and function that lead to irreversible cell damage. Because of the large number of biochemical pathways that exist in cells and the diversity of cell types, there are a multitude of biochemical indicators that can be chosen to study the effects of chemicals or drugs at the cellular, subcellular, and molecular level. A number of factors must be considered when choosing an assay or method for use in an *in vitro* toxicity testing system. Species specificity, sensitivity, reproducibility, and reliability are key factors to consider when

deciding on a particular assay or method. Furthermore, knowledge of the physical properties of the test agent and theoretical predictions of the biochemical pathways affected will also play a role in the decision of which cell type and assay system to use.

IN VITRO/IN VIVO CORRELATION EXAMPLE

Chlorotrifluoroethylene Oligomers

Polychlorotrifluoroethylene (3.1 oil) is a prototype nonflammable hydraulic fluid being evaluated for use in both military and commercial aircraft. This hydraulic fluid is composed of a mixture of chlorotrifluoroethylene (CTFE) oligomers, predominantly C_6 (trimer) and C_8 (tetramer) chain length.

In vivo inhalation and oral gavage studies with this 3.1 oil indicated that the liver was the principal target organ for toxicity [12,13]. Furthermore, the oral gavage study also showed that the tetramer CTFE oligomer was more toxic than the trimer CTFE oligomer component of 3.1 oil as evidenced, in part, by significant body weight loss in orally dosed male Fischer 344 (F-344) rats over 14 days (Figure 17). However, 3.1 oil was not found to be toxic in orally dosed Rhesus (Macaca mulatta) monkeys [14]. Differences between 3.1 oil-exposed rats and primates, with respect to liver peroxisomal β-oxidation rates and peroxisome number, were also found. Rats orally dosed with 3.1 oil indicated significant increases in peroxisome number and rates of β-oxidation when compared to 3.1 oil-exposed primates (Table 19).

In another study, evidence that the trimer and tetramer CTFE oligomers were metabolized to their corresponding oligomer carboxylic acids was provided [15]. These acids were later identified in male F-344 rats by gas chromatography/mass spectroscopy [16]. An oral gavage study using the trimer and tetramer CTFE acids has also shown the tetramer CTFE acid to be more hepatotoxic than the trimer CTFE acid [17].

In Vitro Results

In vitro dose–response studies with the trimer and tetramer CTFE carboxylic acids, using primary rat hepatocytes, also indicated that the tetramer CTFE acid was more toxic than the trimer CTFE acid as determined by significantly increased lactate dehydrogenase enzyme leakage when compared to control (Figure 18). This result correlates very well with the previous *in vivo* study using the CTFE acids mentioned above. Furthermore, rat hepatocytes exposed to the CTFE acids resulted in increased rates of peroxisomal β-oxidation whereas exposed primate hepatocytes did not (Figure 19). This data also correlates well with that found in *in vivo* studies mentioned above with rats and primates exposed to 3.1 oil.

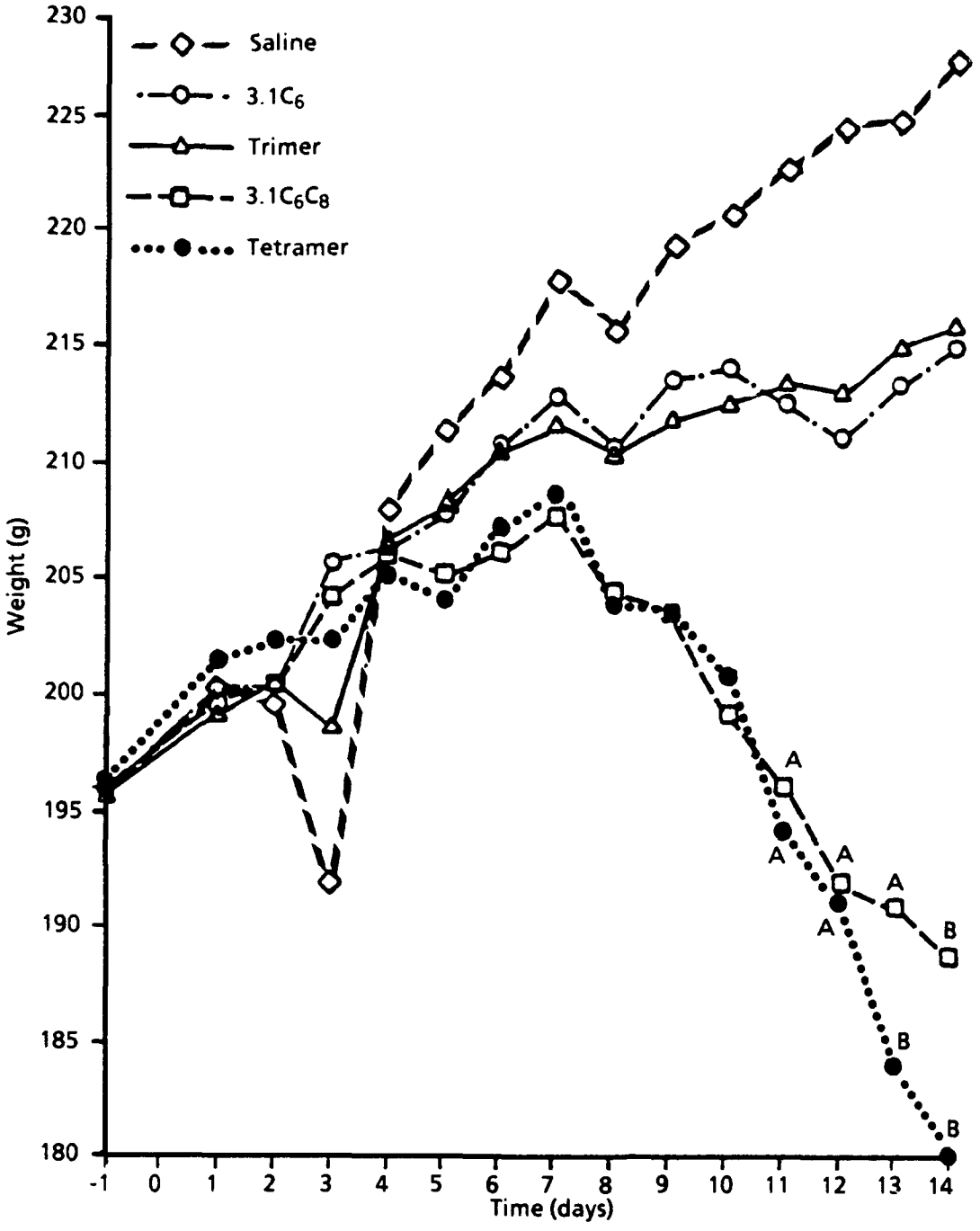

Figure 17. Effect of 14 Days of Repeated Oral Dosing with CTFE Oligomer-Based Compounds on Body Weight Gain. (A) $P<0.05$ as determined by the Bonferroni multiple comparison test when compared to saline control. (B) $P<0.05$ when compared to saline control, 3.1 oil-C6, and trimer. *Data taken from [13].

TABLE 19. *IN VIVO* PEROXISOMAL BETA-OXIDATION IN PRIMATE AND RAT LIVER FOLLOWING EXPOSURE TO PolyCTFE 3.1 OIL FOR TWO WEEKS

	Treatment	Mean Peroxisome Count	β-Oxidation Activity (μmoles/min/g)
Rat	Saline	10.2 ± 3.6	5.6 ± 2.6
	3.1 Oil	17.9 ± 7.8*	10.2 ± 1.2*
Primate	Saline	2.5 ± 0.3	3.0 ± 0.8
	3.1 Oil	3.3 ± 0.4	4.1 ± 2.8

* Sig at $p < 0.05$ by ANOVA.

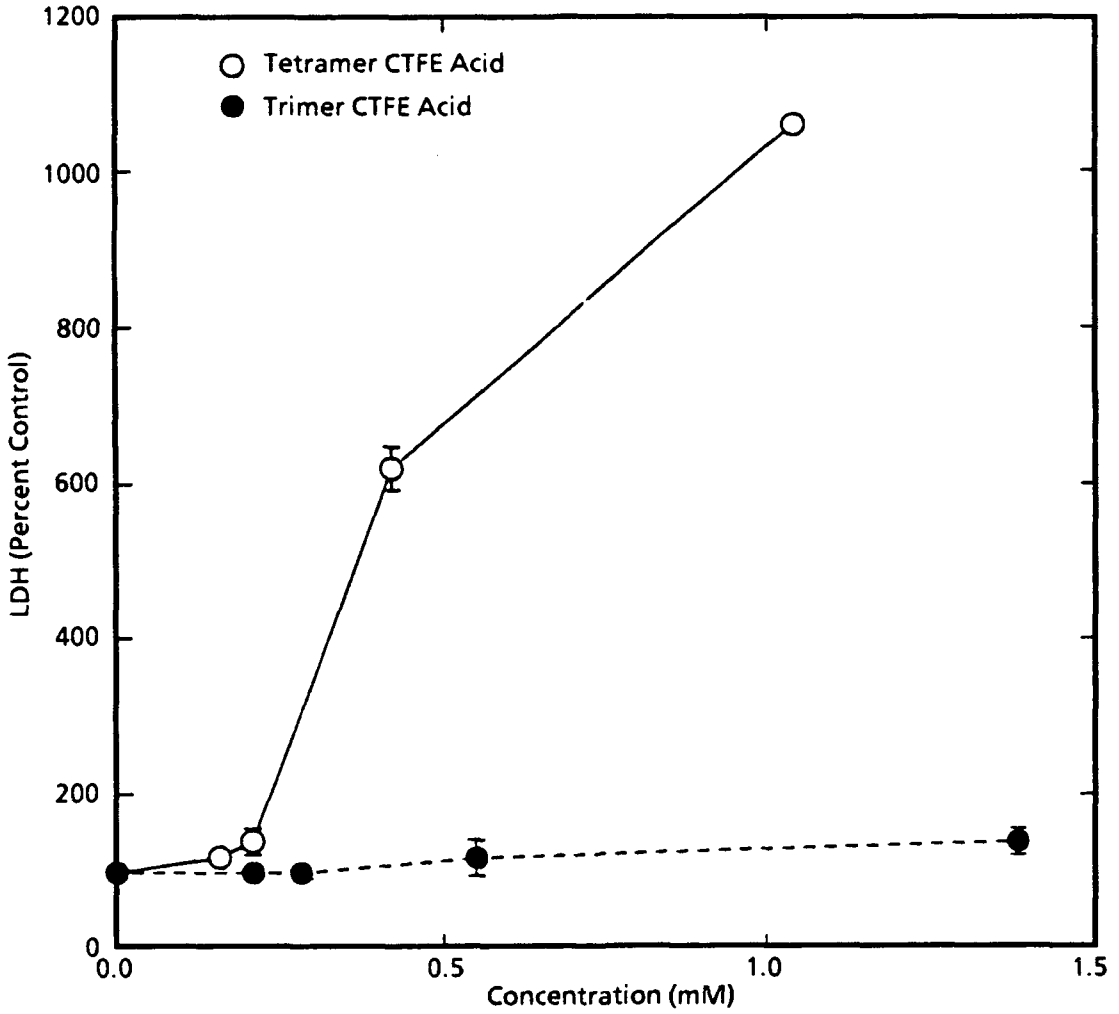

Figure 18. Dose–Response Curves for the Trimer and Tetramer CTFE Oligomer Acids in 24-h Primary Rat Hepatocyte Cultures as Determined by Lactate Dehydrogenase (LDH) Enzyme Leakage. Only control cells exhibiting <25% of the total intracellular LDH were considered valid experiments. Data points represents the average from three experiments and triplicate plates.

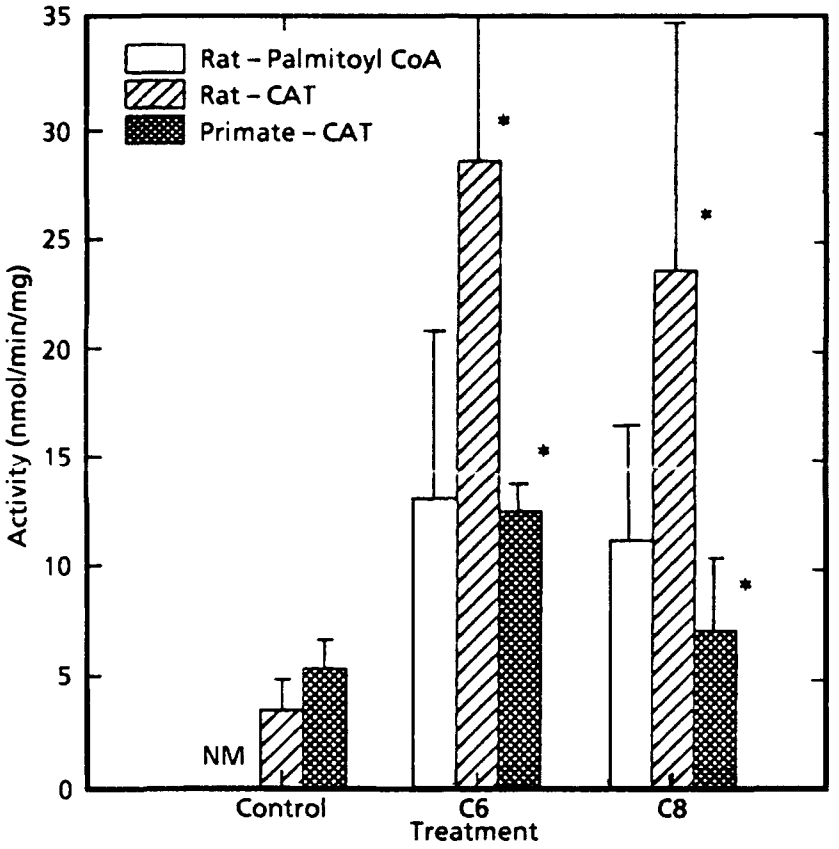

Figure 19. Peroxisomal β-oxidation Activities in Primary Rat and Primate Hepatocytes After Exposure to Trimer and Tetramer CTFE Acids. Bars represent the average from three experiments and triplicate plates. CAT; carnitine acetyltransferase. NM; (not measurable) Peroxisomal β-oxidation of palmitoyl CoA could not be determined in either control rat or primate hepatocytes under experimental conditions. Only rat hepatocytes exposed to the CTFE acids resulted in measurable rates of palmitoyl CoA oxidation. *; Significantly different than control at $P<0.05$ using multifactorial ANOVA.

Interestingly, primary rat hepatocytes exposed to noncytotoxic doses of the trimer CTFE acid indicated a significant increase in P450 activity and no effect on cell glutathione (GSH) levels (Figure 20). In contrast, hepatocytes exposed to the tetramer CTFE acid indicated no effect on P450 activity and exhibited significantly reduced GSH levels. This data indicates that P450 activity and GSH level may play important roles in the determination of CTFE acid hepatotoxicity, and demonstrates how *in vitro* systems can be used to give insight into possible mechanisms of toxicity.

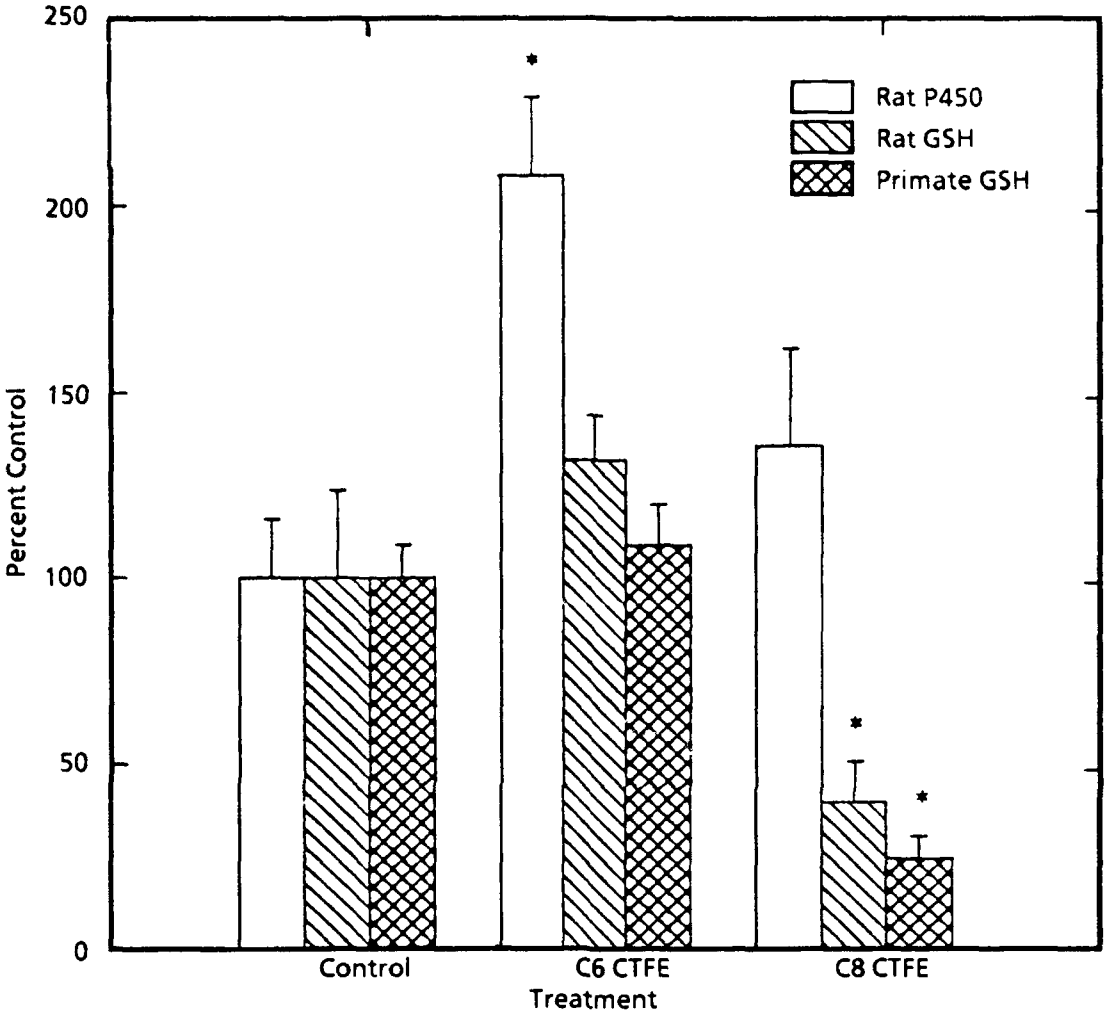

Figure 20. GSH Levels and P450 Activity in Primary Rat and Primate Hepatocytes After Exposure to Trimer and Tetramer CTFE Acids. Rat and primate hepatocytes were dosed with the CTFE acids at ~0.1 mM for 72 h and 96 h, respectively. Bars for rat hepatocytes represent results from a typical experiment with triplicate plates repeated three times. Control values for rat hepatocyte P450 activity and GSH level ranged from 0.25 to 0.52 nmol/min/mg and 10 to 31 nmol/mg, respectively. Bar for primate hepatocytes represents results from a single experiment with triplicate plates. Control primate hepatocyte GSH level was 8.0 ± 0.7 nmol/mg. Primate hepatocyte P450 activity was not determined. * Significantly different from control at $P < 0.05$ using multifactorial ANOVA.

CONCLUSION

The example of *in vitro/in vivo* correlation outlined above using primary hepatocytes exposed to CTFE oligomer acids is just one example of how primary cells can be utilized in toxicology. Recent advances in tissue culture techniques have allowed for the establishment of primary cell cultures from many different organs. Again, it should be noted that no one cell type will substitute for whole animal studies. To make predictions concerning toxicity with a high degree of confidence will require

batteries of *in vitro* assays using multiple cell types. In addition, the advantages and disadvantages of *in vitro* systems must be recognized before they can be effectively utilized in toxicity studies. *In vitro* methods must be properly validated to achieve maximal utility in these type of studies. The process of validation for *in vitro* methods that can be used in toxicity testing has been described [18].

It is not realistic, at this time, to expect that *in vitro* methods will totally replace the whole animal. However, the use of *in vitro* systems to reduce animal numbers and refine *in vivo* experiments have been documented [1]. There are a number of integrated interrelationships between structure, function, and behavior in toxicology that will require whole animal studies. Some examples include: measurements of neurobehavioral effects of toxicants, strain and species differences with respect to metabolic fate of test compounds, the role of intestinal flora and enterohepatic circulation in influencing metabolic pathways of test compounds, and differential distribution of xenobiotic biotransformation due to organ architecture and cell polarity. It is conceivable that advancements in *in vitro* technology may some day be capable of addressing the problems of integrated interrelationships in toxicology and result in the total replacement of animals.

ACKNOWLEDGEMENT

Animals used to provide data in this report were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Uses of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, DHHS, National Institute of Health Publication #85-23, 1985, and the Animal Welfare Act of 1966, as amended.

REFERENCES

1. **Gad, S.C.** 1990. Recent developments in replacing, reducing, and refining animal use toxicologic research and testing. *Fundam. Appl. Toxicol.* 15:8.

2. **Golberg, L.** 1986. Charting a course for cell culture alternatives to animal testing. *Fundam. Appl. Toxicol.* 6:607.

3. **Berry, M.N. and D.S. Friend.** 1969. High-yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* 43:506.

4. **Seglen, P.O.** 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* 13:29.

5. **Guzelian, P.S., D.M. Bissell, and U.A. Meyer.** 1977. Drug metabolism in adult rat hepatocytes in primary culture. *Gastro.* 72:1232.

6. **LeBigot, J.F., J.M. Begue, J.R. Kiechel, and A. Guillouzo.** 1987. Species differences in metabolism of ketotifen in rat, rabbit and man: Demonstration of similar pathways *in vivo* and in cultured hepatocytes. *Life Sciences* 40:883.

7. **Maslansky, C.J. and G.M. Williams.** 1982. Primary cultures and the levels of cytochrome P-450 in hepatocytes from mouse, rat, hamster and rabbit liver. *In Vitro* 18:683.

8. **Paine, A.J. and L.J. Hockin.** 1980. Nutrient imbalance causes the loss of cytochrome P-450 in liver cell culture: formulation of culture media which maintain cytochrome P-450 in rat liver cell culture. *Biochem. Pharmacol.* 31:1175.

9. **Dickins, M. and R. Peterson.** 1980. Effects of a hormone-supplemented medium on cytochrome P450 content and mono-oxygenase activities of rat hepatocytes in primary culture. *Biochem. Pharmacol.* 29:1231.

10. **Benford, D.J.** 1987. Biological models as alternatives to animal experimentation. *ATLA* 14:318.

11. **Bisgaard, H.C.** and H.R **Lam.** 1989. *In vitro* and *in vivo* studies on the metabolism of 1,3-diaminobenzene: comparison of the metabolites formed by the perfused rat liver, primary hepatocyte culture, hepatic microsomes and the whole rat. *Toxicol. In Vitro* 3:167.

12. **Kinkead, E.R., E.C. Kimmel, H.G. Wall, R.S. Kutzman, R.B. Conolly, R.E. Whitmire., and C.D. Flemming.** 1990 Subchronic inhalation studies of polychlorotrifluoroethylene (3.1 oil). *Inhal. Toxicol.* 2:431.

13. **DelRaso, N.J., C.S. Godin, C.E. Jones, H.G. Wall, D.R. Mattie, and C.D. Flemming.** 1991. Comparative hepatotoxicity of two polychlorotrifluoroethylene (CTFE) oligomers in male Fischer 344 rats. *Fundam. Appl. Toxicol.* 17:550.

14. **Jones, C.E., M.B. Ballinger, D.R. Mattie, N.J. DelRaso, C. Seckel, and A. Vinegar.** 1990. Effects of short-term oral dosing of polychlorotrifluoroethylene (PolyCTFE) on the rhesus monkey. *J. Appl. Toxicol.* 11:51.

15. **DelRaso, N.J., K.L. Auten, H.C. Higman, and H.F. Leahy.** 1991. Evidence of hepatic conversion of C_6 and C_8 chlorotrifluoroethylene (CTFE) oligomers to their corresponding CTFE acids. *Toxicol. Lett.* 59:41.

16. **Brashear, W.T., R.J. Greene, and D.A. Mahle.** 1992. Structural determination of the carboxylic acid metabolites of polychlorotrifluoroethylene. *Xenobiotica* 22:499.

17. **Kinkead, E.R., S.K. Bunger, R.E. Wolfe, C.D. Flemming, R.E. Whitmire, and H.G. Wall.** 1991. Repeated-dose gavage studies on polychlorotrifluoroethylene acids. *Toxicol. Indust. Health* 7:295.

18. **Balls, M., B. Blaauboer, D. Brusick, J. Frazier, D. Lamb, M. Pemberton, C. Reinhardt, M. Roberfroid, H. Rosenkranz, B. Schmid, H. Spielmann, A-L. Stammati, and E. Walum.** 1990. Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA* 18:313.

COUPLING OF COMPUTER MODELING WITH *IN VITRO* METHODOLOGIES TO REDUCE ANIMAL USAGE IN TOXICITY TESTING

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SUMMARY

The use of *in vitro* data to support the development of physiologically based pharmacokinetic (PBPK) models and to reduce the requirement for *in vivo* testing is demonstrated by three examples. In the first example, poly-chlorotrifluoroethylene, *in vitro* studies comparing metabolism and tissue response in rodents and primates made it possible to obtain definitive data for a human risk assessment without resorting to additional *in vivo* studies with primates. In the second example, a PBPK model for organophosphate esters was developed in which the parameters defining metabolism, tissue partitioning, and enzyme inhibition were all characterized by *in vitro* studies, and the rest of the model parameters were established from the literature. The resulting model was able to provide a coherent description of enzyme inhibition following both acute and chronic exposures in mice, rats, and humans. In the final example, the carcinogenic risk assessment for methylene chloride was refined by the incorporation of *in vitro* data on human metabolism into a PBPK model.

INTRODUCTION

In vitro techniques have long been used for qualitative purposes, either as a short-term indicator of longer term toxicity potential, as in the case of mutagenicity/genotoxicity screening [1], or as a direct substitute for *in vivo* toxicity testing [2]. However, in recent years another important use of *in vitro* methodologies has emerged: to provide mechanistic and quantitative information needed for the development of physiologically based pharmacokinetic (PBPK) models [3] and their application to quantitative risk assessment [4].

The principal challenge in the use of *in vitro* data for quantitative risk assessment is relating *in vitro* conditions to the *in vivo* situation. Physiologically based pharmacokinetic modeling provides a biologically meaningful quantitative framework within which *in vitro* data can be properly utilized. In contrast with classical pharmacokinetics, which is data based and fairly empirical, in recent years there has been a shift towards a physiologically based approach. In the classical approach, constants often lacked any clear physiological meaning and it would therefore be difficult to design *in vitro* studies to determine them. As a result, in a classical approach it is possible to obtain a good fit to the data without being exactly sure what it means. With a biologically motivated structure, the model structure is dictated to a large extent by known physiological and biochemical relationships: correct

tissue blood-flows and volumes, measured tissue partitioning, and experimentally characterized metabolic pathways with the proper mathematical description (e.g., Michaelis-Menten). This biologically motivated structure greatly simplifies incorporation of *in vitro* results, because if the experiments are done properly, the constants that are obtained *in vitro* can be used directly in the model in the equation that describes the particular process.

There are a number of parameters that are needed for a physiological model but as its name would imply, many of them are available from the physiology literature and the others are often susceptible to direct measurement in an *in vitro* experiment. In many cases there is a choice between designing an *in vivo* study, for example, collecting metabolites, or designing an analogous *in vitro* method to establish the same constants. One of the goals of physiologically based modeling should be to make as much use as possible of *in vitro* studies, or at least of short-term experiments on a small number of animals. Once the model has been developed and validated, which has usually taken a good deal of work, it can be used to design informative, animal-efficient experiments [5], as well as to extrapolate from the scenarios that can be investigated experimentally to those which cannot, in particular to human exposures [6].

Polychlorotrifluoroethylene Oligomer

Polychlorotrifluoroethylene oligomer (pCTFE) is the major component of a nonflammable hydraulic fluid which was under consideration by the Air Force. Although initial acute toxicity screening studies were unremarkable, a subchronic inhalation exposure showed pCTFE to be hepatotoxic in rats [7]. The toxicity, however, appeared to be similar to that of a number of other chemicals that cause peroxisome proliferation in the rodent, and for which the relevance to humans is currently under debate. The key question for the pCTFE risk assessment, then, was whether the toxicity seen in rats was relevant for humans. It seemed appropriate, therefore, to investigate the toxicity of pCTFE in nonhuman primates. Clearly, it would be preferable to limit the number of primates that would have to be used, and so prior to the design of the primate study, efforts focused on the development of a PBPK model that could be used to predict the most informative doses for the primate study, and on the design of *in vitro* studies to characterize interspecies differences in metabolism and tissue response. In this way it was hoped to minimize the amount of information that had to be collected *in vivo* in the primate.

The PBPK model of pCTFE [8] was developed on the basis of the rodent inhalation studies and scaled-up to primates using standard allometric relationships. The model was then used to predict the oral dosing that should be used in primates in order to produce parent concentrations in the liver tissues that would be appropriate for the observance of an effect, if there were one. The success of the model-directed experimental design is shown in Figure 21, which compares the model-predicted

and measured concentrations in fat, liver, and blood for inhalation in the rat (Figure 21a) and for oral dosing in the primate (Figure 21b). Note that in spite of the differences in species and in routes of exposure, the concentrations of pCTFE achieved in the blood and tissues are very similar. Thus, the model was very useful for predicting dose regimens that would produce parent chemical concentrations in the primate comparable to those at which toxicity was observed in the rodent.

Significantly, the peroxisomal proliferation response observed in the rodent was not observed in the primate studies. However, there was still some question whether the absence of this effect was qualitative or quantitative. Comparison of the concentrations of the parent chemical and the metabolites in the livers of rodents versus those of primates showed that even though parent levels for the primates were somewhat higher than those in the rodent, the metabolite levels, particularly for the tetramer acid, were much higher in the rodent. The tetramer acid metabolite accumulated in the liver of the rodent, whereas it was readily excreted in the primate. It still remained, therefore, to establish whether there would be an effect at similar metabolite loadings, since it had been determined in other studies that the metabolites were producing the toxicity.

Rather than conducting additional *in vivo* primate studies, *in vitro* assays were performed in which the trimer acid or the tetramer acid were applied at the same concentration and period of time to cultures of rat liver cells or primate liver cells. There was a significant increase in β-oxidation, a sensitive indicator of the peroxisomal response, in the rodent tissues but not in the primate tissues (Table 20). This result, coupled with the *in vivo* results, provided the necessary information to discount the importance of the rodent-specific peroxisomal response [7]. Instead, a different, milder form of hepatotoxicity seen in the primate in both the *in vivo* and *in vitro* studies will be used to estimate human risk from exposure to pCTFE. The PBPK model has now been expanded to include the species-specific kinetics of the acid metabolites, so that it can be used to estimate metabolite loadings for various human exposure scenarios. By using the PBPK model to compare the metabolite burden for these human exposures with those associated with toxicity in the primate, safe exposure conditions can be determined quantitatively.

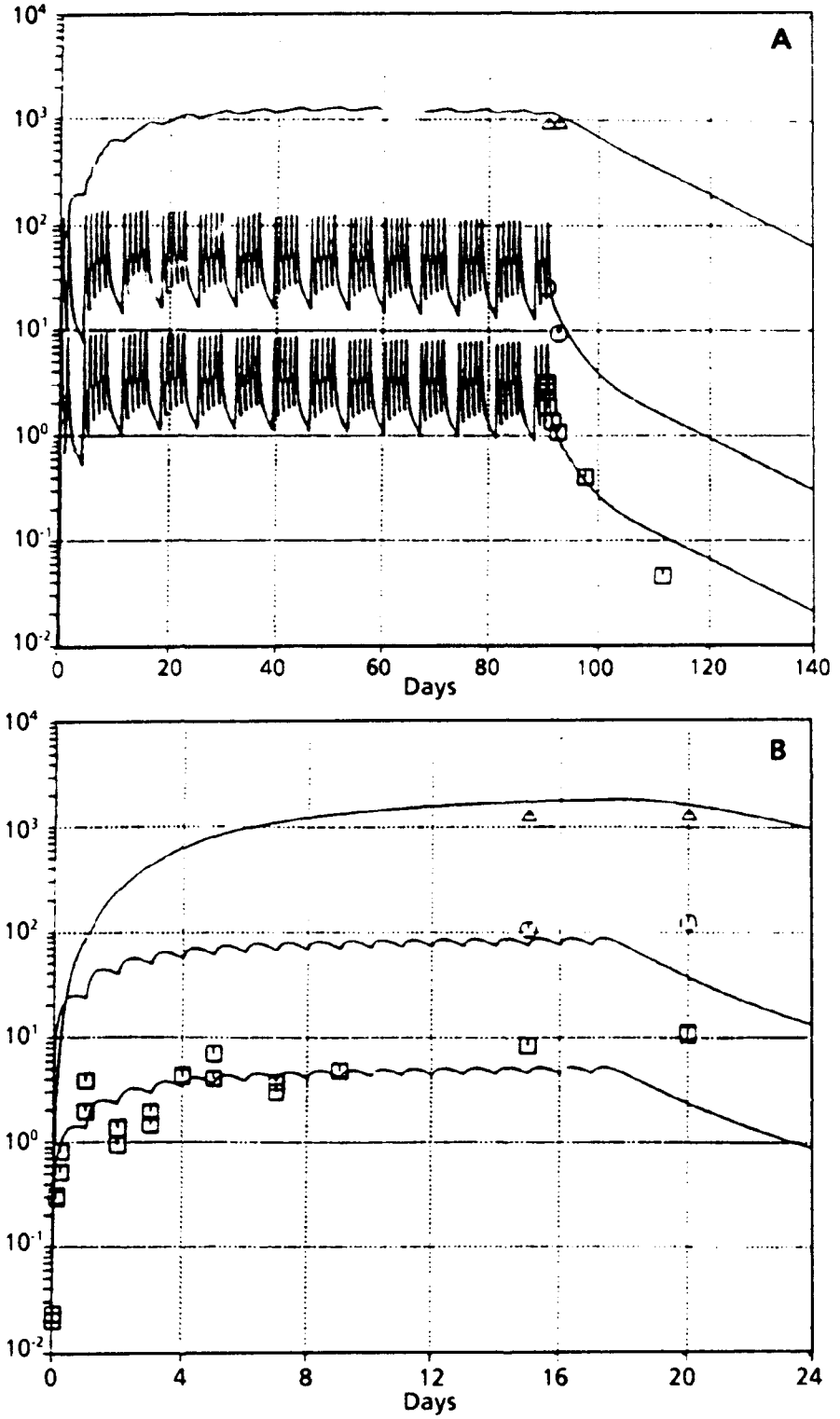

Figure 21. Venous Blood (squares), Liver (circles), and Fat (triangles) Concentrations (mg/L) of pCTFE (A) for Repeated Inhalation Exposure of Rats to 0.5 mg/L pCTFE, 6-h Per Day, 5 Days Per Week, and (B) For a Daily Oral Dose of 725 mg/kg to Primates.

TABLE 20. *IN VITRO* COMPARISON OF BETA-OXIDATION RATES IN HEPATOCYTES FOLLOWING TREATMENT WITH 50 µg/mL pCTFE ACIDS

β-Oxidation Rate (nM/min/mg)	Rodent	Primate
Control	N.D.[a]	N.D.
Trimer Acid	13.2	N.D.
Tetramer Acid	11.3	N.D.

[a] Not detectable (Limit of detection = 3 nM/min/mg)

Organophosphate Esters

The second example provides a demonstration of a PBPK model based almost entirely on *in vitro* information. The model, of the organophosphate ester diisopropylfluorophosphate (DFP), was developed to describe the complex pharmacodynamics (see Figure 22) of acetylcholinesterase (AChE) inhibition [9]. Due to DFP's extreme toxicity, however, it was desirable to avoid any *in vivo* experiments. All of the bimolecular rate constants for inhibition, the regeneration and aging of the esterase, and the metabolism of DFP itself were measured with *in vitro* assays and then used to build a PBPK model. The model had a large number of compartments (Figure 23), but all of the physiological parameters could be obtained from the literature, the tissue partitions from *in vitro* studies, and the metabolism and enzyme kinetics from *in vitro* studies, or on the basis of data from the literature. Figure 24 is an example, one of many, of the ability of the model to reproduce data in the literature for *in vivo* exposures to DFP. In this case, the model was able to describe the inhibition and rebound of AChE levels in the brain of the rat following repeated exposure.

The rodent model was then scaled allometrically to predict human time courses for DFP [10]. Figure 25 shows the agreement of the model with data from the literature for red blood cell AChE, and plasma butylcholinesterase (BChE) activities following administration of DFP to human volunteers. It is important to note that this does not represent a fitting process, but the ability of the physiological model to be scaled across species using standard allometric relationships. The model was next modified to simulate the kinetics for a different chemical, parathion, which is metabolized to the AChE inhibitor paraoxon [10]. Again, the model adaptation was performed without conducting any *in vivo* studies. The modified model was able to satisfactorily describe data from the literature on paraoxon levels in rats following parathion exposure (Figure 26).

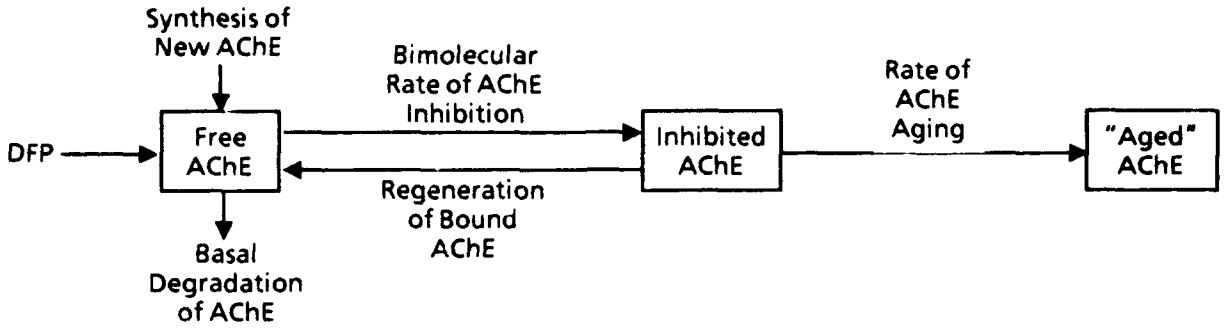

Figure 22. Model for AChE Inhibition, Aging, Regeneration, Synthesis, and Degradation.

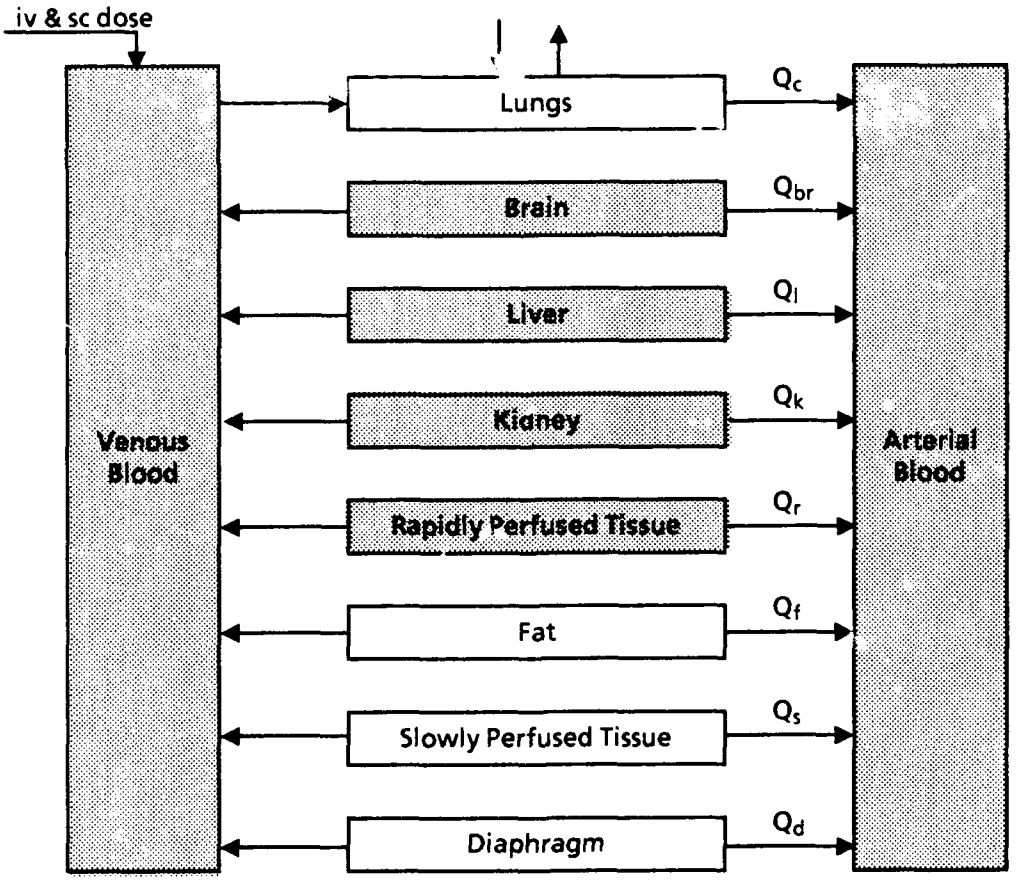

Figure 23. Diagram of the PBPK Model for DFP. The shaded tissue compartments indicates organs within the model in which DFPase activity is described. Arrows in and out of the lung compartment indicate inhalation and exhalation of DFP.

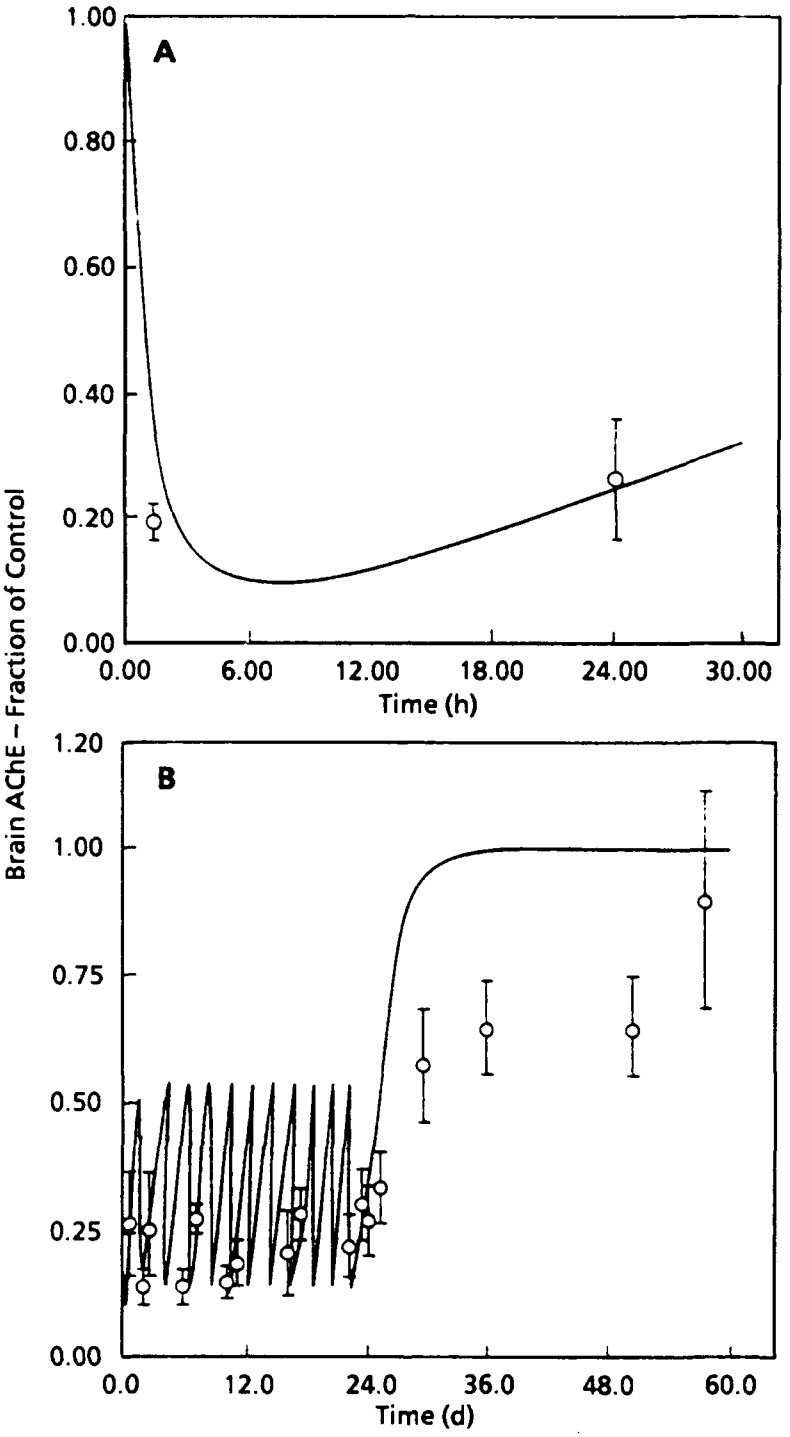

Figure 24. Brain Acetylcholinesterase (AChE) in Male Rats Injected Subcutaneously with DFP (a first dose of 1.1 mg DFP/kg, then 0.7 mg/kg every other day for 22 days). Data are expressed as a fraction of control AChE activity. Brain AChE activity was assayed at 1.5- and 24 h after DFP dosing. Each datum represents (± one standard deviation of the mean) of six animals. Solid line depicts computer simulation.

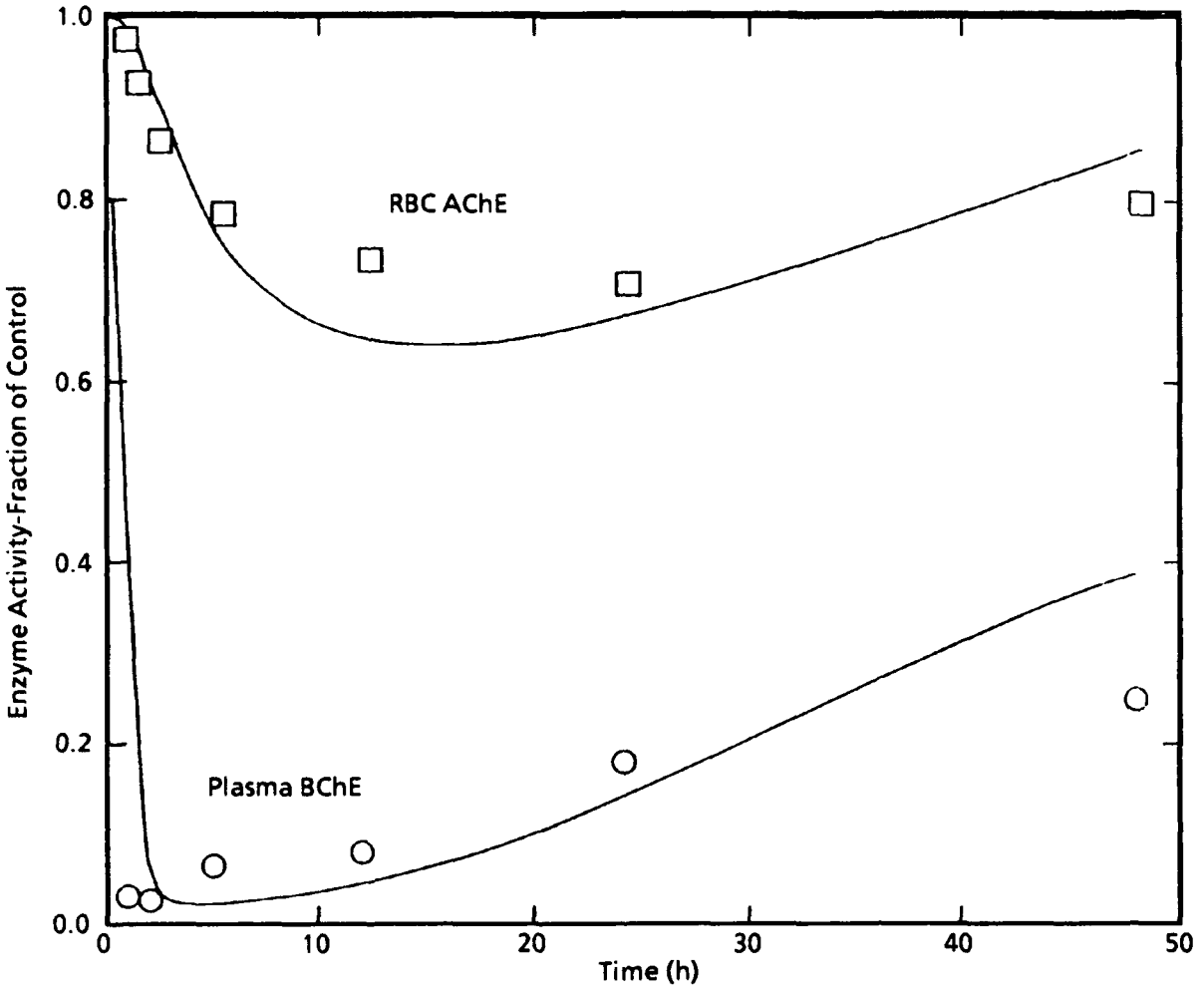

Figure 25. Time-Course of Red Blood Cell AChE and Plasma Butyrylcholinesterase (BChE) Activities in a Human After an Intramuscular Injection with 33 fg DFP/kg. Data are expressed as a fraction of control activity. Solid lines depict computer simulation.

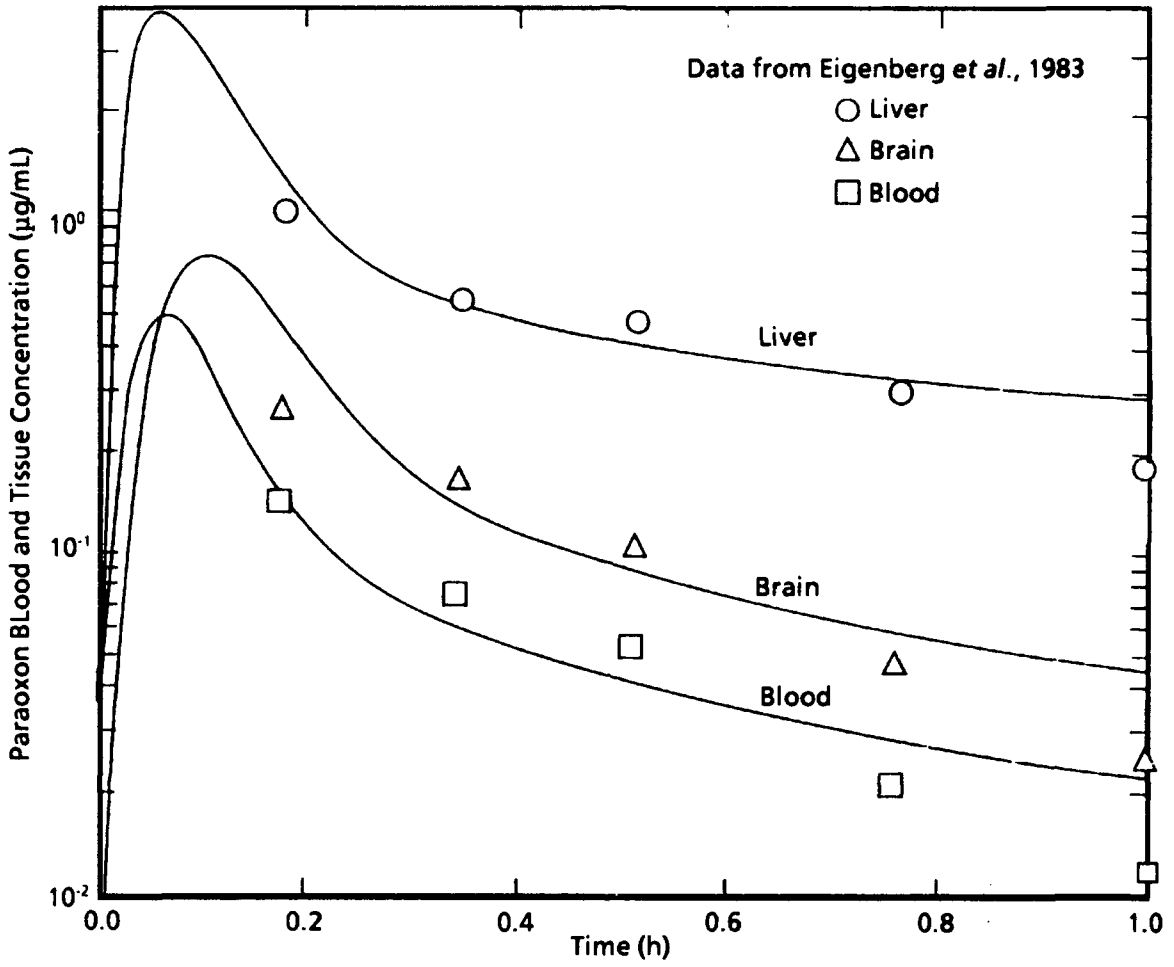

Figure 26. Time-Course of Paraoxon in Liver, Brain, and Blood in mg/L for 1 to 3 h After an Intravenous Injection of Parathion at a Dose of 3 mg/kg. Each data point represents the mean of four rats.

Methylene Chloride

The third example demonstrates the use of *in vitro* data to refine quantitative risk estimates based on a PBPK model. A PBPK model for methylene chloride [11,12] was used by the EPA as the basis for revising the carcinogenic risk assessment for that chemical. Methylene chloride is metabolised by two pathways: a mixed function oxidase (MFO) pathway characterised by a maximum rate (V_{max}) and an affinity (K_m), and a glutathione conjugation (GST) pathway characterised by a first order rate constant (K_f). The risks predicted by the model are based on the assumption that a product of the GST pathway is responsible for the carcinogenicity of methylene chloride [11,13], and are therefore

critically dependent on the activity of the glutathione-dependent metabolic pathway in the mouse and human.

Figure 27 displays the results of an analysis of the sensitivity of the risks calculated by the methylene chloride model to the parameters used in the model [14]. Note that although the model contains 29 parameters (Appendix), only 9 of these have sufficient impact on the predicted risks to merit being displayed in Figure 27. Of those displayed, none have normalized sensitivities significantly greater than one (which would indicate amplification of error). Of the many parameters in the model, only the blood/air partition coefficients, the alveolar ventilation rates and the metabolic parameters play a significant role in the determination of risk. Of these, the human metabolism parameters and the ratios of metabolism in the lung and liver were the least well characterized at the time of the initial model development. Because of the recognized importance of these parameters for the model predicted risks, studies were initiated to measure methylene chloride metabolism in lung and liver tissues from both rodents and humans [15].

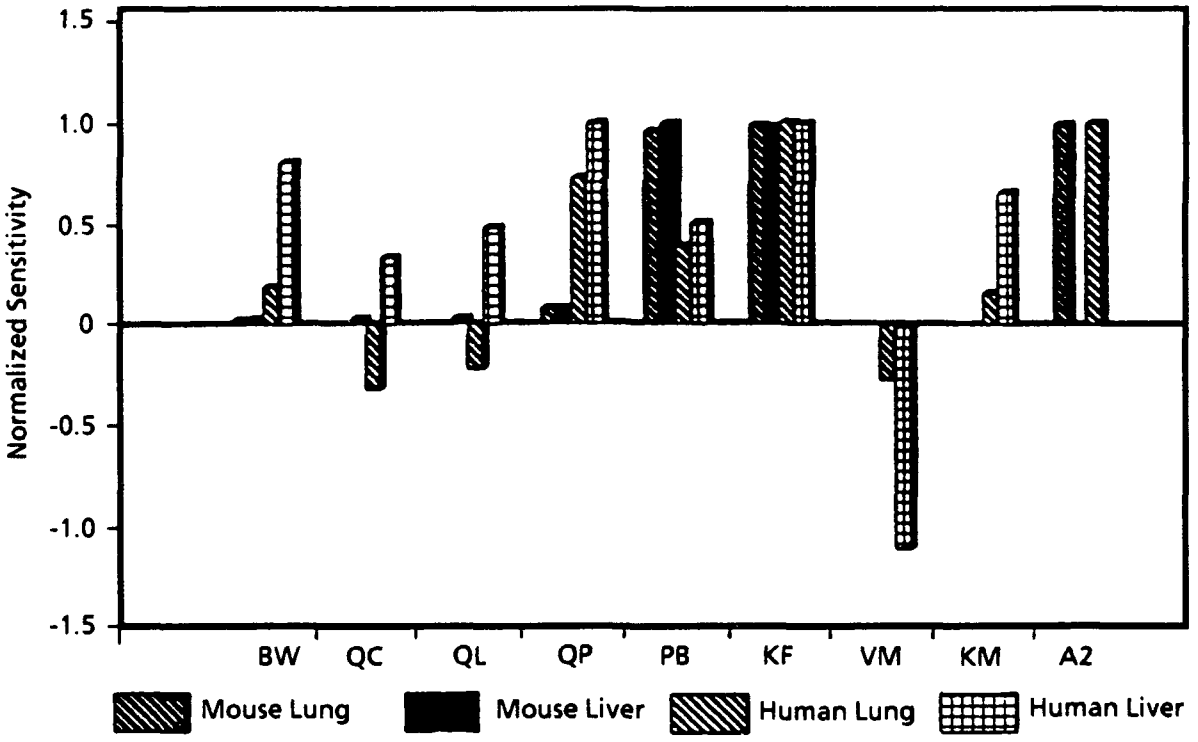

Figure 27. Analytical Sensitivities of Methylene Chloride Model Parameters for the GST Pathway Dose Surrogate. Parameter abbreviations are explained in the Appendix.

Unfortunately, the *in vitro* method used to establish the metabolic parameters was not able to provide results in the rodent liver comparable to those which had been determined by *in vivo* studies, particularly in terms of the enzyme affinity parameter for the MFO pathway (K_m), which was several

orders of magnitude larger (i.e., much lower affinity) than *in vivo* determination. It is not unusual to find that the K_m for the MFO enzyme system appears to be much larger for an *in vitro* assay when compared to an *in vivo* estimate, although in other cases *in vitro* studies have been able to reproduce the *in vivo* parameters quite closely [16]. In the case of methylene chloride, the failure of the *in vitro* results for the rodent to match the *in vivo* determinations made it necessary to apply a ratio analysis, in which the ratio of the *in vivo* and *in vitro* activity in the rodent is use to correct the *in vitro* measured activity in human tissue [15].

Table 21 shows the human metabolic parameters determined on the basis of *in vivo* measurements with humans (for V_{max} and K_m), or on the basis of allometric scaling of *in vivo* measurements with rodents (for K_f), for comparison with the human parameters estimated from the *in vitro* assays using the ratio analysis described above. The *in vitro* estimate of 0.43/h for K_f in the human agrees very well with the value of 0.53/h obtained by allometric scaling of the rodent *in vivo* K_f, whereas the *in vitro* estimate of V_{max} (40) is about 1/3 of the value (118.9) estimated from human *in vivo* exposures. In the same study [15], the relative activity of the two metabolic pathways in lung and liver was determined using methylene chloride as the substrate. These results are also shown in Table 21 for comparison with the values used in the original modeling [11], which were based on other substrates.

TABLE 21. COMPARISON OF METABOLIC PARAMETER ESTIMATES FOR METHYLENE CHLORIDE

	V_{max} Liver	K_m	K_f Liver	V_{max} Lung/Liver	K_f Lung/Liver
Mouse					
In Vivo	1.054	0.4	4.01		
In Vitro	1.054[c]	–	4.01[c]	0.41	0.28
				0.416[a]	0.137[b]
Human					
In Vivo	118.9	0.56	0.53[d]		
In Vitro	40.	–	0.43	<0.03	0.18
				.00143[a]	0.0473[b]

[a] Based on *in vitro* studies with 7-ethoxycoumarin.
[b] Based on *in vitro* studies with 2,5-dinitrochlorobenzene.
[c] *In vivo* value used as reference point for in vitro extrapolation.
[d] Scaled allometrically from mouse *in vivo* estimates.

In vitro data on human metabolism can also be used with the PBPK model to to evaluate the impact of human variability on the risk assessment. Table 22 presents the results of the MFO and GST assays performed on tissues from the livers of different individuals [15]. Activities in the lung were too low to measure in individual tissues, so the tissues were pooled for the analysis. The capacity of the MFO pathway (V_{max}) in the four individuals spans more than an order of magnitude. This variability

should not be surprising given the inducibility of the cytochrome P450IIE1 enzyme (the MFO isozyme which plays a major role in the metabolism of methylene chloride and many other low molecular weight carcinogens [17]) by chemicals to which humans are frequently exposed, such as ethanol [18]. Moreover, a polymorphism of the human P450IIE1 gene has been detected for which an association with susceptibility to lung cancer has been suggested [19]. In the case of GST activity, three of the individuals show fairly similar activity, whereas one shows no evidence of GST activity at all. Again, such interindividual differences, whether acquired (due to environmental exposures or disease states) or inherited (due to genetic polymorphism) may influence an individual's risk for development of cancer [20].

TABLE 22. *IN VITRO* MEASURED HUMAN METABOLIC ACTIVITY FOR METHYLENE CHLORIDE

	V_{max} (liver)	V_{max} (lung)	GST (liver)	GST (lung)
Human				
99	5.27		2.62	
103	1.53		0.0	
105	13.0		2.71	
109	6.24		3.03	
pooled		<0.1		0.37

The PBPK model provides a method to assess the quantitative impact of this variability on individual risk (as opposed to population risk) by comparing model-predicted risks for each individual's parameter values. In this case, the risk predicted for individuals 99, 105, and 109 would be fairly close to the population risk, whereas the risk for individual 103 (Table 22) would be zero, because the risk is based on products of GST metabolism. The model could also be exercised to determine the quantitative impact on individual risk of other sources of variability, such as differences in body weight or breathing rate. For example, a higher breathing rate and cardiac output should be used for occupational exposures involving heavy exertion [21].

In vitro data can also be used to provide estimates of parameter uncertainty (that is, the confidence limits on the central estimate, as contrasted with variability, the potential range of individual parameter values in a population, discussed above). In the case of methylene chloride, information from repeated *in vitro* assays of metabolic parameters [15] and partition coefficients [22] was used together with estimates from the literature of uncertainty in the physiological parameters to characterize the uncertainty in the input parameters for the methylene chloride model. The range of uncertainties used for the various types of model input parameters is shown in Figure 28, as are the resulting ranges of uncertainty in several model dose surrogates (Appendix), as estimated by a Monte

Carlo analysis [23]. As was seen for the sensitivity analysis, the uncertainty in the model input parameters is not greatly amplified in the model outputs.

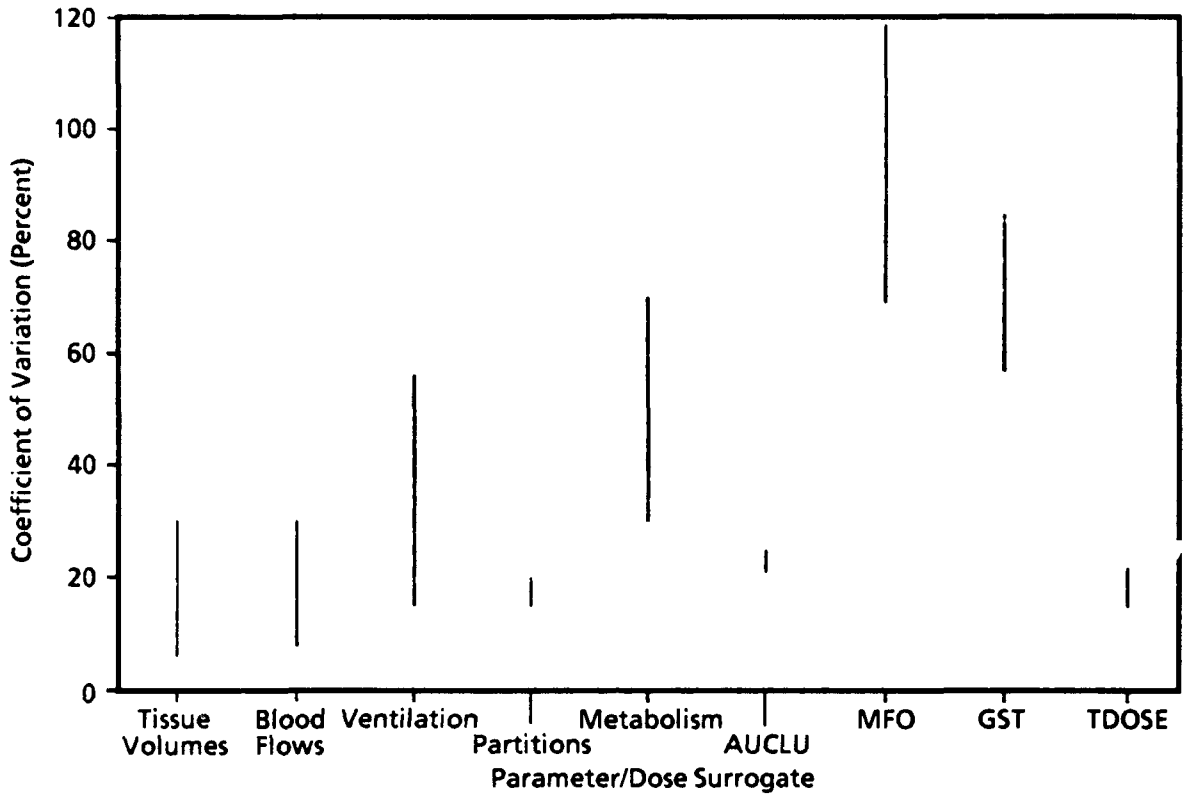

Figure 28. Range of Coefficients of Variation (CVs) for PBPK Model Input Parameters and Dose Surrogates. (CVs for dose surrogates based on Monte Carlo sampling with 500 cases.) Dose surrogates are defined in the Appendix.

Additional Monte Carlo analysis was then performed [23] to determine the resulting uncertainty in the lung cancer risks predicted for a 45 year occupational exposure to methylene chloride. The results of the analysis are shown in Figure 29, which displays the estimated extra lifetime risk of lung cancer from exposure to methylene chloride 8-h per day, 5-day per week, for 45 years at concentrations ranging from 1 to 500 ppm. The uppermost two lines represent the Occupational Safety and Health Administration (OSHA) risk estimates [24], which are based on a dose measure of milligram per kilogram per day (calculated as the product of the ventilation rate, exposure duration, and exposure concentration), and the maximum likelyhood estimate (MLE) or 95% upper confidence limit (UCL) for the multistage model. The two lower lines embrace the range of PBPK model-estimated risks considering the impact of uncertainty in the PBPK model parameters. The PBPK model used in this analysis [23] is similar to the original model [11] except for the preferred (estimate of central tendency) parameter values. As in the original model, the GST pathway was used as the dose surrogate.

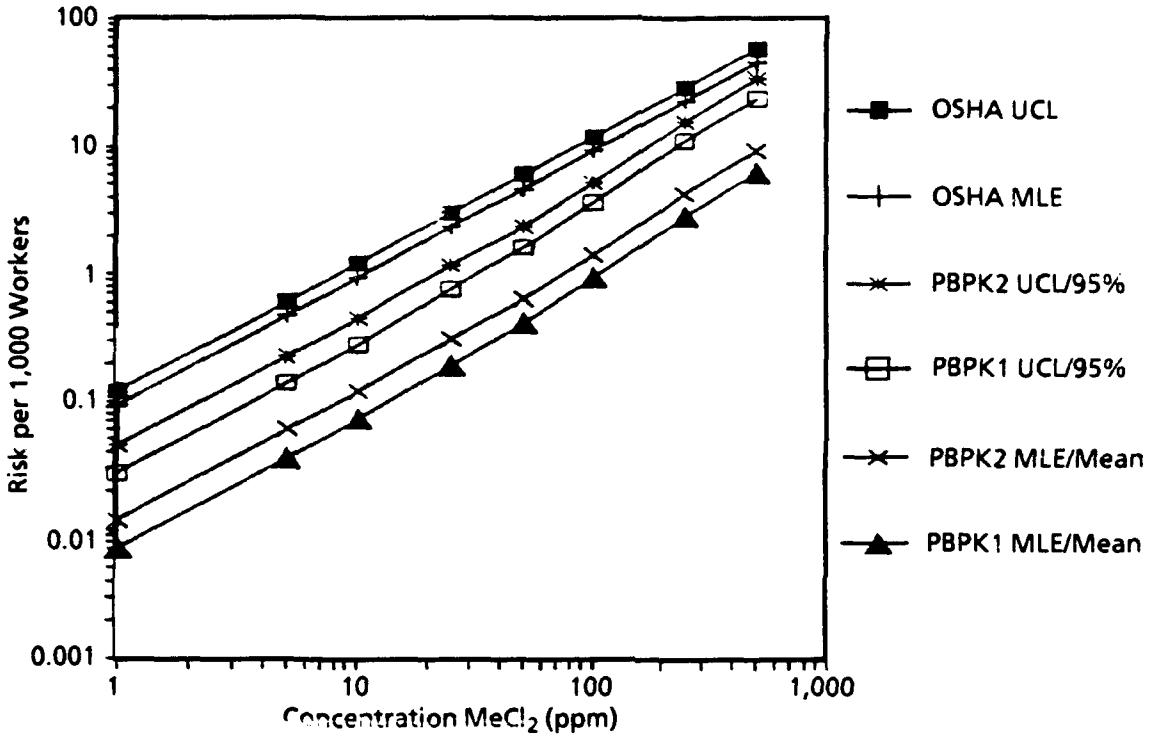

Figure 29. Comparison of OSHA and PBPK-Based Risk Estimates for 45-Year Occupational Exposure to MC Based on Total Female Mouse Lung Tumors. UCL = 95% Upper Confidence Limit on Risk, MLE = Maximum Likelihood Estimate, UCL/95% = 95th percentile of distribution of UCLs produced by variation of PBPK parameters independently in the mouse and the human, and MLE/MEAN = mean of distribution of MLEs produced by variation of PBPK parameters.

The two lines for the PBPK model were chosen to display the range of risk estimates, based on 500 Monte Carlo simulations, from the mean estimate of the MLE (MLE/MEAN) to the upper 95th percentile of the distribution of predicted UCL's (UCL/95%). This range of risk estimates reflects the potential impact of uncertainty as to the "true" values of the PBPK parameters in the bioassay animals and in humans. Even with a highly conservative measure of uncertainty (the upper 95th percentile of the distribution of risks for randomly selected parameter values in both animals and humans), the UCL's based on PBPK modeling are a factor of 2 to 5 lower than the UCL's calculated by OSHA.

Moreover, it is important to understand that the use of the PBPK model did not increase the uncertainty in the risk assessment; it brought it to light. That is, differences in pharmacokinetics across species result in different risks, whether or not the methodology is able to incorporate them.

The same is true of individual risks. The true risk for humans is critically dependent on their relative metabolic capabilities. Current default risk assessment procedures ignore this dependence.

Of greater concern than the impact of uncertainty in the PBPK model parameters is the impact of uncertainty regarding the choice of dose surrogate. The dose measure used by OSHA (milligram per kilogram per day), which provides a meaningful measure of parent chemical exposure in the case of oral dosing or for inhalation of a water soluble chemical, does not have a physiological basis in the case of inhalation exposure to methylene chloride, a lipophilic chemical which rapidly attains a steady-state concentration in the blood. The best-estimate (MLE/MEAN) risks predicted by the PBPK model for the GST pathway are substantially lower (at least a factor of five) than those based on milligram per kilogram per day. Any meaningful measure of parent chemical exposure, on the other hand, (see Figure 30) produces risk estimates substantially higher than those based on milligram per kilogram per day; for example, a factor of four for time-weighted average exposure concentration (TWA PPM).

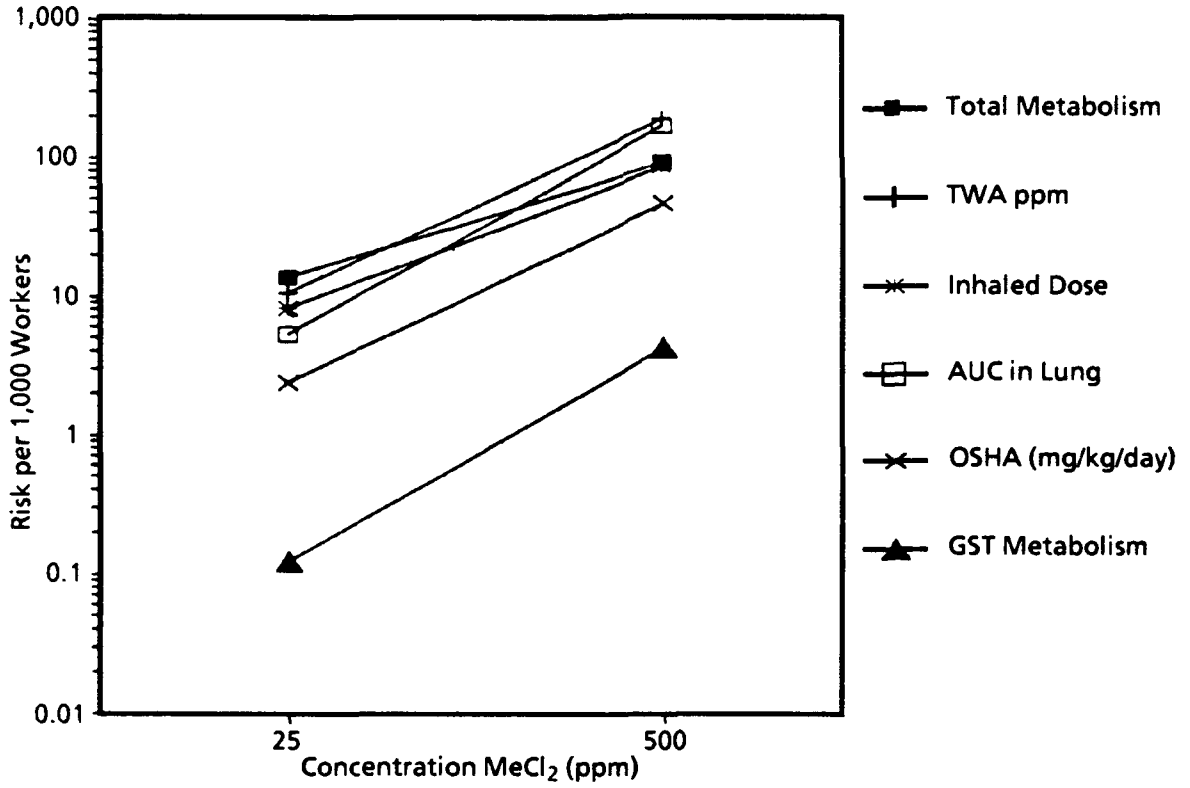

Figure 30. Comparison of Risk Estimates with Various Potential Dose Surrogates, Based on Total Female Mouse Lung Tumors. TWA ppm = Time-weighted average parts per million, AUC = Area under the curve.

CONCLUSION

Three examples have been described to demonstrate the use of *in vitro* studies in the development of PBPK models and to supplement and refine the models in their application for human risk assessment. The use of *in vitro* studies in this manner hopefully provides dual benefits in terms of increased accuracy of the risk estimate and reduced requirements for *in vivo* studies. In the first example, pCTFE, the PBPK model was developed on the basis of *in vitro* assays and on *in vivo* studies which were already required to support the hazard identification process. The ability of the PBPK model to extrapolate across routes of administration and across species was then used to help design *in vivo* primate experiments with a minimum number of animals. When additional information on the primate response was needed, it was possible to incorporate *in vitro* studies instead of performing additional *in vivo* experiments.

In the second example, the organophosphate esters, a PBPK model was developed solely on the basis of *in vitro* studies and existing information from the literature. The resulting model not only is able to reproduce single and repeated exposure data by several routes in several species, but should be useful in establishing safe human exposure conditions.

In the third example, the application of *in vitro* assays to refine a PBPK model for methylene chloride increased the understanding of the quantitative implications of the underlying uncertainty in the processes determining methylene chloride toxicity. The *in vitro* data, as analyzed with the PBPK model, makes visible (as opposed to increases) some of the sources of uncertainty in estimating human risk on the basis of animal studies. Use of *in vitro* data in the PBPK model can also provide information on the impact of human interindividual variability on risk to an individual versus the average risk.

Future studies to characterize the human risk from chemical exposure should feature early and extensive use of *in vitro* studies and PBPK modeling both to limit the requirement for *in vivo* animal studies and to improve the accuracy of the extrapolation to human risk. In particular, the use of *in vitro* metabolism assays should be emphasized both for the identification of isozyme-specific metabolic parameters for use in PBPK models, and for evaluation of tissue dose-response. For example of the first use, the relative contribution of the various cytochrome P450 isozymes to the metabolism to a chemical such as trichloroethylene is a function of both dose and enzyme status (e.g., induced or inhibited by other chemicals) [18]. Carefully conducted *in vitro* studies with both chemical and antibody probes are often needed to provide a definitive assessment of a particular chemical's biotransformation. As an example of the second use, the response of a particular enzyme may provide a useful biomarker for specific effects of a chemical (e.g., P450IVA1 induction by rodent

peroxisomal proliferators, P450IA induction by dioxin-like chemicals, and P450IIB1 induction by phenobarbital-like chemicals).

REFERENCES

1. **Brusick, D.** 1989. A computer-assisted procedure for the assembly and analysis of short-term genotoxicity test data. *Toxicol. Lett.* 49:123-138.

2. **Frazier, J.M.** 1992. *In vitro* models for toxicological research and testing. *Toxicol. Lett.*

3. **Wilkinson, G.R.** 1987. Prediction of *In Vivo* Parameters of Drug Metabolism and Distribution From *In Vitro* Studies. In: *Drinking Water and Health.* Volume 8, pp. 80-95. Pharmacokinetics in Risk Assessment. National Academy Press, Washington, DC.

4. **Clewell, H.J., III and M.E. Andersen.** 1985. Risk assessment extrapolations and physiological modeling. *Toxicol. Indust. Health* 1:111-131.

5. **Clewell, H.J., III and M.E. Andersen.** 1989. Improving toxicology testing protocols using computer simulations. *Toxicol. Lett.* 49:139-158.

6. **Clewell, H.J. and M.E. Andersen.** 1989. Biologically motivated models for chemical risk assessment. *Health Physics* 57(Suppl. 1):129-137.

7. **Mattie, D.R., H.J. Clewell, and M.E. Andersen.** 1992. Issues surrounding comparative risk assessments of operational materials: An example with Air Force hydraulic fluids. In: H.J. Clewell, ed., *Proceedings of the Conference on Chemical Risk Assessment in the Department of Defense (DoD): Science, Policy, and Practice*, pp. 000-000. American Conference of Government Industrial Hygienists, Cincinnati, OH.

8. **Vinegar, A., C.S. Seckel, D.L. Pollard, E.R. Kinkead, R.B. Conolly, and M.E. Andersen.** 1992. Polychlorotrifluoroethylene (pCTFE) oligomer pharmacokinetics in Fischer 344 rats: Development of a physiologically based model. *Fundam. Appl. Pharmacol.* 18:504-514.

9. **Gearhart, J.M., G.W. Jepson, H.J. Clewell, M.E. Andersen, and R.B. Conolly.** 1990. Physiologically based pharmacokinetic and pharmacodynamic model for the inhibition of acetylcholinesterase by diisopropylfluorophosphate. *Toxicol. Appl. Pharmacol.* 106:295-310.

10. **Gearhart, J.M., G.W. Jepson, H.J. Clewell, M.E. Andersen, and R.B. Conolly.** In Press. Physiologically based pharmacokinetic model for the inhibition of acetylcholinesterase by organophosphate esters. *Environ. Health Perspect.*

11. **Andersen, M.E., H.J. Clewell,III, M.L. Gargas, F.A. Smith, and R.H. Reitz.** 1987. Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol. Appl. Pharmacol.* 87:185-205.

12. **Andersen, M.E., H.J. Clewell, M.L. Gargas, M.J. Mac Naughton, R.H. Reitz, R. Nolan, and M. McKenna.** 1991. Physiologically based pharmacokinetic modeling with dichloromethane, it's metabolite carbon monoxide, and blood carboxyhemoglobin in rats and humans. *Toxicol. Appl. Pharmacol.* 108:14-27.

13. **Casanova, M., H. d'Heck, and D.F. Deyo.** 1991. Dichloromethane: Metabolism to Formaldehyde and Formation of DNA-Protein Crosslinks in Mice and Hamsters. *Toxicologist* 11:655.

14. **Lee, T., H.J. Clewell, J.W. Fisher, and R.L. Carpenter.** 1992. Parameter sensitivity in PBPK models of methylene chloride. In: *Proceedings of the Conference on Chemical Risk Assessment in the Department of Defense (DoD): Science, Policy, and Practice*. Armstrong Laboratory Technical Report 92-00, pp. 000-000, Wright-Patterson Air Force Base OH.

15. **Reitz, R.H, A.L. Mendrala, and F.P. Guengerich.** 1989. *In vitro* metabolism of methylene chloride in human and animal tissues: use in physiologically based pharmacokinetic models. *Toxicol. Appl. Pharmacol.* 97:230-246.

16. **Gargas, M.L. and M.E. Andersen.** 1992. Kinetic constants for biotransformation reactions of volatile organic chemicals (VOCs): *In vivo/in vitro* comparisons. *The Toxicologist* 12, 1:347.

17. **Guengerich, F.P., D. Kim, and M. Iwasaki.** 1991. Role of cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.* 4:168-179.

18. **Nakajima, T., R. Wang, N. Murayama, and A. Sato.** 1990. Three forms of trichloroethylene-metabolizing enzymes in rat liver induced by ethanol, phenobarbital, and 3-methylcholanthrene. *Toxicol. Appl. Pharmacol.* 102:546- 552.

19. **Uematsu, F., H. Kikuchi, M. Motomiya, T. Abe, I. Sagami, T. Ohmachi, A. Wakui, R. Kanamaru, and M. Watanabe.** 1991. Association between restriction fragment length polymorphism of the human cytochrome P450IIE1 gene and susceptibility to lung cancer. *Jap. J. Cancer Res.* 82:254-256.

20. **Harris, C.C.** 1989. Interindividual variation among humans in carcinogen metabolism, DNA adduct formation and DNA repair. *Carcinogenesis* 10, 9:1563-1566.

21. **Astrand, P. and K. Rodahl.** 1970. Textbook of Work Physiology, pp. 158-208. McGraw-Hill, New York.

22. **Seckel, C.S., C.L. Flemming, and J.M. Gearhart.** 1992. Variability of partition coefficients and its effect on physiologically-based pharmacokinetic model behavior. *The Toxicologist* 12, 1:347.

23. **Clewell, H.J.** 1992. The use of physiologically based pharmacokinetic modeling in risk assessment: a case study with methylene chloride. In: Cancer Dose- Response, ILSI Press, Washington DC, in press.

24. **Occupational Safety and Health Administration.** 1991. Occupational exposure to methylene chloride; proposed rule. *Fed. Reg.* 56, 216:57036-57141.

APPENDIX

METHYLENE CHLORIDE MODEL PARAMETERS AND DOSE SURROGATES

UNSCALED PARAMETERS

BW	Body Weight (kg)
QPC	Alveolar Ventilation (L/h, 1 kg animal)
QCC	Cardiac Output (L/h, 1 kg animal)

Tissue Blood Flows (Fraction of Cardiac Output):

QGC	Flow to GI Tract
QLC	Flow to Liver
QFC	Flow to Fat
QSC	Flow to Slowly Perfused Tissues
QMC	Flow to Moderately Perfused Tissues
QRC	Flow to Rapidly Perfused Tissues

Tissue Volumes (Fraction of Body Weight):

VGC	Volume of GI Tract
VLC	Volume of Liver
VFC	Volume of Fat
VSC	Volume of Slowly Perfused Tissues
VMC	Volume of Moderately Perfused Tissues
VRC	Volume of Rapidly Perfused Tissues
VLUC	Volume of Lung Tissue

Partition Coefficients:

PB	Blood/Air
PG	GI Tract/Blood
PL	Liver/Blood
PF	Fat/Blood
PS	Slowly Perfused Tissue/Blood
PM	Moderately Perfused Tissue/Blood
PR	Richly Perfused Tissue/Blood
PLU	Lung/Blood

Metabolic Parameters:

VMAXC	Maximum Velocity of Saturable Pathway (mg/h, 1 kg animal)
KM	Affinity of Saturable Pathway (mg/L)
KFC	Rate Constant for 1st Order Pathway (/h, 1 kg animal)
A1	Vmaxc(Lung) / Vmaxc(Liver)
A2	KFC(Lung)/KFC(Liver)

SCALED PARAMETERS

VG = VGC*BW

VL = VLC*BW

VF = VFC*BW

VS = VSC*BW

VM = VMC*BW

VR = VRC*BW

VLU = VLUC*BW

V_{max} = VMAXC*BW**0.75

KF = KFC/BW**.25

QP = QPC*BW**0.75

QC = QCC*BW**0.75

QG = QGC*QC

QL = QLC*QC

QF = QFC*QC

QS = QSC*QC

QM = QMC*QC

QR = QRC*QC

DOSE SURROGATES

AUCL	Area under the curve of liver concentration of MeCl2
Risk1L	Amount metabolized by the liver linear pathway / VL
Risk2L	Amount metabolized by the liver saturable pathway / VL
AUCLU	Area under the curve of lung concentration of MeCl2
Risk1LU	Amount metabolized by the lung linear pathway /VLU
Risk2LU	Amount metabolized by the lung saturable pathway /VLU
CV	Mixed venous blood concentration of parent compound
Tdose	Total amount inhaled during exposure = AUC of QP*(CI-CX)
Tmet	Total amount metabolized (liver plus lung) / BW

REGULATORY AGENCY CONSIDERATIONS AND REQUIREMENTS FOR VALIDATION OF TOXICITY TEST ALTERNATIVES

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SUMMARY

When developing an alternative toxicity test, one must first determine whether the alternative assay is to be used as a screen or as a replacement for the traditional toxicity test. An assay used as a screen will require less stringent acceptance criteria, for it is designed to answer fewer and less complex questions (e.g., the assessment of only potential teratogenicity). An assay used as a replacement will be used to establish hazard or lack thereof (safety). In other words, a replacement assay must clearly establish whether or not a chemical is a teratogen. One should also have knowledge of and experience with the *in vivo* assay to be replaced. This knowledge should be of not only the procedural aspects of the test but also the regulatory information it provides (i.e., how the results are used for hazard determination). Thorough consideration of the regulatory information is critical for a test intended to be used as a replacement. Validation should include intralaboratory and interlaboratory reproducibility of results from a standard protocol, an assessment of the qualitative and quantitative aspects of the test responses, and the use of a sufficient number of chemicals representative of the defined category of interest.

INTRODUCTION

It is not possible for a regulatory agency to precisely prescribe requirements for validation of a test or method, because there are a variety of purposes for which tests are used. What could suffice as a validation test for one purpose may not be satisfactory for another.

There are, however, guiding principles or steps that are necessary before an alternative test can be accepted as valid by the scientific community. If a method or test is to be used to make regulatory decisions, which I define as establishing potential hazard or lack thereof to humans, a few additional steps must be taken.

Validational Guidelines for Screens

The term "validational guidelines" rather than "validation requirements" describes the subject matter of this paper. The extensiveness of such guidelines should relate to how the alternative test is to be used. Broadly speaking, alternative tests are used in two fashions: as screens or as replacements.

An assay used as a screen will require less stringent acceptance criteria than are required for a replacement test. A screen is a test that is used for making preliminary decisions or for establishing the potential toxicity of an agent and the need for further testing. Screens answer fewer and less complex questions than do replacement tests, and the results from screens must usually be confirmed by more definitive testing. A replacement test, however, must provide the same information and data as *in vivo* methods for a definitive toxicological assessment to be made

An alternative assay used as a replacement would probably be used to establish hazard, which is an intricate process and must be accomplished with the best science available. In an alternative test, the *in vitro* end point should have some biological or physiological relevance to that observed *in vivo* [1]. For example, in teratology tests, ideally there should be some relevance of the *in vitro* end points to skeletal or soft-tissue developmental effects. However, relevance is probably not an absolute requirement for a screen but it is necessary for a replacement assay.

If the end point is satisfactory, the procedural aspects of the test should be standardized. The time of chemical exposure and culture media composition must be standardized to obtain reproducible results among laboratories [2].

The selection of agents is also important, and one should consider the following when selecting chemicals: (1) the chemicals should represent a range of toxicity (i.e., some should be highly toxic, some mildly toxic, and some relatively nontoxic; (2) the selection should also represent a range of structurally related chemicals from defined chemical classes to provide some information about structure and activity relationships; (3) some agents or formulations should be included that will provide data on mixtures; and (4) there should be correlative data on the effects of these chemicals in whole animals [2].

When the protocol is developed and the agents are selected, intralaboratory and interlaboratory testing must be conducted. In the published literature there are some suggestions as to the number of agents that should be tested and the number of laboratories that should participate. However, there is no logical reason for the choice of these numbers, and thus investigators should include as many agents and laboratories as practically possible, given the type and quantity of information the test will provide.

Because validation is a process and is not subject to a precise definition, I have for convenience divided it into two components: qualitative and quantitative aspects. The qualitative aspect provides information as to whether the assay responds in the same manner as the more traditional assay. In this respect, one should test a spectrum of chemical classes to objectively evaluate the new assay. In this instance I am referring to the correlation only (i.e., sensitivity and specificity).

The quantitative aspect could involve determining whether a rank order of the positive agents in terms of toxicity or other measured effect is the same or similar in the alternative and traditional assays. There is some uncertainty as to whether the quantitative aspect of validation is a concern for screens used for toxicological tests. The quantitative aspect is important (1) when decisions will be made relative to the next level of testing on the basis of the degree of first level toxicity or activity and (2) when results among laboratories are compared, because the rank order of the positive agents in terms of toxicity or effect should be fairly similar. Thus, for intralaboratory testing, quantitative validation may not be necessary, but for interlaboratory testing it is highly desirable or required.

Finally, the statistical evaluation of the data should be perfected, as these data will be used by regulators to interpret and evaluate the results. Table 23 summarizes the guidelines for validation of screens.

TABLE 23. GUIDELINES FOR SCREENS

Revelance or End Point to Effect to be Detected	Not Required
Test Standardization/Technology Transfer	Required
Selection of Agents	Required
Intralaboratory Testing (Qualitative and Quantitative)	Quantitative Not Required
Interlaboratroy Testing (Qualitative and Quantitative)	Required
Statistical Evaluation	Required

Validational Guidelines for Replacements

With respect to guidelines for replacement assays (Table 24), it is essential that the end point is relevant to the effect observed *in vivo*. For example, an assay that uses human embryonic palatal mesenchyme cells to screen for potential teratogens would not provide any direct information about skeletal or soft-tissue effects because the end point of the assay is cellular inhibition. Cellular proliferation is essential to normal development and its interruption could lead to depressed growth and possibly to teratogenesis. However, how does this assay differentiate between a general cytotoxic agent and one that produces clear-cut terata? If this assay is to be used as a replacement test, the biological or physiological end points that indicate that this assay is indeed identifying teratogens must be defined. The correlation between known teratogens and nonteratogens in the assay must be established, but without the basic scientific foundation underlying the end point, the conclusions reached are suspect. It is far simpler to determine and express correlation in terms of an end point (e.g., to say, "This teratogen inhibits cellular proliferation," than "Because this agent inhibits cellular proliferation it is a suspected teratogen"). The latter implies a relationship between the end point in the alternative test and the effect that is found in the animal.

TABLE 24. GUIDELINES FOR REPLACEMENTS

Revelance of End Point to Effect to be Detected	Required
Revelant Data to Make Regulatory Decisions	Required
Test Standardization/Technology Transfer	Required
Selection of Agents	Required
Intralaboratory Testing (Qualitative and Quantitative)	Required
Interlaboratory Testing (Qualitative and Quantitative)	Required
Statistical Evaluation	Required

The extent to which the end point is relevant to the effect to be measured strengthens or weakens the interpretations and conclusions drawn from such an alternative assay, and consequently its regulatory usefulness. The end points used in various toxicologic tests provide specific information, which is used by a regulatory agency to make decisions about the hazardous nature of a substance. It is imperative, therefore, that alternative tests provide analogous information. Most toxicologic tests provide no observed adverse effects levels (NOAEL) from which safety factors are set for environmental agents and food additives. It would be almost impossible to set a safety factor based on inhibition of cellular proliferation in the human embryonic palatal mesenchyme assay, because there would not be a NOAEL for skeletal and soft-tissue damage.

In acute studies, symptomatology based on observations of live laboratory animals and necropsy information are used to derive information about the relative hazard of various types of substances. These end points allow regulators to make decisions about indirect food additives (e.g., migration of a package material into food). Alternative assays to date have not been able to provide this type of information and, consequently, have not replaced animals in toxicity testing. Similar examples can be cited for subchronic, chronic, and reproduction studies. Until alternative assays can provide end points analogous to those of traditional assays, replacing animals with alternative *in vitro* assays will be very difficult, if not impossible.

A definitive protocol must be developed to facilitate reproducibility among laboratories and transfer of technology (Table 24). This, however, is not as easy as one might envisage, for invariably as soon as a protocol is developed, a determination is made that a certain category of agents is not detected and the protocol must be modified. Validation is a dynamic process and cannot be described by a rigid set of guidelines or rules.

Considerations mentioned earlier regarding selection of agents for screens also apply for replacement assays (Table 24). With respect to intralaboratory testing, the qualitative requirement is the same for screens and replacements, but quantitative validation data are essential for the

replacement assay for intralaboratory as well as interlaboratory testing. If there are wide and disparate results in the rank order of toxicity between an alternative assay and the traditional assay, how much confidence would be placed in that alternative test? Such results would suggest mechanistic differences that should be studied, and a determination must be made as to whether safety would be compromised.

Previous discussions pertaining to interlaboratory testing and statistical evaluation for screens also apply for replacement assays. Thus, there are many similarities between guidelines for screens and guidelines for replacements. The major differences in the guidelines, or lack thereof, relate to the ability of each type of assay to provide information that is useful for safety evaluation.

REFERENCES

1. **Green, S.** 1987. Regulatory issues associated with use of alternative tests. In: M. Mehlman, ed. *Advances in Modern Environmental Toxicology*, Vol 10, pp. 107-116. Princeton, NJ: Princeton Scientific.

2. **Frazier, J.M., S.C. Gad, A.M. Goldberg, and J.P. McCulley.** 1987. *A Critical Evaluation Of Alternatives to Acute Ocular Irritation Testing*, pp. 113-117. New York: Mary Ann Liebert, Inc.

SESSION III

APPLICATIONS OF PHARMACOKINETICS IN RISK ASSESSMENT

ESTIMATING THE RISKS OF LIVER AND LUNG CANCER IN HUMANS EXPOSED TO TRICHLOROETHYLENE USING A PHYSIOLOGICAL MODEL

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ABSTRACT

Trichloroethylene (TCE) is a common and persistent environmental contaminant found in groundwater near most large cities and at Superfund landfill sites. In many cases, the groundwater is the primary source for water consumption. Because of the widespread distribution of TCE in the environment, a significant fraction of the population may ingest or inhale TCE over an extended period of time. Typically, environmental concentrations of TCE are in the ppb range [1]. Health concerns for environmental exposure to TCE stem largely from positive outcomes in laboratory cancer bioassay studies with rodents at relatively high exposure concentrations [2]. Epidemiological evidence that TCE is a human carcinogen is equivocal [2].

Metabolic activation is apparently required for TCE to exert its carcinogenic effect. Recent cancer studies with rodents have linked two metabolites of TCE, dichloroacetic acid (DCA) and trichloroacetic acid (TCA), with hepatocellular carcinoma formation [3,4]. Trichloroethylene is metabolized by the cytochrome P450 system. Trichloroacetaldehyde is formed, possibly via a transient epoxide which rapidly undergoes an intramolecular rearrangement [5]. Trichloroacetaldehyde is either oxidized TCA or reduced to trichloroethanol. Other minor metabolites are formed via dechlorination reactions (oxalic acid, carbon dioxide, and DCA) [6].

The United States Environmental Protection Agency (U.S. EPA) [2] has estimated the health risks of cancer in humans exposed to low concentrations of TCE by using data from rodent cancer bioassay studies. In their risk analysis approach, a nonthreshold extrapolation model (linearized multistage model) was used in the estimation of an excess cancer risk of 1 in 1 million based on the amount of TCE metabolized. Various target organs and types of cancer were included in their calculations. More recently, to estimate the health risks from inhalation exposure to TCE in humans, a classical compartmental model was developed by the U.S. EPA and used to estimate the amount of TCE metabolized for chronic TCE vapor exposures in rodents [7]. Both of these risk analyses for TCE were conducted without the advantage of proper laboratory studies with rodents to quantitatively characterize the metabolism of TCE. That is, metabolic capacity (e.g., V_{max}, the maximum metabolic rate) was not determined for each strain and species of bioassay rodent. In addition, no attempts

were made by the U.S. EPA to develop a human kinetic model for TCE and estimate the metabolic capacity for TCE in humans.

In this research effort, liver and lung cancer risks are presented for lifetime human exposure by ingestion of drinking water containing TCE and inhalation of TCE vapors. A generic, multiroute physiologically based pharmacokinetic exposure model for TCE was constructed for mice and humans. It was used in conjunction with a linearized multistage model to calculate lung and liver cancer risks. The linearized multistage model was used for low dose extrapolation to maintain consistency with U.S. EPA methodology. These cancer risk calculations were based on quantitative metabolic studies carried out in the laboratory using TCE-exposed B6C3F1 mice [8] and published cancer bioassay findings in which B6C3F1 mice developed liver and lung cancer [2,7]. This risk analysis represents a refinement in the U.S. EPA's risk assessment methodology for TCE. It does not address issues related to the appropriateness of the B6C3F1 mouse as a surrogate for estimating human cancer risks. The results of these studies are described in detail elsewhere [9,10]. Based on this analysis, TCE concentrations of 7.0 µg/L in water and 10.0 ppb in air, respectively, correspond to excess liver and lung cancer risks of 1 in 1 million.

REFERENCES

1. **Coleman, W.E., R.D. Lingg, R.G. Melton, F.C. and Kopfler.** 1976. The occurrence of volatile organics in five drinking water supplies using gas chromatography/mass spectrometry. In: L. H. Keith, ed. *Identification and Analysis of Organic Pollutants of Water*, pp. 305-327. Ann Arbor, MI: Ann Arbor Science Publishers.

2. **United States Environmental Protection Agency.** 1985. *Health Assessment Document for Trichloroethylene.* EPA/600/8-82/006F, PB-249696. Washington DC: Office of Health and Environmental Assessment.

3. **Bull, R.J., I.M. Sanchez, M.A. Nelson, J.L. Larson, and A.J. Lansing.** 1990. Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacetate. *Toxicology* 63:341-359.

4. **Herren-Freund, S.L., M.A. Pereira, M.D. Khoury, M.and G. Olson.** 1987. The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid, in mouse liver. *Toxicol. Appl. Pharmacol.* 90:183-189.

5. **Daniel, J.W.** 1963. The metabolism of ^{36}Cl-labelled trichloroethylene and tetrachlorothylene in the rat. *Biochem. Pharmacol.* 12:795-802.

6. **DeKant, W., A. Schultz, M. Metzler, and D. Henschler.** 1986) Absorption, elimination, and metabolism of trichloroethylene: A quantitative comparison between rats and mice. *Zenobiotica* 16:143-152.

7. **United States Environmental Protection Agency.** 1987. *Addendum to the Health Assessment Document for Trichloroethylene: Updated Carcinogenicity Assessment for Trichloroethylene.* Draft, EPA/600/8-82/006FA. Washington, DC: Office of Health and Environmental Assessment.

8. **Fisher, J.W., M.L. Gargas, B.C. Allen, and M.E. Andersen.** 1991. Physiologically based pharmacokinetic modeling with trichloroethylene and its metabolite, trichloroacetic acid, in the rat and mouse. *Toxicol. Appl. Pharmacol.* 109:183-195.

9. **Allen, B.C. and J.W. Fisher.** In Press. Pharmacokinetic modeling of trichloroethylene and trichloroacetic acid in humans. *Risk Anal.*

10. **Fisher, J.W. and B.C. Allen.** 1993. Estimating liver cancer risks in humans exposed to trichloroethylene using physiological models. *Risk Anal.*

VARIABILITY OF PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL PARAMETERS AND THEIR EFFECTS ON PBPK MODEL PREDICTIONS IN A RISK ASSESSMENT FOR PERCHLOROETHYLENE (PCE)

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SUMMARY

When used in the risk assessment process, the output from physiologically based pharmacokinetic (PBPK) models has usually been considered as an exact estimate of dose, ignoring uncertainties in the parameter values used in the model and their impact on model predictions. We have collected experimental data on the variability of key parameters in a PBPK model for tetrachloroethylene (PCE) and have used Monte Carlo analysis to estimate the resulting variability in the model predictions. Blood/air and tissue/blood partition coefficients and the interanimal variability of these data were determined for PCE. The mean values and variability for these and other published model parameters were incorporated into a PBPK model for PCE and a Monte Carlo analysis (n = 600) was performed to determine the effect on model predicted dose surrogates for a PCE risk assessment. For a typical dose surrogate, area under the blood time curve for metabolite in the liver (AUCLM), the coefficient of variation was 25% and the mean value for AUCLM was within a factor of two of the maximum and minimum values generated in the 600 simulations. These calculations demonstrate that parameter uncertainty is not a significant potential source of variability in the use of PBPK models in risk assessment. However, we did not in this study consider uncertainties as to metabolic pathways, mechanism of carcinogenicity, or appropriateness of dose surrogates.

INTRODUCTION

Physiologically based pharmacokinetic (PBPK) models have become useful tools for deriving internal dose estimates from exposure to chemicals by integration of information on the administered dose, the physiological structure of the mammalian species, and the physiochemical properties of the specific chemicals [1,2,3]. While these PBPK models provide a simplified description

of a much more complex physiological system, the mammalian body, they still result in 30 to 40 parameters in the model structure. Fortunately, for most chemicals, only 25 to 30% of these parameters have significant enough impact on the model prediction to warrant consideration. Because these most critical parameters are chemical-specific, they have to be determined for each chemical. In order for these models to find their application in determining excess risk from exposure to a chemical, the variability of the model parameters used in describing the pharmacokinetic data should be incorporated into the overall simulation of the pharmacokinetic data.

Previous studies have been conducted to address these issues in PBPK model parameter variability. Bois et al. [4] reported a risk assessment for tetrachloroethylene (PCE) exposures they conducted to consider the precision and sensitivity of pharmacokinetic models for mice, rats, and humans. They studied the precision of their risk estimate by treating PBPK model parameters as random variables and determined the range of risk estimates when parameter uncertainties were considered via Monte Carlo simulations. The results of the Monte Carlo simulations were analyzed to determine the pharmacokinetic model sensitivity to its parameters. The kinetic parameters defining the metabolic rate were the most important for the cases studied.

Farrar et al. [5] performed an uncertainty analysis on the input parameters for PBPK models and the resulting uncertainty in the output in a risk assessment of PCE. Using data from the literature as the basis for setting the preferred PBPK model parameter values, they developed probability distributions to express uncertainty in the model parameters. Each PBPK model parameter value had an accompanying uncertainty factor which ensured that values for any model parameter were within 95% of the parameter mean. By using Monte Carlo simulation with the Latin-hypercube procedure they were able to derive risk estimates for human PCE exposure at 50 ppm, the current Occupational Safety and Health Administration (OSHA) standard, based on three different dose surrogates.

In this study, the interanimal variability was determined for the blood/air and tissue/air partition coefficients of PCE. These data were then used with the published variation of body organ volumes and flow rates in a PBPK model for PCE and trichloroacetic acid (TCA) to determine the extent this parameter variation would have on model parameter output. These calculations demonstrate that the uncertainty associated with these particular parameters is within the range of other physiological parameters and most likely not a significant potential source of variability in the use of PBPK models in risk assessement. This study demonstrates the need for focusing on uncertainties as to metabolic pathways, mechanisms of carcinogenicity, appropriateness of dose surrogates, and interspecies differences in sensitivity to carcinogensis.

MATERIALS AND METHODS

Determination of Tissue and Blood Partition Coefficients

A variation of the vial equilibrium method of Gargas et al. [6] was used to determine PCE blood/air or tissue/air partition coefficients in individual animals. Fresh tissues were homogenized and then ≈ 100 mg of muscle, liver, or kidney was smeared on the wall of a 25 mL liquid scintillation vial. Because of the high affinity of fat for PCE, only 50 mg of adipose tissue was used. Each vial was then injected with PCE from a standard bag and incubated for 3 h at 37 °C, except fat, which was incubated for 4 h. For blood, 250 µL was placed in the scintillation vial with 250 µL of chemical and was incubated at 37 °C with vortexing for 3 h. A headspace sample (1 mL) was injected onto a gas chromatograph (GC) equipped with a flame ionization detector (FID).

To validate the smear method, the vial equilibration method was used [6]. Rat tissues were used because they provided enough material to run both methods on the same sample. All incubation conditions were the same as the smear method except that the samples were prepared differently. The tissues were removed after the animals were euthanized, they were then homogenized in 0.09% physiological saline (1:3 w/v), and 2 mL of the mixture was pipetted into the 25 mL scintillation vials. Each vial was then injected with PCE from a standard bag and incubated for 3 h at 37 °C, except fat, which was incubated for 4 h. The blood/air and tissue/air partitions were then calculated according to Gargas et al. [6].

Determination of *In Vivo* Metabolism by Gas Uptake

Individual mice were exposed to PCE vapor using a 0.7-L glass and aluminum closed nonrecirculating inhalation exposure chamber. Starting concentrations of PCE in the chamber were 200, 1000, and 3500 ppm. The decrease in PCE chamber concentration with time was monitored by hand sampling and injection on a GC every 5 min for the first hour, every 15 min until 4 h and every 30 min for the remainder of the exposure. To determine metabolic constants from these data, the decrease in chamber concentration with time for each starting concentration was simulated with the PBPK model and the model parameters describing metabolism of PCE [V_{max} [mg/h], K_m [mg/L] and KFC [h^{-1}] were varied to achieve the best model prediction of the data at all concentrations.

Acute Oral Gavage of PCE in Corn Oil

Ten male B6C3F1 mice (30 to 35 g) per dose level received an acute oral gavage of PCE in corn oil (≈ 200 µL) at the two doses 0.536 and 1.072 g/kg/day, as used in the National Cancer Institute (NCI) [7] bioassay and five at a third dose (0.1 mg/kg) which would allow validation of the model at lower doses. Repeated blood samples were taken at 1,2,3,5,7,24,26,28 and 48 h after dosing to determine the kinetics of PCE and the major metabolite of PCE metabolism, trichloroacetic acid (TCA).

Blood Sample Preparation and Analysis

Repeated blood samples were taken from the lateral tail vein of B6C3F1 mice using a 25-gauge needle attached to a hematocrit tube. Blood samples were extracted by vortexing for 1 h with 1 mL of hexane. One microliter of the hexane extract was injected onto a GC equipped with an electron capture detector (ECD). TCA blood samples were collected in 1 mL of cyclohexane. One hundred microliters of methanolic HCl derivatizing agent was then added to the blood samples and they were heated at 100 °C for 30 min. The vials were then vortexed for 1 h and centrifuged. One microliter of the cyclohexane extract was injected onto the GC.

Statistical Analysis

A one factorial analysis of variance with appropriate test of assumptions was done to determine the tissue/air partitions variability among animals, with the factor being animal (mice). To compare the methods (smear and saline) of obtaining tissue/air partitions, data taken from 2- to 3-month-old rats was analyzed using a two sample independent t-test with appropriate tests of assumptions.

A Monte Carlo simulation of 500 oral exposures to PCE was conducted for mice at 536 and 1072 mg PCE/kg body weight. The human oral exposure was 1 ppb. The dose surrogates area under the curve for blood (AUCB), area under the curve for liver (AUCL), and area under the curve for liver metabolite (AUCLM) for the above exposures were compared against various simulated independent variables. The comparison was done using Pearson's Correlational Analysis.

RESULTS

Blood and Tissue Partition Coefficients

The mean and standard deviation for mouse and human PCE blood/air and tissue/air partition coefficients are shown in Table 25. There was a significant difference ($p \leq 0.05$) between the mouse versus human blood/air partition coefficients, with the mouse value being almost twice that of the human value. The tissue/air partitions for the kidney, muscle, liver, and fat could not be statistically tested, because the human tissue samples were from one human subject, but the human values for the later three tissues were greater than for mouse, while the fat/air partition for mouse was greater than the human value.

TABLE 25. BLOOD/AIR, TISSUE/AIR, AND TISSUE/BLOOD PCE PARTITION COEFFICIENTS FOR MICE AND HUMANS

Mouse Partition Coefficients[a]				
Blood/Air	Fat/Air	Kidney/Air	Muscle/Air	Liver/Air
21.47 ± 3.6	1510.8 ± 147	79.14 ± 12.32	79.14 ± 15.63	48.8 ± 5.6
	Fat/Blood	Kidney/Blood	Muscle/Blood	Liver/Blood
	70.37 ± 7.33	2.34 ± 0.26	3.69 ± 0.45	2.34 ± 2.53
Human Partition Coefficients				
Blood/Air	Fat/Air	Kidney/Air	Muscle/Air	Liver/Air
11.58 ± 2.28	1450	58.64	70.45	61.10
	Fat/Blood	Kidney/Blood	Muscle/Blood	Liver/Blood
	125.2	5.06	6.11	5.28

[a] One standard deviation. N = 7 for mice; N = 9 for human blood/air values.

The quotient calculated tissue/blood partitions were much larger for human tissues than for the mouse tissues because of the significantly lower human blood/air partition coefficient. This could not be statistically tested because the human tissue/air partitions were obtained from one human cadaver.

Saline Dilution Versus Smear Partition Coefficients

The determination of tissue/air partition coefficients by the two different methods produced partitions that were not statistically different for all four tissues examined. There was a nonsignificant difference in the fat/air partition coefficient, even though the saline method produced a lower partition than the smear method (Table 26). The kidney/air and muscle/air partitions as determined by the two methods were almost identical, whereas the liver/air partition by the saline dilution method was about 23% higher than the smear method.

Variation In Partition Coefficients in B6C3F1 Mice

The mean values for blood/air and tissue/blood partition coefficients for PCE in mice all had coefficients of variation of less than 20% (Table 25). The liver/blood coefficent was the highest (17.5), whereas the fat/blood was the lowest (9.7%). The measured blood/air partition coefficient (21.5) was representative of the range of values for PCE in mice reviewed in the literature [8] by Hattis et al.

TABLE 26. COMPARISON OF PARTITION COEFFICIENT METHODS IN RATS

Partition		Smear vs. Saline Technique	
		Smear	Saline
Fat/Air	N*	5	5
	Mean	1437.200	1236.670
	SD	193.720	238.782
	CV	0.135	0.193
Kidney/Air	N	5	5
	Mean	51.260	51.660
	SD	27.196	13.240
	CV	0.531	0.256
Liver/Air	N	5	5
	Mean	50.200	61.960
	SD	24.260	27.310
	CV	0.483	0.441
Muscle/Air	N	5	5
	Mean	21.619	18.070
	SD	8.168	8.077
	CV 0.378		0.447

* N = number of animals, SD = standard deviation, CV = coefficient of variation.

Determination of *In Vivo* Metabolism by Gas Uptake

The rate of *in vivo* metabolism of PCE determined by gas uptake was 0.2 mg/h-kg for the V_{max}, 2.0 mg/L for the K_m, with a first order metabolism rate of 2.0 $h^{-1}kg^{-1}$. Figure 31a depicts the PBPK analysis of the gas uptake curves without including a first order rate of metabolism. By setting the V_{max} and K_m to 1.0 mg/L/h and 0.45 mg/L, the computer optimized values for these data. It was not possible to predict the decrease in chamber concentration in the two higher exposure groups. Adding a first order metabolic rate constant of 2.0 $h\text{-}L^{-1}$ (Figure 31b) decreased the initial rate of decrease for the chamber concentration so that the simulation of 3500 ppm exposure provided a very good fit of the data.

PCE and TCA Kinetics after Acute Oral Gavage of PCE in Corn Oil

Blood kinetics of PCE with the model simulations of the data for three dose levels are shown in Figure 32a. The model provided excellent predictions of PCE blood levels for the first 10 h of the two highest oral doses, but underpredicted the presence of PCE still found in the blood of experimental animals past 24 h of dosing. The peak blood concentration of the 100 mg/kg dose group was underpredicted, but the data points from ≈ 5 to 30 h were predicted by the model simulation.

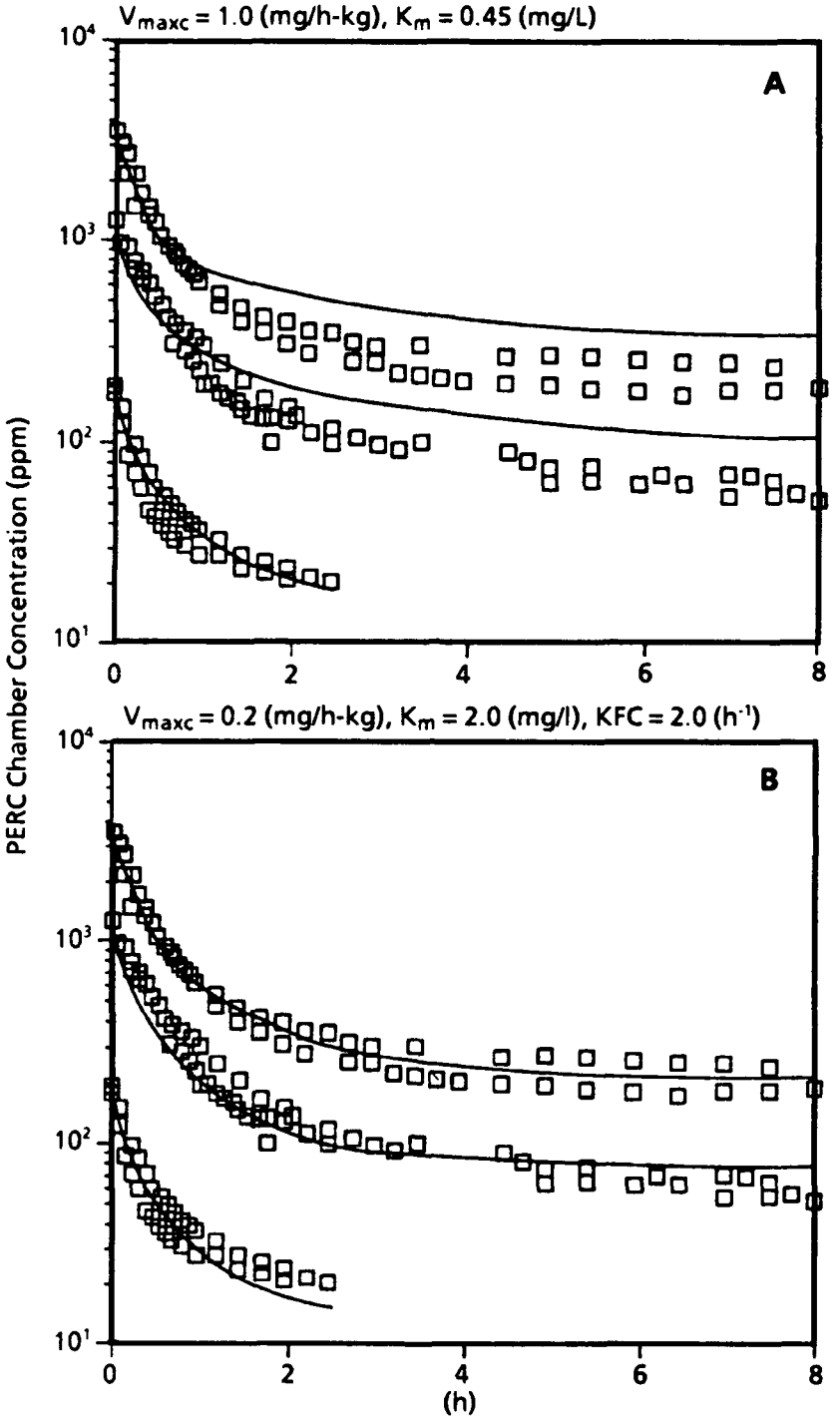

Figure 31. (A and B). Gas-Uptake Studies Utilized to Estimate the Kinetic Constants for Saturable Metabolism in Mice. Data are ppm of tetrachloroethylene in the chamber atmosphere as a function of time. Experimental data are shown as symbols, whereas the computer simulations are presented as solid lines. (A) simulation of the data with only saturable metabolism, (B) simulations of the data with saturable and first order rates of metabolism.

Blood kinetics of TCA with the model simulations of the data for the three PCE dose levels are shown in Figure 32b. The model provided an excellent prediction of the TCA blood time course for the entire period of measurement of TCA blood levels. The peak concentration of TCA in blood in the lowest dose group again was underpredicted, as would be expected because there was an underprediction of the substrate for this metabolite. The model did provide a very good estimate of the TCA blood concentrations from ≈ 5 to 30 h after dosing, as it did with the PCE kinetic data.

Simulation of Human Exposure Data

The ability of the model to predict human exposures to PCE was tested by analyzing some human data sets available in the literature. Stewart et al. [9] exposed human subjects to 101 ppm PCE for 6 h and then collected PCE in the expired breath. Figure 33a shows model simulations with the data of the human exposures from immediately following exposure out to ≈ 120 h after the end of the inhalation exposure. The model provided a good overall prediction of the general shape of the expired breath time course and was actually within the mean and standard deviation of most time points. The ability of the model to predict excretion of the primary metabolite of PCE, TCA, was examined (Figure 33b) by simulating urinary excretion data for human subjects exposed to a concentration of PCE of 150 ppm for 8 h [9]. The model parameter responsible for controlling urinary excretion was fit to the available data to help set the human metabolism parameters for PCE.

Fernandez et al. [10] exposed humans to 100 ppm for varying lengths of time (1, 2, 4, and 8 h) and measured the exhaled concentration of PCE during and after exposure (Figures 34 and 35). There was a very good agreement between the simulation and data for the 1-, 2-, and 4-h inhalation and postinhalation exhaled breath concentrations. The simulation of the 8-h inhalation exposure provided a good prediction of the data, but the simulation and data of the postexposure exhaled breath concentration did not agree with the simulation overpredicting the amount of exhaled PCE.

Correlation Of Model Parameters With Output Variables

The correlation of nine model parameters with three representative dose surrogates for oral exposure of mice to PCE in corn oil at 536 and 1072 mg/kg and humans exposed at a steady state to 1 ppb PCE are shown in Tables 27 through 29. For each model parameter and dose surrogate, the larger the correlation value, and the greater the effect of the model parameter on the dose surrogate calculated by the model. For the 536 mg/kg dose, the most significant negative correlation was the effect of pulmonary ventilation (QPC) on area under the curve for PCE in blood. QPC also had a significant negative correlation with concentration of PCE in the liver and area under the curve for metabolite in liver (AUCLM). The strongest positive correlation due to an input model parameter occurred with the blood/air partition coefficient (PB). The same trend of negative correlation of QPC with AUCB was true for both the 1072 mg/kg dose and the 1 ppb human simulation.

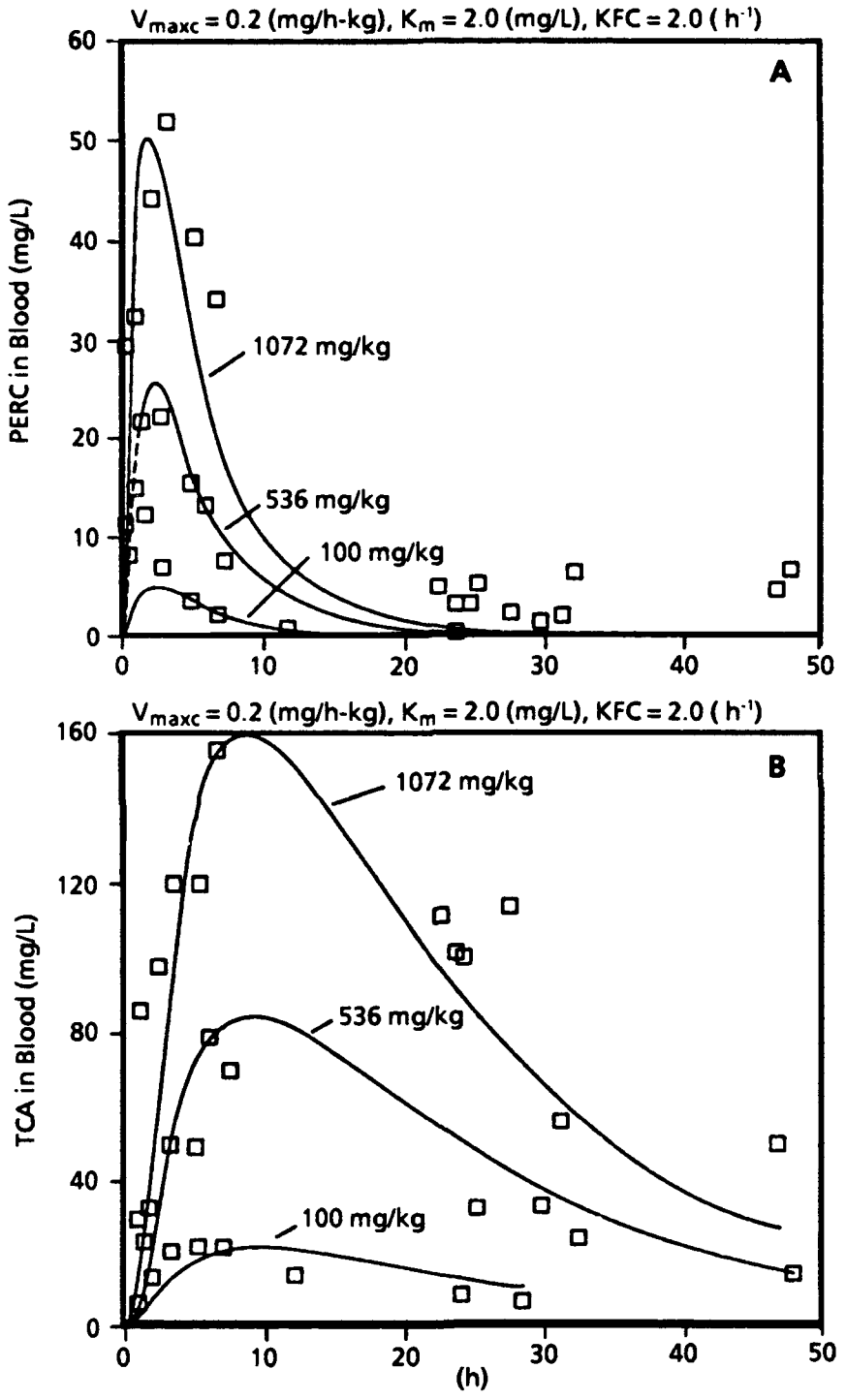

Figure 32. (A and B). Corn Oil Gavage Studies of Tetrachloroethylene at 0.1, 0.536, and 1.072 mg/kg Doses of PCE. Experimental data are shown as symbols, whereas the computer simulations are presented as solid lines. (A) Simulation of PCE blood concentrations, (B) simulation of TCA blood concentrations. Other symbols and abbreviations as in Figure 37.

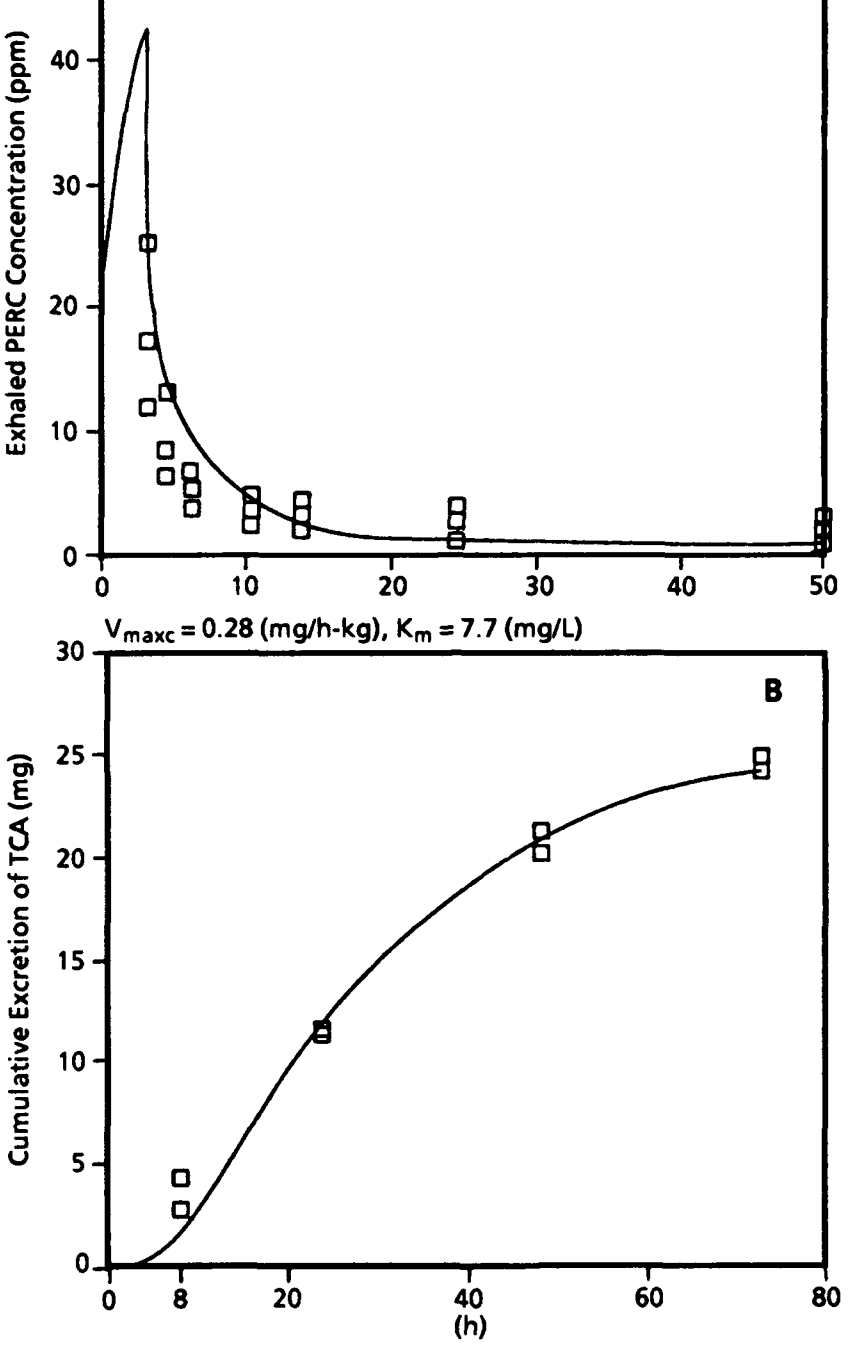

Figure 33. (A and B). Simulation (solid line) of Human Inhalation Exposure Data (symbols) from Stewart et.al. [9]. (A) Concentration of PCE in exhaled breath of human subjects exposed to 101 ppm PCE for 6 h, (B) Cumulative amount of TCA excreted in urine by human subjects exposed to 150 ppm PCE for 8 h. Other symbols and abbreviations as in Figure 37.

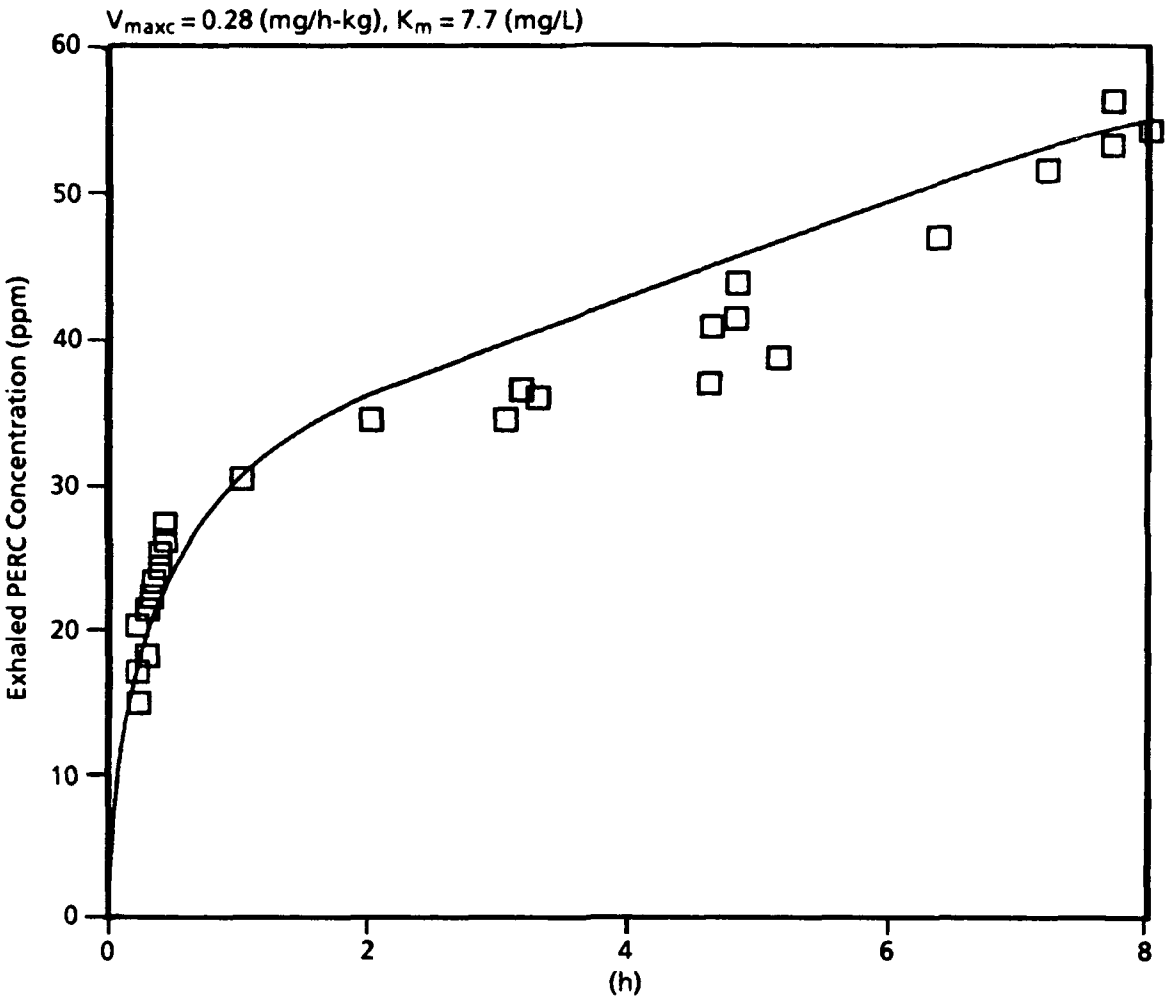

Figure 34. Simulation (solid line) of Exhaled PCE in Human Breath During and After Inhalation Exposure (data-symbols) to 100 ppm PCE for 8 h. Fernandez et al. [10].

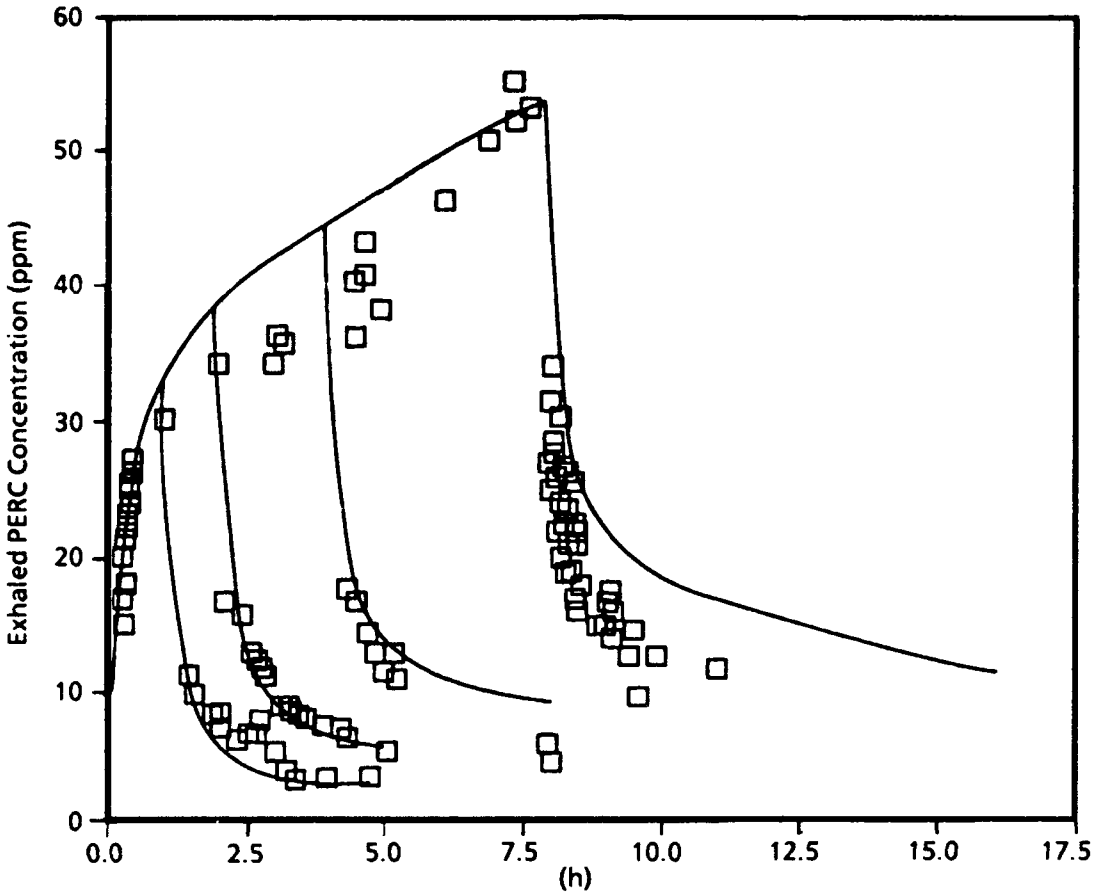

Figure 35. Simulation (solid line) of Exhaled PCE in Human Breath During and After Inhalation Exposure (data-symbols) to 100 ppm of PCE for 1, 2, 4, or 8 h. Fernandez et al. [10].

TABLE 27. CORRELATIONAL TABLE OF MODEL PARAMETERS AND OUTPUT FOR A GAVAGE DOSE OF 536 mg/kg IN MICE EXPOSED TO PCE

	AUCB	AUCL	AUCLM
QPC	-0.75[a]	-0.55[a]	-0.43[a]
QCC	0.07	-0.08[b]	-0.48[a]
BW	0.17[a]	0.14[a]	0.04
PL	-0.07	0.59[a]	-0.09[b]
PB	0.57[a]	0.44[a]	0.38[a]
VLC	-0.05	-0.04	0.37[a]
VSC	0.04	0.02	-0.12[a]
AUCB		0.74[a]	0.57[a]
AUCL			0.46[a]

[a] the row variable is highly correlated with column variable ($p<0.01$).
[b] the row variable is correlated with column variable ($0.01<p<0.05$).
QPC - pulmonary ventilation (L/h/kg)
QCC - cardiac output (L/h/kg)
BW - body weight (kg)
PL - liver/air partition coefficient
PB - blood/air partition coefficient
VLC - volume of the liver as percent body weight
VSC - volume of the slowly perfused tissue as percent body weight
AUCB - area under the curve for blood
AUCL - area under the curve for liver

TABLE 28. CORRELATIONAL TABLE OF MODEL PARAMETERS AND OUTPUT FOR A GAVAGE DOSE OF 1072 mg/kg IN MICE EXPOSED TO PCE

	AUCB	AUCL	AUCLM
QPC	-0.78[a]	-0.62[a]	-0.50[a]
QCC	0.05	-0.07	-0.50[a]
BW	0.10[a]	0.06	-0.02
PL	-0.03	0.61[a]	-0.05
PB	0.60[a]	0.45[a]	0.39[a]
VLC	-0.06	-0.05	0.36[a]
VSC	0.01	0.03	-0.14[a]
AUCB		0.74[a]	0.58[a]
AUCL			0.49[a]

[a] the row variable is highly correlated with column variable ($p<0.01$).
QPC - pulmonary ventilation (L/h/kg)
QCC - cardiac output (L/h/kg)
BW - body weight (kg)
PL - liver/air partition coefficient
PB - blood/air partition coefficient
VLC - volume of the liver as percent body weight
VSC - volume of the slowly perfused tissue as percent body weight
AUCB - area under the curve for blood
AUCL - area under the curve for liver

TABLE 29. CORRELATIONAL TABLE OF MODEL PARAMETERS AND OUTPUT FOR HUMANS CONSUMING DRINKING WATER AT A CONCENTRATION OF 1 ppb OF PCE

	AUCB	AUCL	AUCLM
QPC	-0.72[a]	-0.62[a]	-0.44[a]
QCC	-0.06	-0.50[a]	-0.57[a]
BW	-0.15[a]	-0.15[a]	-0.43[a]
PB	0.69[a]	0.57[a]	0.45[a]
VKC	-0.09[b]	-0.07	-0.31[a]
VSC	0.078[b]	0.06	0.13[a]
AUCB		0.88[a]	0.71[a]
AUCL			0.89[a]

[a] the row variable is highly correlated with column variable ($p<0.01$).
[b] the row variable is correlated with column variable ($0.01<p<0.05$).
QPC - pulmonary ventilation (L/h/kg)
QCC - cardiac output (L/h/kg)
BW - body weight (kg)
PB - blood/air partition coefficient
VLC - volume of the liver as percent body weight
VSC - volume of the slowly perfused tissue as percent body weight
AUCB - area under the curve for blood
AUCL - area under the curve for liver

DISCUSSION

The blood/air partition coefficients measured for mice in our laboratory were approximately twice the human values. The mean mouse blood/air partition coefficient we measured (21.5) was in the middle of the range of values reported in the literature for mice (16.9 to 24.4), as reviewed by Hattis et al. [8]. If the range of blood/air partitions was calculated as the mean ± one standard deviation of the mean, then this range would encompass the range of values reported in the literature. It should be noted also that the published range includes other strains of mice in addition to those used in this study and also includes values determined for female mice.

As with the mouse blood/air partition coefficients, the human blood/air mean value we have determined experimentally (11.56) is very representative of the reported range of human values of 10.3 to 14.0 [8]. Again, if the mean ± one standard deviation of the mean were calculated, then this range would encompass the range of values reported in the literature. The reasons for this significant species difference are not known. Lam et al. [11] have proposed that the rat versus human species differences in affinity of some organic solvents for red blood cells versus plasma is a function of the greater affinity of rat versus human hemoglobin for hydrophobic solvents. Of greater importance than knowing the actual mechanism for the blood/air partition coefficient difference is that for modeling purposes. The rodent model can be scaled to human physiological values and we can then use the actual human blood/air partition values collected in the laboratory.

The *in vivo* metabolism of PCE as determined by gas uptake in this study required the addition of a first order metabolic rate constant to provide an adequate fit of the data. It is known that PCE forms a conjugate with glutathione at a rate that appears first order, so that adding a first order rate constant to improve prediction of gas-uptake data is not mechanistically unreasonable. The difficulty arises when the first order pathway is used to generate TCA and not glutathione. This has not been a proposed pathway for the generation of TCA from PCE metabolism, yet the oral pharmacokinetic data indicates the necessity of continued production of TCA at oral doses as great as 1072 mg/kg/day. If no first order rate of PCE metabolism to TCA is incorporated into the metabolic analysis of the kinetic data, there is saturation of the Michaelis-Menten kinetics near the 0.536 mg/kg oral dose of PCE and not enough TCA is produced at the higher oral doses to reach the levels of TCA measured in the actual experimental studies. The urinary excretion data of Buben and O'Flaherty [12] support the necessity of continued TCA production at higher dose levels. Their studies showed that mice dosed with PCE continued to produce metabolites without complete saturation of metabolic pathways up to ≈ 2.0 g/kg, almost twice the concentration used in the bioassay and in this kinetic study.

One of the technical objectives of this research project was to develop a method to determine interanimal and intraanimal variability of tissue/air and blood/air partition coefficients. The

determination of interanimal variability of each partition coefficient was necessary for setting the error bounds on the mean of each respective partition coefficient. The interanimal variability was necessary to allow a Monte Carlo simulation of the distribution of all partition coefficients. Because of the small tissue volumes of mice relative to rats, the species in which most partition coefficients has been determined, a technique was developed to measure partition coefficients in small tissue and blood volumes. This technique involved homogenizing the fresh tissue and smearing an appropriate volume on the inner wall of a tared vial. For tissues that do not distribute uniformly after homogenization in water and/or the amount of tissue per animal is small, it is possible to determine a partition coefficent.

Simulation of PCE and TCA after oral dosing at three different levels of PCE shows the ability of the PBPK model to not only predict concentration of the parent compound, PCE, but also to provide a very good description of the TCA kinetics. A validated PBPK model makes it possible to consider different dose surrogates to correlate with the cancer bioassay data for mice exposed by oral exposure. This will produce a risk assessment based more strongly on the biology and physiology of the species used in the cancer bioassay, while at the same time providing a rational means of incorporating human physiological and biochemical parameters into a mathematical framework for extrapolation to low-dose human exposures.

The usefulness of PBPK models in the risk assessment process can be strongly dependent on the variation of the basic model parameters. In this study, we have determined that the variation of blood/air and tissue/blood partition coefficients, key parameters in the simulation of pharmacokinetic data, is well within the variation of normal physiological variability. In this particular study of PCE, the analysis of the correlation of model parameters has shown that the pharmacokinetics of PCE is predominately controlled by the two main parameters which control PCE entry into the body by inhalation, the blood/air partition coefficient, PB, and pulmonary ventilation, QPC. The rate of exhalation of a compound is dependent upon the affinity of the compound for the blood, the PB, and the amount of air exchanged with the blood flowing to the lungs, the QPC.

A preliminary calculation of the risk to humans consuming 2 L of water per day contaminated with 1 μg/L of PCE is compared to two other previously calculated risk estimates (Table 30). The risk calculation presented in this paper (Table 30) is based on the animal kinetic/PBPK studies described and the PBPK simulation of human exposure data used to estimate human metabolism of PCE. The U.S.Environmental Protection Agency (EPA) [13] risk estimate is based on the urinary excretion of TCA from the study of Buben and O'Flaherty [12], so as to utilize the available human urinary excretion of TCA. The Chen and Blancato [14] risk estimate is based on a PBPK model, which was used to analyze the data of Pegg et al. [15] and Schumann et al. [16] The EPA [13] risk estimate is ten times higher than that calculated by Chen and Blancato [14] (Table 30) and approximately thirty times higher than the

estimate of risk determined in this study. The major differences between the EPA estimate and that of Chen and Blancato [14], or the present risk estimates is in the use of a PBPK model to obtain data-based estimates of total parent absorption, which is less than 100%. In the classical EPA method, 100% of the parent chemical is assumed to be absorbed, while for PCE, experimental human inhalation exposures have shown that at higher concentrations, most of what is inhaled is exhaled

TABLE 30. COMPARISON OF PCE RISK AT A UNIT DOSE 1 µg/L IN WATER

EPA (Classical - total metabolism)	1.5×10^{-6}
Chen and Blancato (PBPK - total metabolism)	1.5×10^{-7}
Gearhart et al. (PBPK - total metabolism)	4.9×10^{-8}

The major differences between the Chen and Blancato risk estimate versus our analysis is the model parameter values and the sex of the animals used for the cancer dose–response relationship. Chen and Blancato [14] based their risk estimate on the data from female mice, whereas our analysis used male mice for the kinetic data collection and the male mouse NCI [7] cancer dose–response data. Also, Chen and Blancato [14] fit their PBPK model metabolism to predict the amount of PCE metabolites excreted in the urine. In the present study, we based our estimate of metabolism on disappearance of the PCE in gas-uptake studies, and by predicting the blood time-course of PCE and TCA.

REFERENCES

1. **Andersen, M.E., H.J. Clewell, III, M.L. Gargas, F.A. Smith, and R.H. Reitz.** 1987. Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol. Appl. Pharmacol.* 87:185-205.

2. **Reitz, R.H., P.S. McCroskey, C.N. Park, M.E. Andersen, and M.L. Gargas.** 1990. Development of a physiologically based pharmacokinetic model for risk assessment with 1,4-dioxane. *Toxicol. Appl. Pharmacol.* 105:37-54.

3. **Frederick, C.B., D.W. Potter, M.I. Chang-Mateu, and M.E. Andersen.** 1992. A physiologically based pharmacokinetic and pharmacodynamic model to describe the oral dosing of rats with ethyl acrylate and its implications for risk assessment. *Toxicol. Appl Pharmacol.* 114:246-260.

4. **Bois, F.Y., L. Zeise, and T.N. Tozer.** 1990. Precision and sensitivity of pharmacokinetic models for cancer risk assessment: Tetrachloroethylene in mice, rats, and humans. *Toxicol. Appl. Pharmacol.* 102:300-315.

5. **Farrar, D., B. Allen, K. Crump, and A. Shipp.** 1989. Evaluation of uncertainity in input parameters to pharmacokinetic models and the resulting uncertainity in output. *Toxicol. Letters* 49:371-385.

6. **Gargas, M. L., R.J. Burgess, D.E. Voisard, G.H. Cason, and M.E. Andersen.** 1989. Partition coefficients of low- molecular weight volatile chemicals in various liquids and tissues. *Toxicol. Appl Pharmacol.* 98:87-99.

7. **NCI (National Cancer Institute).** 1977. Bioassay of tetrachloroethylene for possible carcinogenicity. DHEW Pub. No. (NIH) 77-813. Bethesda, MD: Public Health Service, National Institutes of Health, U.S. Department of Health, Education, and Welfare.

8. **Hattis, D., P. White, L. Marmorstein, and P. Koch.** 1990. Uncertainties in pharmacokinetic modeling for perchloroethylene. I. Comparison of model structure, parameters, and predictions for low-dose metabolism rates for models derived by different authors. *Risk Anal.* 10:449- 457.

9. **Stewart, R.D., E.D. Baretta., and H.C. Dodd.** 1970. Experimental human exposure to tetrachloroethylene. *Arcn. Environ. Health* 20:224-229.

10. **Fernandez, J., E. Guaberan, and J. Caperos.** 1976. Experimental human exposures to tetrachloroethylene vapor and elimination in breath after inhalation. *Am. Ind. Hyg. Assoc. J.* 37:145-150.

11. **Lam, C-W., T.J. Galen, J.F. Boyd, and D.L. Pierson, D.L.** 1990. Mechanism of transport and distribution of organic solvents in blood. *Toxicol. Appl. Pharmacol.* 104:117-129.

12. **Buben, J.A. and E.J. O'Flaherty.** 1985. Delineation of the role of metabolism in the hepatotoxicity of trichloroethylene and perchloroethylene: A dose-effect study. *Toxicol. Appl. Pharmacol.* 78:105-122.

13. **United States Environmental Protection Agency (EPA).** 1985. *Health Assessment Document for Tetrachloroethylene (Perchloroethylene): Final Report.* EPA/600/8-82/005f, PB85-249704. Washington, DC: Office of Health and Environmental Agency.

14. **Chen, C.W. and J.N. Blancato.** 1987. Role of pharmacokinetic modeling in risk assessment: Perchloroethylene as an example. In: *Pharmacokinetics in Risk Assessment, Drinking Water, and Health 8.* Washington, DC: National Academy Press.

15. **Pegg, D.G., J.A. Zempel, W.H. Brown, and P.G. Watanabe.** 1979. Deposition of tetrachloro(^{14}C)ethylene following oral and inhalation exposure in rats. *Toxicol. Appl. Pharmacol.* 51:465-474.

16. **Schumann, A.M., T.F. Quast, and P.G. Watanabe.** 1980. The pharamacokinetics of perchloroethylene in mice and rats as related to oncogenicity. *Toxicol. Appl. Pharmacol.* 55:207-219.

ACKNOWLEDGMENTS

This research was conducted under Department of the Air Force Contract No. F33615-90C-0532. The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institue of Laboratory Animals Resources, National Research Council, Department of Health and Human Services, National Insitute of Health Publication No. 86-23, 1985; and the Animal Welfare Act of 1966, as amended.

A PHARMACOKINETIC MODEL FOR CHROMIUM

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SUMMARY

Reduction of hexavalent chromium (Cr(VI)) to trivalent chromium (Cr(III)) and differential kinetics of Cr(III) and Cr(VI) are important determinants of the disposition and toxicity of chromium. A physiologically based model of chromium disposition in the rat has been developed. The model takes into account different absorption and reduction rates in the lung and gastrointestinal tract; different efficiencies of transfer of Cr(III) and Cr(VI) into tissues including erythrocytes, where Cr(VI) is reduced to Cr(III) and retained for an extended period of time; uptake and storage in bone; and reabsorption of chromium from the gastrointestinal tract. The model is shown to be capable of generating the observed distributions of chromium between plasma and erythrocytes in rats given Cr(VI) intragastrically, intraduodenally, or intratracheally.

This work was supported by Contract No F33615-85-C-0532 from the U.S. Air Force.

A PHARMACOKINETIC MODEL FOR CHROMIUM

INTRODUCTION

Chromium is an essential metal. Reduction in intake of chromium below a critical level consistently leads to biochemical or physiological functional deficits, and supplementation consistently reverses these deficits. The signs of chromium deficiency are similar in experimental animals and humans. They include impairment of glucose tolerance and elevation of serum cholesterol. Chromium appears to act by potentiating the action of insulin. In laboratory rats fed a low-chromium diet, impairment of glucose tolerance is promptly reversed by chromium supplementation. In humans, chromium deficiency has been found in association with malnourishment and with total parenteral alimentation. Chromium supplementation sometimes, but not always, improves glucose tolerance in diabetic subjects, presumably partly because not all subjects were chromium-deficient to begin with. However, in documented instances of chromium deficiency associated with impaired glucose and lipid metabolism, chromium supplementation has normalized the functional deficits. These functions of chromium are due to trivalent chromium (Cr(III)) as a constituent of a low-molecular-weight complex called the Glucose Tolerance Factor.

Trivalent chromium is not known to have systemic toxic effects. Compounds of both Cr(III) and hexavalent chromium, Cr(VI), cause allergic contact dermatitis, and Cr(VI) is locally irritating and corrosive [1]. Historically, industrial exposure to Cr(VI) by inhalation was commonly associated with nasal septal ulceration and perforation, although these effects are rarely seen today. The corrosive action of Cr(VI) is associated with oxidative effects linked with its reduction to Cr(III). In all biological systems and in most environmental settings, Cr(VI) is rapidly reduced to Cr(III) by a variety of enzymatic and nonenzymatic mechanisms [2]. The ubiquity and rapidity of this reduction, and the multiplicity of mechanisms that can mediate it, complicate efforts to model chromium kinetics.

In the acid environment of the stomach, Cr(VI) is rapidly reduced, apparently largely by nonenzymatic mechanisms. In other biological settings including the lung, the reduction appears to be principally an enzyme-mediated process. Among several intermediates that may be generated in the course of reduction of Cr(VI) to Cr(III) are reactive oxygen species and pentavalent chromium (Cr(V)) [3]. Reviews of a large number of epidemiological studies of cohorts of workers employed in a variety of industries using chromium compounds have led to the conclusion that workers exposed to Cr(VI) as chromates, dichromates, or chromic trioxide are at risk for development of lung cancer [4]. It is believed that one or more of the reactive intermediates generated in the reduction process are responsible for the carcinogenicity of Cr(VI).

Whether Cr(VI) is systemically absorbed from either the lung or gastrointestinal tract and penetrates to peripheral tissues before its reduction to Cr(III) is not known. There is evidence that when a soluble salt of Cr(VI) is inhaled, a larger fraction of the dose reaches the systemic circulation as Cr(VI) than when it is ingested. Hexavalent chromium attaches strongly to the erythrocyte, and persists in this association for a substantial fraction of the cell's life span. (Chromium is not a satisfactory marker for the life span of the erythrocyte, as is sometimes stated.) It is believed that this prolonged association is due to the relative inability of Cr(III) to cross cell membranes. Chromium readily enters the erythrocyte as Cr(VI), is reduced to Cr(III), and is subsequently slowly lost from the cell as Cr(III). Therefore, the relative amount of chromium in association with the erythrocyte fraction of the blood serves as an index of the amount of chromium that was absorbed as Cr(VI). Comparison of the plasma:erythrocyte chromium concentration ratio after administration of Cr(VI) into the stomach, into the intestine (from which it is presumably absorbed unreduced to a greater extent), and into the lung (Table 31) shows that a much larger fraction of the chromium in the blood is associated with the erythrocyte after intratracheal exposure than after oral exposure. It can be inferred that a significant fraction of inhaled Cr(VI) is also absorbed unreduced from the lung.

TABLE 31. PLASMA:ERYTHROCYTE CHROMIUM AFTER ADMINISTRATION OF Cr(VI)

Administration	Time post-administration	Plasma: RBC concentration ratio	Reference
Stomach tube	4 h	8.8:1	MacKenzie et al. [11]
Intestinal injection	4 h	3.2:1	MacKenzie et al. [11]
Intratracheal instillation	24 h	1.2:1	Edel and Sabbioni [16]
Intratracheal instillation	4 h	1.2:1	Weber [13]
Intratracheal instillation	24 h	0.6:1	Weber [13]

Differential solubility of chromium compounds is also critical to chromium kinetics. Both Cr(VI) and Cr(III) form compounds that range from highly water soluble to those that are essentially insoluble in water. This range of solubility behaviors has complicated efforts to evaluate chromium toxicity. Solubility is particularly important with respect to the pulmonary carcinogenicity of Cr(VI). Relatively soluble compounds may generate higher concentrations of available chromium in the lung than relatively insoluble compounds, but they are also more rapidly cleared. Evidence from experimental animal studies and from epidemiological studies of workers in chromium-related industries supports the hypothesis that the moderately soluble chromium salts are the most potent respiratory carcinogens, whereas the highly soluble salts are much less active [5,6]. Presumably, the less-soluble salts serve as persistent sources of low concentrations of locally available chromium [1].

A third factor important to chromium kinetics was identified above. This is the much greater ability of Cr(VI) than of Cr(III) to penetrate membranes. Hexavalent chromium is readily absorbed, whereas Cr(III) is poorly absorbed from both the gastrointestinal tract and the lung [7]. This relative inability to reach the intracellular milieu or even to reach the systemic circulation has contributed to the general impression that systemic Cr(III) toxicity is unlikely to be of concern. From this point of view, the reduction of Cr(VI) to Cr(III) is a detoxification mechanism. The rapidity and essentially universal distribution of chromium reduction processes throughout the body have suggested to some the existence of a threshold in Cr(VI) dose–response relationships [2]. However, it is now known that Cr(III) can cross cell and other membranes when the ligand environment is favorable. Penetration of Cr(III) into tissues was at one time suggested to be due to trapping of colloidal chromium by the reticuloendothelial system [8], but it is now believed that excretion of Cr(III) in bile and its appearance in erythrocytes and tissues are facilitated by the formation of diffusible complexes [9]. Thus, solubility, reduction of Cr(VI) to Cr(III), and differential kinetics of Cr(III) and Cr(VI) are all important factors determining the kinetic behavior of chromium.

The purpose of this paper is to review the structure and to present partial validation of a physiologically based model of chromium kinetics in rats. The model consists of parallel subunits for Cr(III) and Cr(VI) kinetics, linked by reduction in all tissues except bone, but it does not take into consideration any interaction between solubility of chromium salts and absorption or reduction processes.

CHROMIUM KINETICS

Absorption. Trivalent chromium and Cr(VI) are absorbed to different extents from soluble salts in the gastrointestinal tract of rats. By comparison of concentrations of ^{51}C in the blood 4 to 10 days after intravenous or oral administration of a soluble ^{51}C(III) salt, Mertz et al. [10] judged that 2 to 3% of the oral dose had been absorbed. MacKenzie et al. [11] monitored radiolabeled chromium in blood, tissues, urine, and feces of adult rats given a soluble ^{51}C(III) or ^{51}C(VI) salt by stomach tube. Comparison of average levels of radiolabeled chromium in blood, erythrocytes, and plasma demonstrated that Cr(III) was absorbed only 1/10 or less as well as Cr(VI).

Uptake and clearance from the lung are determined by many factors. The range of half-lives reported for clearance of chromium from the lung [12, 13] suggests that chromium is present in the lung in different states or compartments from which it is cleared at different rates. That lung-to-gastrointestinal tract transfer is important was shown by Langard et al. [14], who observed rapid increases in fecal chromium during 6 h inhalation exposures of rats to dusts of a moderately soluble Cr(VI) salt. Bragt and van Dura [12] showed that the amount of chromium appearing in the feces increased sharply as the solubility of the Cr(VI) salt decreased.

Distribution. In a variety of distribution studies, slow loss of chromium from kidney, spleen, testis, bone marrow, and to a lesser extent liver have consistently been noted [15, 11, 12, 16]. This general distribution gave rise to the early suggestion that chromium is trapped by the reticuloendothelial system [8]. Whatever the mechanism for the delay, which may simply reflect the poor mobility of Cr(III), it is clear that clearance of chromium even from well-perfused tissues can be slow.

Chromium also accumulates in the bone, although not to the same extent as other bone-seeking elements such as lead. Hopkins [15] observed that the bone of young, growing rats tended to concentrate chromium with time (7% increasing to 12% of the dose) after an intravenous injection of a soluble ^{51}C(III) salt, whereas the bone of mature rats acquired less chromium initially (4% of the dose) and the amount acquired did not increase with time. It may be inferred that rapid surface exchange of blood and bone chromium accounted for approximately 4% of the dose whereas the additional incorporation of chromium into the bone of the young rats was due to deposition with

mineralizing new bone. Slow loss of chromium from bone is probably the primary determinant of its biological half-life.

Excretion. Approximately 21 to 22% of an intravenous dose of a soluble salt of either Cr(III) or Cr(VI) is excreted in the urine in the 24 h following administration, irrespective of oxidation state. Not all chromium is excreted in the urine. Studies by Cikrt and Bencko [17] and by Cavalleri et al. [18] have demonstrated that 2 to 3.5% of an intravenous dose of Cr(VI) is excreted in the bile, whereas less than 1% of an intravenous dose of Cr(III) is excreted in the bile. Larger fractions are excreted in the feces, demonstrating that there is significant transfer of chromium from the intestine to the intestinal tract contents. If the gastrointestinal and biliary excretion data of Cikrt and Bencko [17] are combined with the urinary excretion data of MacKenzie et al. [11] to estimate fractional absorption from the gastrointestinal tract, the calculation suggests approximately 17% absorption of soluble Cr(VI) and 6 to 7% absorption of soluble Cr(III) by fasted rats.

GENERAL MODEL OUTLINE

The basis of the chromium model is a general model of rat body and bone growth from birth to maturity, which was developed as the foundation of a physiologically based model of lead kinetics [19,20]. The chromium model (Figure 36) consists of liver, kidney, gastrointestinal tract, lung, bone, other well-perfused tissues, and other poorly perfused tissues. In accordance with the observations, discussed above, on localization and rates of uptake of chromium in bone of juvenile and mature rats, mechanisms of chromium uptake into bone are considered to be rapid surface exchange with calcium and incorporation into forming bone.

Exchange of Cr(VI) between blood and tissues other than bone is assumed to be flow-limited; that is, to be more rapid than the rate of blood flow through the tissues. Partition coefficients are not used for Cr(VI). There is no evidence that Cr(VI) is selectively bound or sequestered in tissues in quantities sufficiently great to have an impact on overall kinetic behavior.

Exchange of Cr(III) between blood and tissues is assumed to be diffusion-limited. This assumption is consistent with the poor mobility of Cr(III), as well as with kinetic observations [10] showing that intravenously administered Cr(III) persists for an extended period even in well-perfused tissues.

Reduction of Cr(VI) to Cr(III) is presumed to take place in all tissues with the exception of bone, as well as in the gastrointestinal tract and lung. Reduction is modeled as a first-order process with a rate constant whose magnitude is dependent on the tissue or fluid in which reduction is taking place. No binding of chromium to plasma or tissue proteins is considered. Such binding has been neither qualitatively nor quantitatively characterized, and there is no evidence to suggest that it is rate-limiting in general. Slower return from certain tissues, such as the kidney, than from other well-perfused tissues is handled by assigning a smaller clearance value to these tissues.

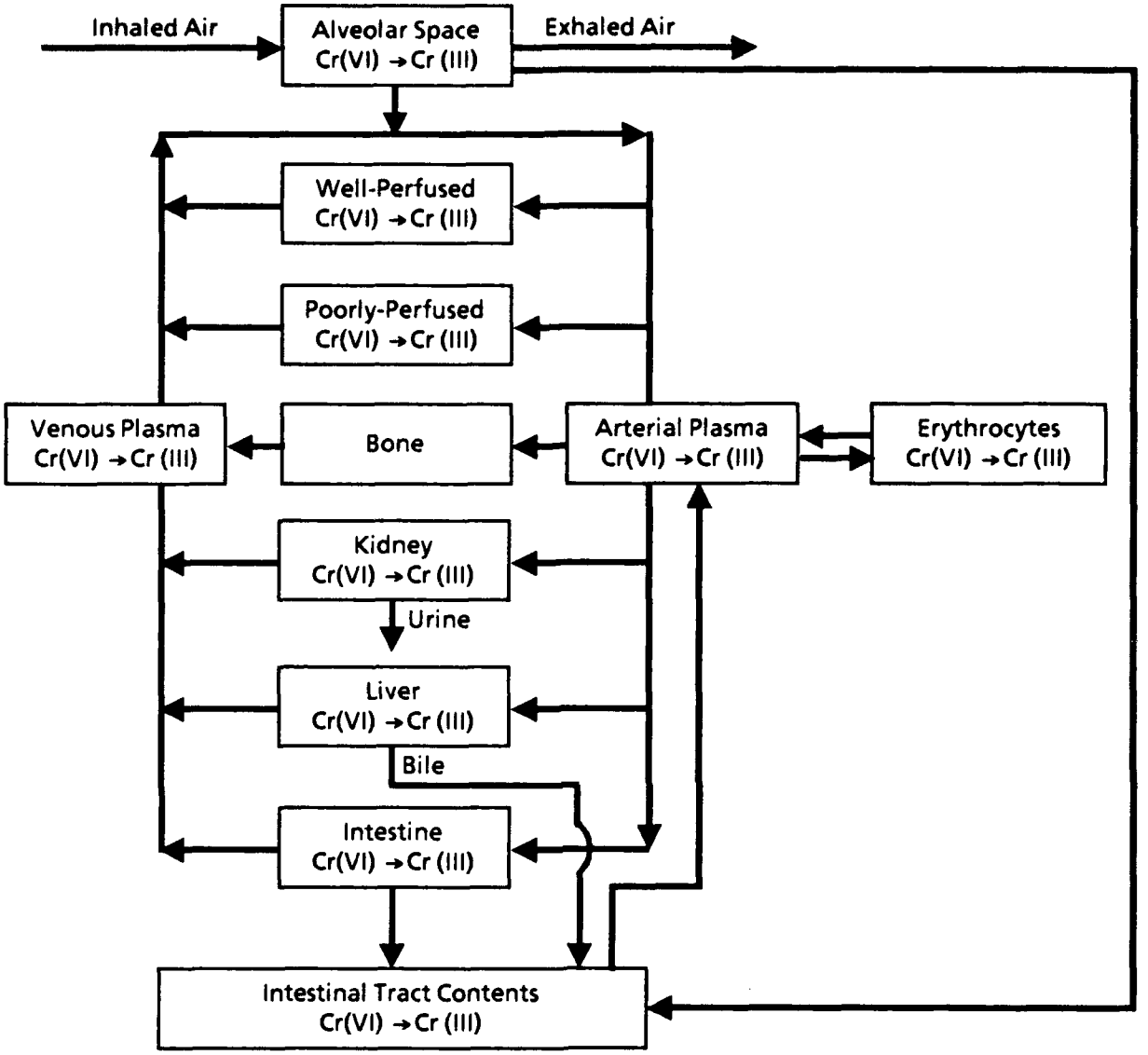

Figure 36. Schematic Diagram of Chromium Model.

Absorption of chromium from the gastrointestinal tract is modeled into the liver via the portal vein. Absorption from the lung is modeled directly into arterial blood. Absorption from the lung, whose rate is oxidation state-specific, competes in the model with mucociliary clearance from the lung to the gastrointestinal tract contents, a process whose rate is not oxidation state-specific. Chromium entering the gastrointestinal tract contents from the lung is subject to reduction and/or absorption as appropriate to its oxidation state.

Excretion of both Cr(III) and Cr(VI) from the body is modeled as first-order loss from the blood into urine, bile, and intestinal tract contents. Reabsorption of chromium from the intestinal contents (enterohepatic recirculation) is included. Figure 36 is a schematic diagram of the model.

PERFORMANCE OF THE MODEL

Chromium(III) and Cr(VI) have been administered to rats by most of the conventional routes of administration (Table 32). The exceptions are intravenous administration of Cr(VI), which would cause localized toxicity, and administration of Cr(III) by inhalation, perhaps because of the expectation that little would reach the systemic circulation. In developing the model, the published data sets were considered in order from the kinetically simplest (intravenous administration of Cr(III)) to the kinetically most complex (inhalation of Cr(VI)). As the model was expanded to accommodate greater kinetic complexity and new parameters were added, the values of these parameters were assigned by visual optimization of model simulations to the new data sets. For example, an initial value for whole-body clearance was estimated from the second term of the three-term sum-of-exponential fit to the chromium body burden data of Mertz et al. [10] as follows:

$$t_{1/2} = 5.9 \text{ days}$$

$$\text{Clearance volume} = (0.8)(\text{Body weight})$$

$$\text{For a 250-g rat,}$$

$$\text{Clearance} = (.250 \text{ L})(0.8)/5.9 \text{ days}$$

$$= 0.034 \text{ L/day}$$

Scaling in accordance with the 0.75 power of body weight, whole-body clearance of Cr(III) would be 0.096 L/day/kg. This figure was used as the initial value of the clearance of Cr(III) from the plasma. This parameter value and the value of fractional uptake of chromium into forming bone were set by visually optimizing the model to the data of Mertz et al. [10] for whole-body loss of Cr(III) from a single intravenous injection. Model predictions were compared to the data of Hopkins [15] to obtain rough estimates of Cr(III) clearances between blood and soft tissues and of the rapid exchange of Cr(III) at bone surfaces. Values of the Cr(III) distribution parameters were adjusted by comparison of predicted with observed tissue concentrations at 4 and 42 days after a single dose [21]. Because these tissue distribution data are so limited and because of concern that the behavior of Cr(III) salts injected intravenously may not be typical of the kinetic behavior of chromium salts administered by other routes, the estimated values of the soft-tissue distribution clearances were further adjusted throughout the models development process as suggested by fits of model predictions to other data sets.

TABLE 32. CHROMIUM KINETIC STUDIES IN RATS

Oxidation State	Administration	References
Cr(III)	Intravenous	Mertz et al. [10] Hopkins [15]
Cr(VI)	Intravenous	none
Cr(III)	Stomach tube*	MacKenzie et al. [11]
Cr(VI)	Stomach tube*	MacKenzie et al. [11]
Cr(III)	Drinking water; chronic	MacKenzie et al. [22]
Cr(VI)	Drinking water; chronic	MacKenzie et al. [22]
Cr(III)	Intratracheal	Edel and Sabbioni [16]
Cr(VI)	Intratracheal	Weber [13] Bragt and van Dura [12] Edel and Sabbioni [16]
Cr(III)	Inhalation	none
Cr(VI)	Inhalation	Langard et al. [14]

*Plasma:RBC ratio only

The model was opened up to Cr(VI) at this stage by the addition of a first-order reduction of Cr(VI) to Cr(II) in all tissues except bone. The first-order rate constant for this process was assigned an initial value of 250 $days^{-1}$ based on the reduction rate in rat blood *in vivo* reported by Cavalleri et al. [18] This value was adjusted downward to bring Cr(VI) absorption and distribution into line with the observations of Cikrt and Bencko [17] and Edel and Sabbioni [16]. The next step was to expand the model to accommodate uptake from the gastrointestinal tract. The first-order rate constants for absorption of Cr(III) and Cr(VI) were initially set equal to the fraction of a single oral dose absorbed [11, 17] on the simplifying assumption that a single dose passes by the absorbtion sites in the intestine within a day. They were adjusted in order to reconcile model predictions with the data of MacKenzie et al. [11].

In this way, the model was developed by adding levels of complexity while leaving the previously optimized parameter values unchanged unless there was reason to doubt their approximate correctness. Initial value estimates were often taken from experimental studies of the particular process (as with the initial estimates of absorption from the gastrointestinal tract), and

optimized by fitting the model simulations to the appropriate data set(s) from the list in Table 2. Three examples of model fits to different data sets will be given.

Figure 37 shows the fit to the data of Mertz et al. [10]. In this study, $^{51}C(III)$ was administered intravenously to male Sprague-Dawley rats, and whole-body retention was monitored for 6 weeks thereafter. Mertz and his coworkers fit the retention curve with a sum of three exponential terms. The half-life of the second of these terms was used as the initial value of the half-life of elimination in fitting the physiologically based model to the data. The third exponential term is related to loss of chromium from bone and its subsequent loss from the body.

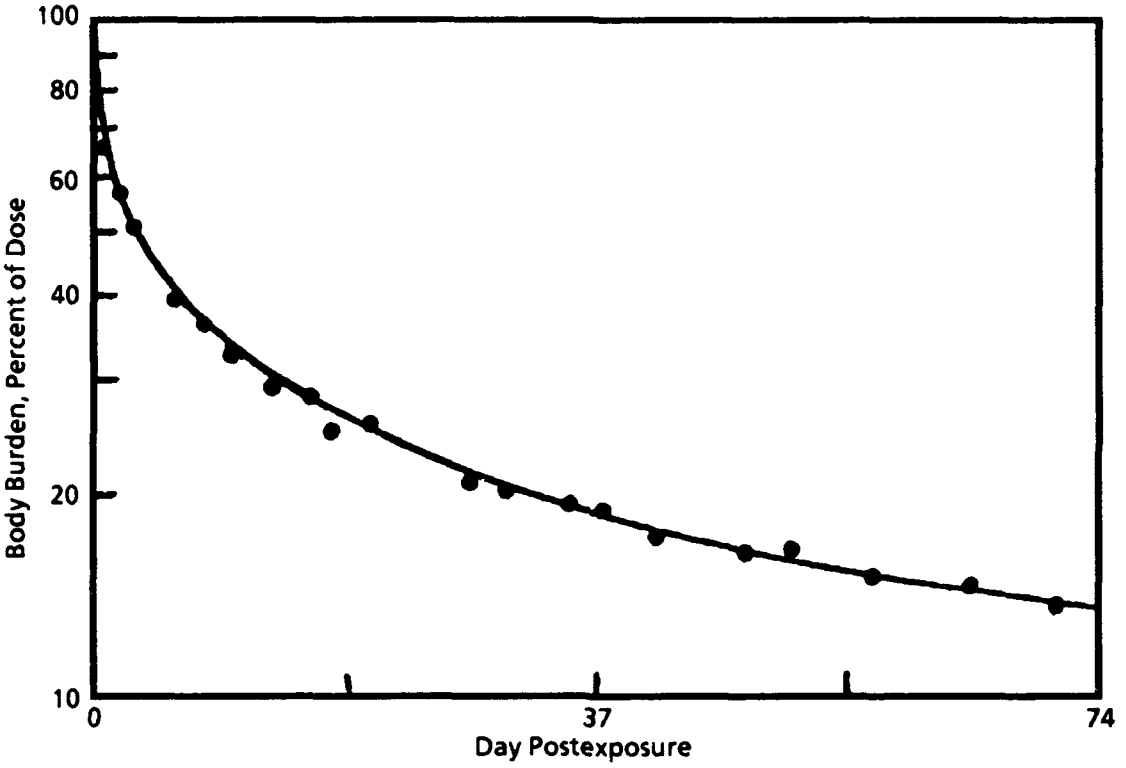

Figure 37. Whole-Body Retention, as Percent of Dose, of a Soluble Cr(III) Salt Administered Intravenously to Rats. Data points are read from Figure 1 of Mertz et al. [10]. The curve is the simulation.

Although the physiologically based model can readily be tuned to fit these data well, as Figure 37 illustrates, heavy emphasis should not be placed on this data set. Table 33 shows that the kinetics of Cr(III), administered intravenously to rats, are strongly dependent on the chemical milieu even of a soluble chromium salt. It would not be surprising if tissue distribution and persistence of Cr(III) in tissues were to be found to be quite different when the soluble Cr(III) salt is given intragastrically or intratracheally.

TABLE 33. CHROMIUM IN TISSUES AND EXCRETA OF RATS 4 DAYS AFTER INTRAVENOUS ADMINISTRATION OF C(III) IN DIFFERENT CHEMICAL FORMS

	Chemical Form		
	$CrCl_3$	$CrCl_3$/acetate	$CrCl_3$/citrate
Excreted in urine, %	15	56	75
Excreted in feces, %	20	8	17
Liver, %/g	5.5	0.16	0.31
Kidneys, %/g	0.44	0.62	0.13
Bone (tibia epiphysis), %/g	1.1	1.0	0.20
Blood, %/g	0.05	0.03	<0.02

Visek et al. [21]

MacKenzie et al. [22] administered Cr(VI) as a soluble salt in the drinking water of Sprague-Dawley rats at five concentrations up to 25 ppm for 1 year. To accommodate these data, the model was expanded to include reduction of Cr(VI) to Cr(III) as shown in the schematic diagram in Figure 36, as well as absorption of Cr(III) and Cr(VI) from the gastrointestinal tract and elimination of Cr(VI) as well as Cr(III). The predictions of the model agree reasonably well with the kidney concentration data, as illustrated in Figure 38. Some dose-dependence is suggested by the curvilinearity of the relationship of kidney concentration to dose rate. This dose-dependence is made very clear by the marked curvilinearity of the liver concentration data in Figure 39. As Figure 39 shows, the predictions of the model agree well with the liver concentration data only up to about 0.3 mg/L. Above the dose level associated with this liver concentration of chromium, there is a systematic curvature of the dose-concentration relationship that is not suggested by the observations of other investigators after single exposures to Cr(III) or Cr(VI) by any route of administration, and that is not at this time accounted for in the model. It is interesting that the simulated relationship of kidney concentration to dose rate is a better representation of the experimental curve than is the simulated relationship of liver concentration to dose rate. It appears that a dose-related increase in chromium retention in the liver during chronic exposure invalidates the values of the liver distribution parameters that were determined on the basis of single exposures.

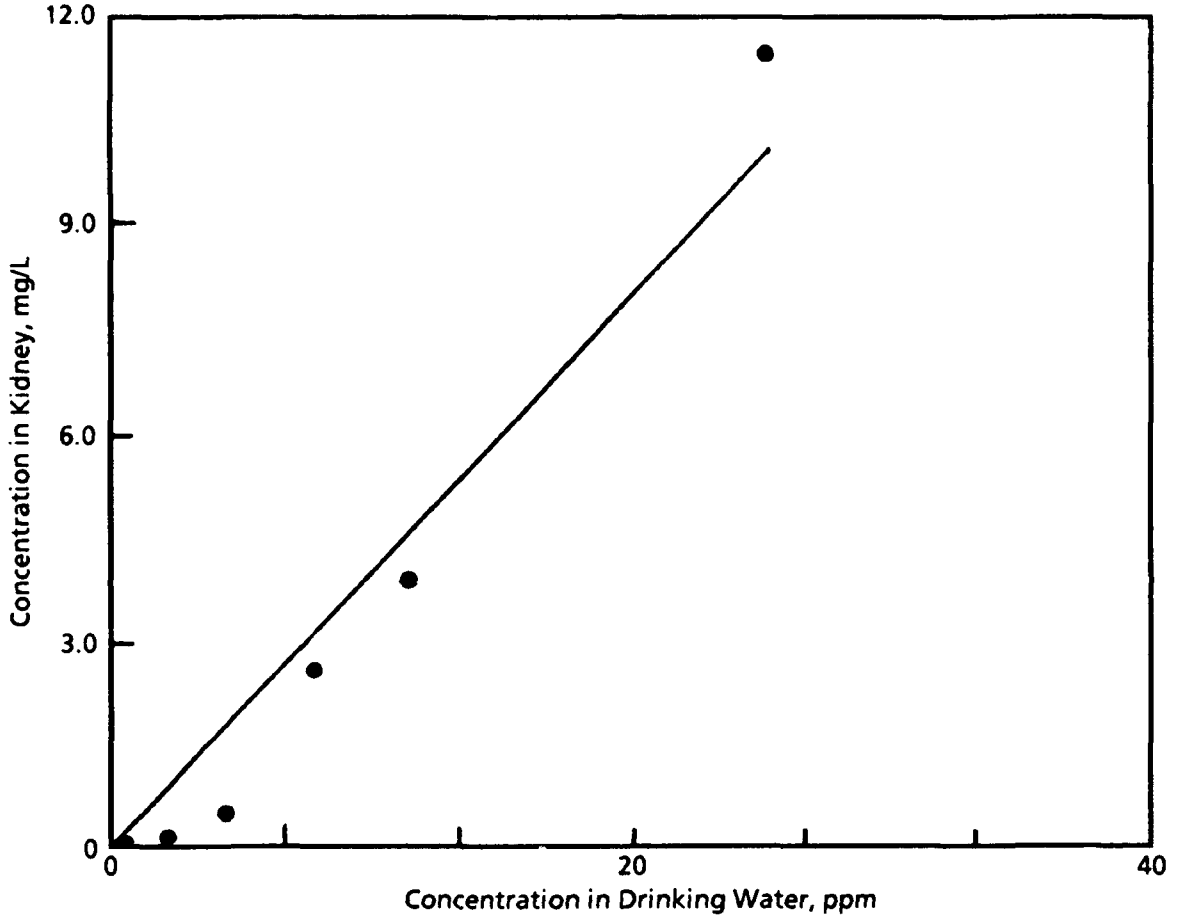

Figure 38. Amount of Chromium in Kidneys of Rats Given a Soluble Cr(VI) Salt in Their Drinking Water for 1 Year. Data points are taken from MacKenzie et al. [22]. The line is the simulation.

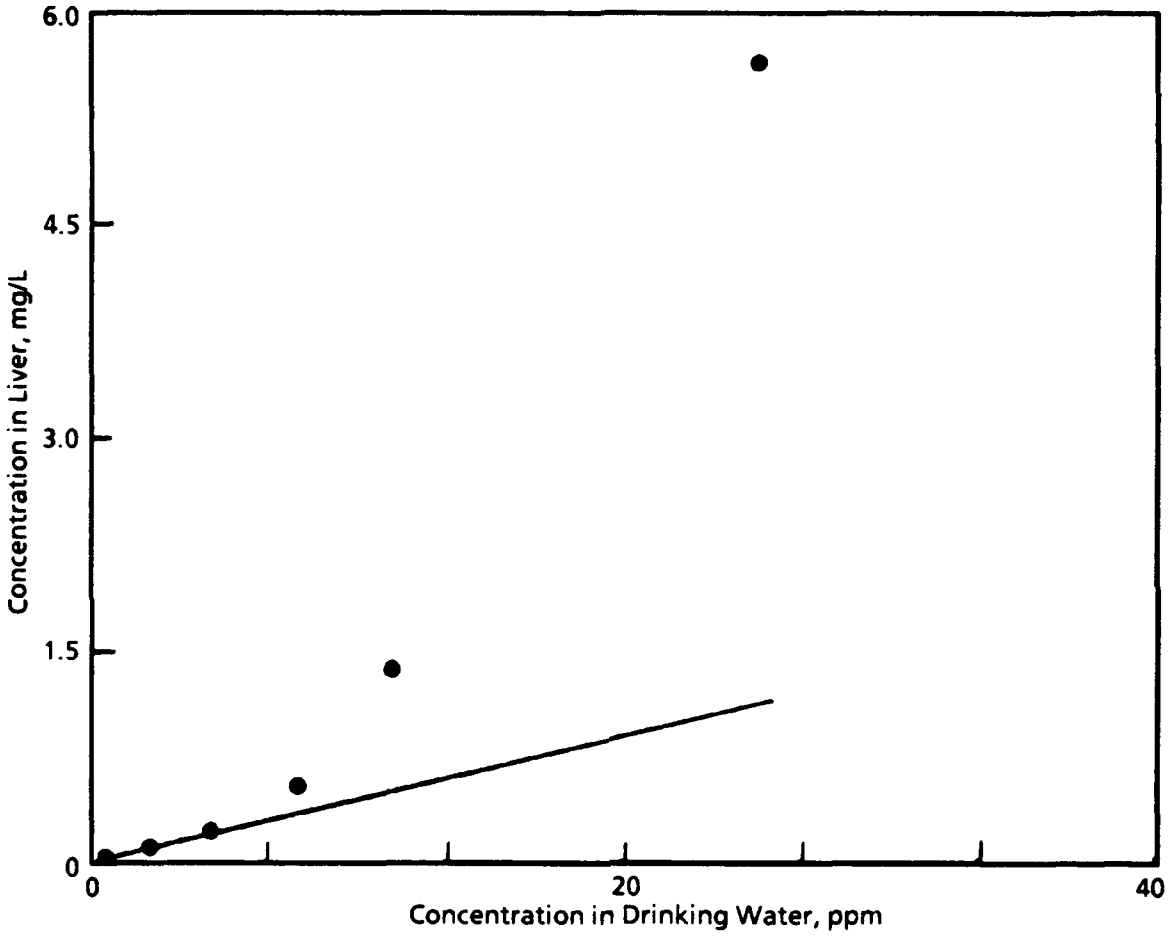

Figure 39. Amount of Chromium in Liver of Rats Given a Soluble Cr(VI) Salt in Their Drinking Water for 1 Year. Data points are taken from MacKenzie et al. [22]. The line is the simulation.

The fits of the model to liver and kidney data from a study in which Cr(VI) was administered intratracheally to male Sprague-Dawley rats [13] are shown in Figures 40 and 41. Although the kidney data are fit quite well (Figure 40), the liver data display a much more rapid fall during the first week following exposure than the model accommodates. Figure 42 shows that the liver is probably more representative of other tissues than the kidney, because whole-body chromium also is lost more rapidly during the first week after exposure than the model simulation would have suggested. Other measurements made in this study suggest that the discrepancy is due to failure to simulate an initial, brief, and very rapid loss from the systemic circulation into the intestinal tract contents and the feces. The model can be refined to account for this loss.

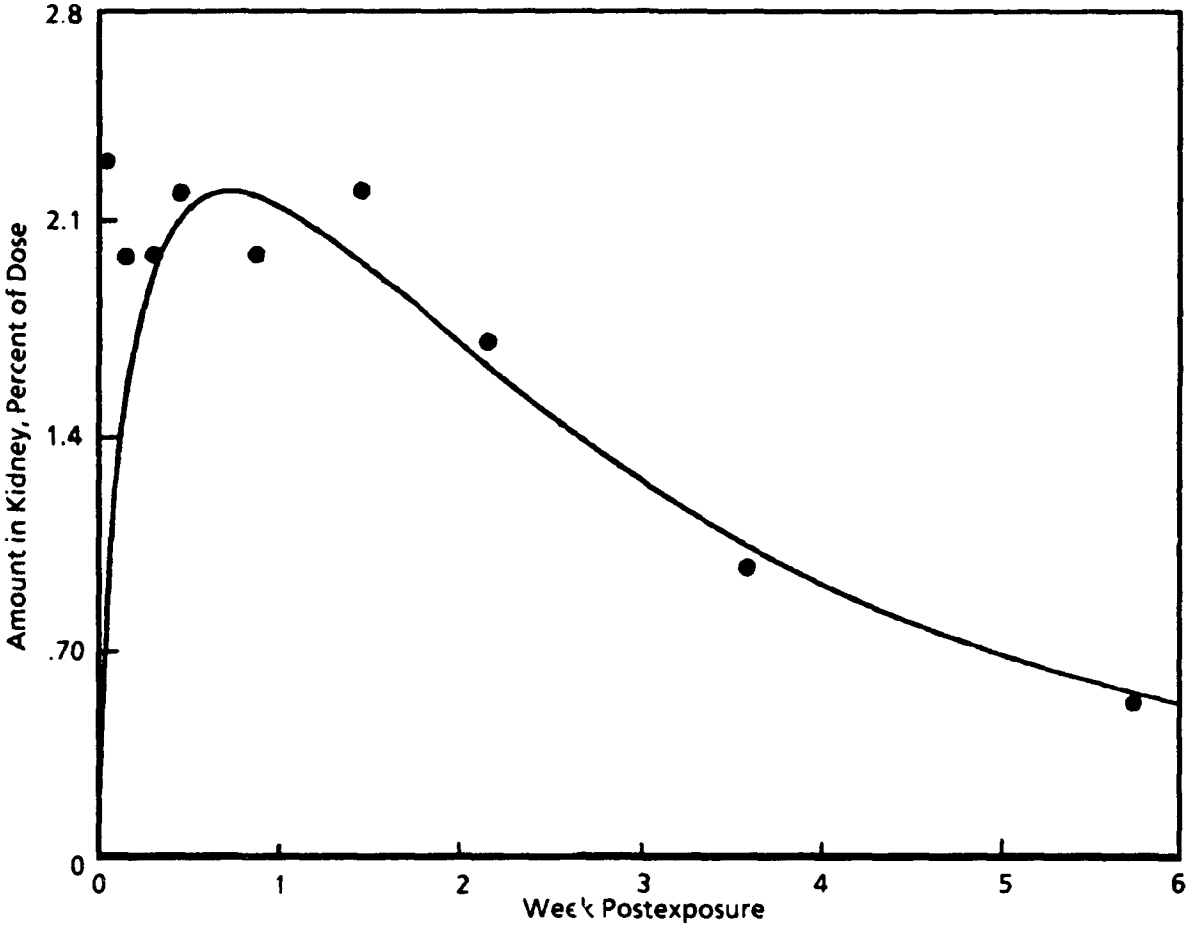

Figure 40. Amount of Chromium, as Percent of Dose, in Kidneys of Rats Following Intratracheal Administration of a Soluble Cr(VI) Salt. Data points are taken from Weber [13]. The curve is the simulation.

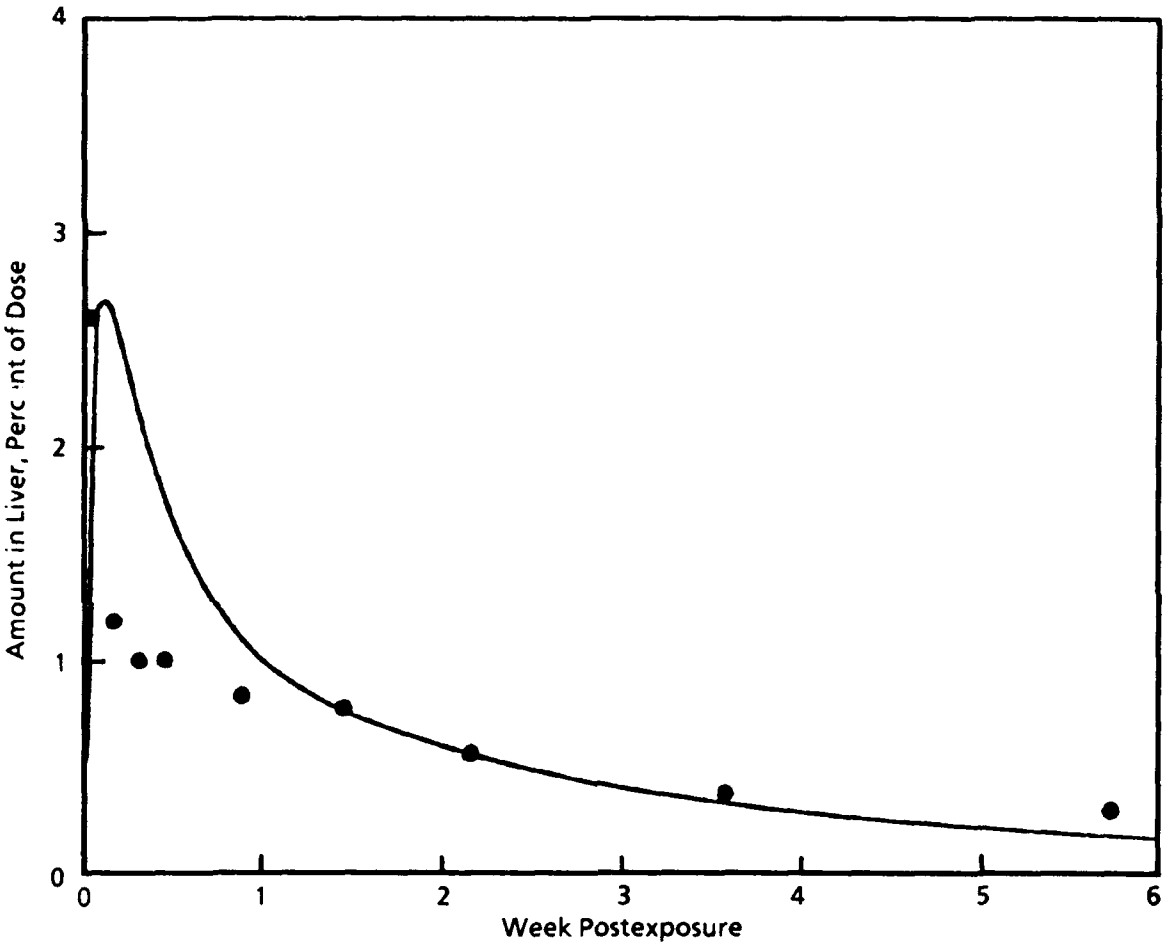

Figure 41. Amount of Chromium, as Percent of Dose, in Liver of Rats Following Intratracheal Administration of a Soluble Cr(VI) Salt. Data points are taken from Weber [13]. The curve is the simulation.

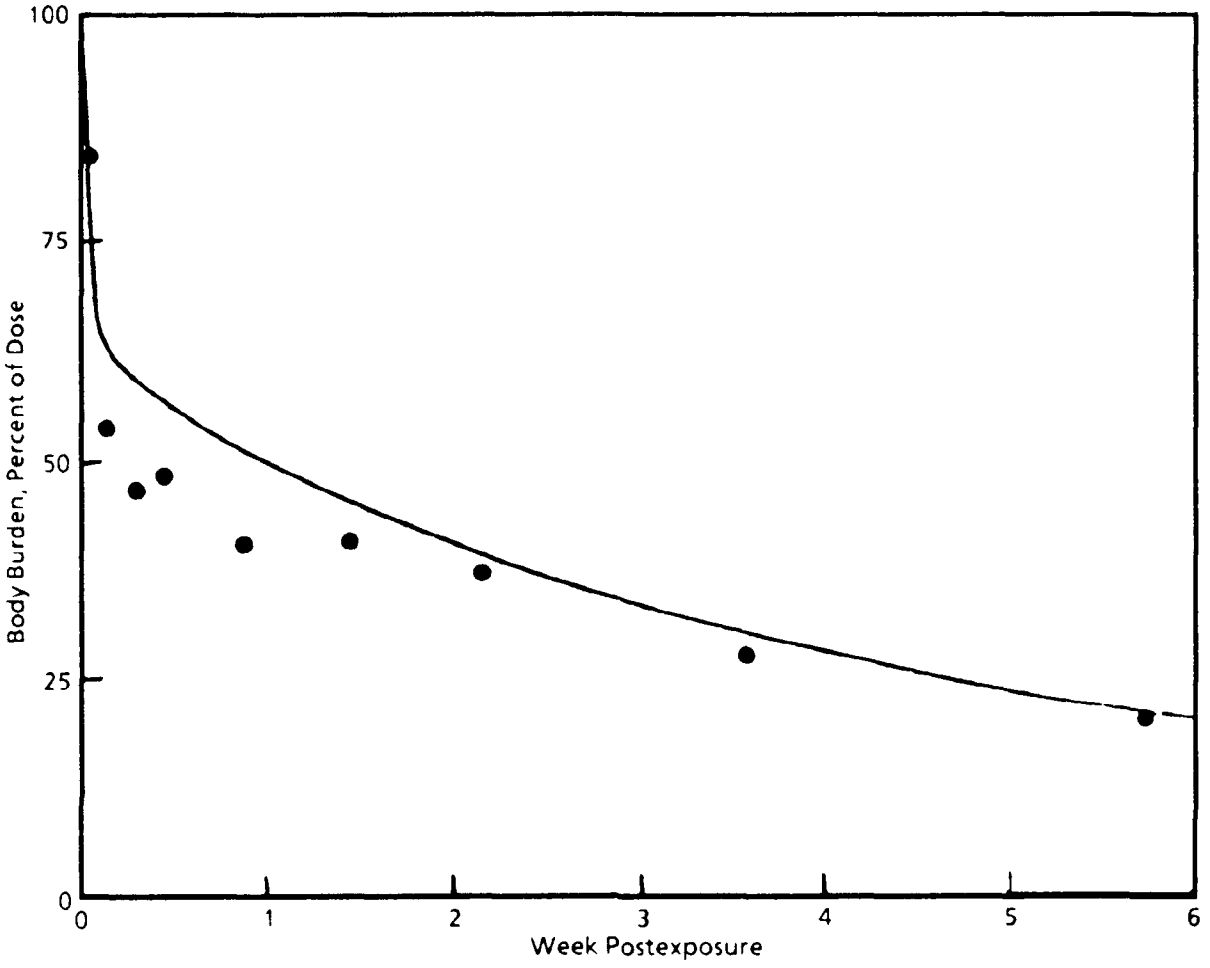

Figure 42. Body Burden of Chromium, as Percent of Dose, in Rats Following Intratracheal Administration of a Soluble Cr(VI) Salt. Data points are taken from Weber [13]. The curve is the simulation.

Finally, it is of interest to determine what the model predicts to be the fraction of a single dose of Cr(VI) that would be absorbed unreduced from the lung and from the gastrointestinal tract. The model in its current form predicts that if Cr(VI) is given intragastrically, a few percent at most will be absorbed as Cr(VI); whereas if Cr(VI) is given intratracheally, as much as 50% will be absorbed as Cr(VI). As remarkably different as they are, these predictions are consistent with the plasma:erythrocyte ratios given in Table 31. The difference between the two predicted absorptions is so great that it is unlikely that they will be greatly altered by further refinement of the physiologically based model.

The kinetic behavior of chromium, while it is influenced by solubility and oxidation state, is consistent with our understanding of the physiological factors that determine absorption and disposition. The several studies by different investigators in which chromium was administered to

rats by different routes appear also to be reasonably consistent with each other and with a coherent, single, physiologically based model of chromium kinetics. This model, when refined and more fully validated, should be capable of illuminating some of the issues related to chromium kinetics and to human chromium exposure.

REFERENCES

1. **Gad, S.C.** 1989. Acute and chronic systemic chromium toxicity. *Sci. Total Environ.* 86:149-157.

2. **Petrilli, L. and S. DeFlora.** 1988. Metabolic reduction of chromium as a threshold mechanism limiting its in vivo activity. *Sci. Total Environ.* 71:357-364.

3. **DeFlora, S., D. Serra, C. Basso, and P. Zanacchi.** 1989. Mechanistic aspects of chromium carcinogenicity. *Arch. Toxicol.* Suppl. 13:28-39.

4. **Langård, S.** 1990. One hundred years of chromium and cancer: A review of epidemiological evidence and selected case reports. *Am. J. Industr. Med.* 17:189-215.

5. **Hathaway, J.A.** 1989. Role of epidemiologic studies in evaluating the carcinogenicity of chromium compounds. *Sci. Total Environ.* 86:169-179.

6. **Gibb, H.J. and C. Chen.** 1989. Evaluation of issues relating to the carcinogen risk assessment of chromium. *Sci. Total Environ.* 86:181-186.

7. **Mertz, W.** 1969. Chromium occurrence and function in biological systems. *Physiol. Rev.* 49:165-239.

8. **Kraintz, L. and R.V. Talmage.** 1952. Distribution of radioactivity following intravenous administration of trivalent chromium 51 in the rat and rabbit. *Proc. Soc. Exp. Biol. Med.* 81:490-492.

9. **Bianchi, V. and A.G. Levis.** 1988. Review of genetic effects and mechanisms of action of chromium compounds. *Sci. Total Environ.* 71:351-355.

10. **Mertz, W., E.E. Roginski, and R.C. Reba.** 1965. Biological activity and fate of trace quantities of intravenous chromium (III) in the rat. *Am. J. Physiol.* 209:489-494.

11. **MacKenzie, R.D., R.A. Anwar, R.V. Byerrum, and C.A. Hoppert.** 1959. Absorption and distribution of ^{51}C in the albino rat. *Arch. Biochem. Biophys.* 79:200-205.

12. **Bragt, P.C. and E.A. van Dura.** 1983. Toxicokinetics of hexavalent chromium in the rat after intratracheal administration of chromates of different solubilities. *Ann. Occup. Hyg.* 27:315-322.

13. **Weber, H.** 1983. Long-term study of the distribution of soluble chromate-51 in the rat after a single intratracheal administration. *Toxicol. Environ. Health* 11:749-764.

14. **Langård, S., N. Gundersen, D.L. Tsalev, and B. Glyseth.** 1978. While blood chromium level and chromium excretion in the rat after zinc chromate inhalation. *Acta Pharmacol. Toxicol.* 42:142-149.

15. **Hopkins, L.L., Jr.** 1965. Distribution in the rat of physiological amounts of injected Cr^{51}(III) with time. *Am. J. Physiol.* 209:731-735.

16. **Edel, J. and E. Sabbioni.** 1985. Pathways of Cr(III) and Cr(VI) in the rat after intratracheal administration. *Human Toxicol.* 4:409-416.

17. **Cikrt, M. and V. Bencko.** 1979. Biliary excretion and distribution of ^{51}Cr(III) and ^{51}Cr(VI) in rats. *J. Hyg. Epidemiol. Microbiol. Immunol.* 23:241-246.

18. **Cavalleri, A., C. Minoia, P. Richelmi, C. Baldi, and G. Micoli.** 1985. Determination of total and hexavalent chromium in bile after intravenous administration of potassium dichromate in rats. *Environ. Res.* 37:490-496.

19. **O'Flaherty, E.J.** 1991a. Physiologically based models for bone-seeking elements. I. Rat skeletal and bone growth. *Toxicol. Appl. Pharmacol.* 111:299-312.

20. **O'Flaherty, E.J.** 1991b. Physiologically based models for bone-seeking elements. II. Kinetics of lead disposition in rats. *Toxicol. Appl. Pharmacol.* 111:313-331.

21. **Visek, W.J., I.B. Whitney, U.S.G. Kuhn III, and C.L. Comar.** 1953. Metabolism of Cr^{51} by animals as influenced by chemical state. *Proc. Soc. Exp. Biol. Med.* 84:610-615.

22. **MacKenzie, R.D., R.U. Byerrum, C.F. Decker, C.A. Hoppert, and R.F. Langham.** 1958. Chronic toxicity studies. II. Hexavalent and trivalent chromium administered in drinking water to rats. *Arch. Industr. Health* 18:232-234.

LIMITING THE UNCERTAINTY IN RISK ASSESSMENT BY THE DEVELOPMENT OF PHYSIOLOGICALLY BASED PHARMACOKINETIC AND PHARMACODYNAMIC MODELS

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SUMMARY

Analysis of the default cancer risk assessment methodology suggests that the confidence interval usually associated with the prediction of an upper bound on risk underestimates the uncertainty in the risk estimate. This underestimate of uncertainty is based on the use of a large number of policy decisions or professional judgements that are incorporated into the methodology as exact values with no estimate of error. An alternative approach is to develop a comprehensive biologically based risk assessment that provides scientific data to substitute for many of the policy decisions of the default methodology.

INTRODUCTION

The routine incorporation of chronic rodent bioassays into chemical safety evaluations has revealed that a relatively high percentage of the compounds evaluated are rodent carcinogens. Many of these rodent carcinogens are considered to be important in the production of various components of modern society (e.g., vinyl chloride for the production of a wide variety of domestic plastic products, butadiene for the production of synthetic rubber products such as automobile tires, etc.). The continued production (or restrictions on production) of these materials has significant economic implications. However, adverse toxicological effects associated with industrial and consumer exposure to toxic substances also have significant ethical, political, and economic implications. A reasonably accurate estimate of the economic impact of restrictions (or the lack of restrictions) on chemical exposure versus the impact of exposure on human health requires an accurate estimate of toxicological risk. The following discussion will describe some recent efforts to provide improved assessments of carcinogenic risk relative to the default quantitative risk assessment methodology.

DEFAULT CANCER RISK ASSESSMENT METHODOLOGY

To understand the need to develop more accurate estimates of carcinogenic risk, it is important to understand the current default quantitative risk assessment methodology used in a variety of government regulatory agencies (outlined in Figure 43). This process was generally described by Anderson and the Carcinogen Assessment Group of the Environmental Protection Agency [1] and has been detailed in a variety of specific regulatory activities. In this context, a quantitative estimate of carcinogenic risk is defined relative to a specific population. Population risk is based on the

distribution of chemical exposure of the population and the distribution of individual biological responses resulting from exposure. The following discussion will primarily address efforts to model the distribution of individual responses following exposure and will not substantively address efforts to model the distribution of possible environmental exposures.

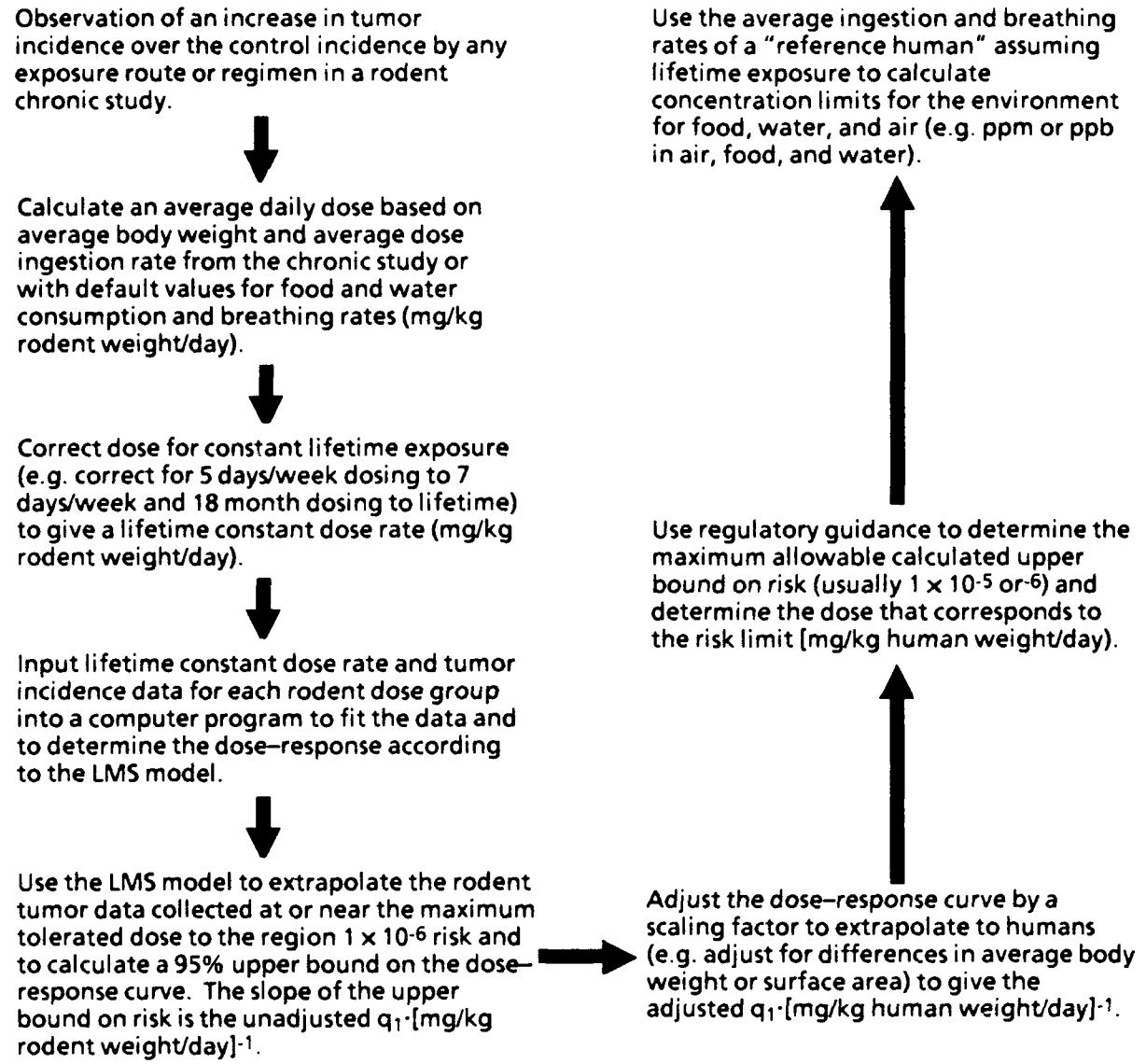

Figure 43. Steps in the Conduct of the Default Risk Assessment Methodology Used by a Variety of Regulatory Agencies. The risk assessment is generally based on a chronic rodent bioassay conducted at or near the maximum tolerated dose for the rodents. In practice, this process may be conducted with a computer program with which all of the inputs (e.g., chronic study dose concentrations, number of animals in each dose group, average animal weight for all tested animals, average food and water ingestion for each dose group, etc.) are entered into the program initially. The program may then calculate human risk estimates for various exposure routes and concentrations using logical steps similar to that described in this figure.

The default methodology is based upon the assumption that minimal data are available to describe the biochemical events, extended dose response, and interspecies extrapolability of the mechanism by which a rodent carcinogen induces the tumors that were observed in a chronic study. To address a pragmatic societal need for quantitative risk assessment, the default methodology blends the dose–response for rodent tumor incidence with a series of policy decisions to provide what has been described as "a plausible upper bound" on human carcinogenic risk from exposure to the chemical of concern [1]. (In this context, a policy decision is defined as a consensus of scientific experts that has been adopted to bridge a gap in knowledge to achieve a societal goal. In the absence of appropriate scientific data, no value judgement is assumed or implied with regard to its accuracy.) Regulatory decisions based on the default methodology often state that the actual human risk under low-dose environmental exposure conditions may be as low as zero, but it is assumed to not exceed the upper bound described by the default methodology.

In more detail, the default methodology consists of the initial observation of a statistically significant dose-related increase in tumor incidence in a rodent chronic bioassay. The dose measure used in the study (e.g., ppm in inhaled air, food, etc.) is converted to an average daily body burden expressed in milligrams per kilogram rodent body weight per day. The dose and tumor incidence data are entered into a computer program (usually the linearized multistage model, LMS) that performs a nonlinear curve fit on the data based upon a polynomial equation that describes multistage carcinogenesis and calculates a projected upper bound on rodent carcinogenic risk in the region of 1 in 10^6 risk. The dose–response on the upper bound of predicted risk in this region is referred to as the unadjusted q_1^*. A correction for the interspecies extrapolation of risk is then applied. Historically, this correction is based on the difference in body surface area or body weight; although the adoption of an intermediate value, body weight to the 0.75 power, is under consideration [2]. The resulting dose–response on the upper bound of projected human carcinogenic risk is referred to as the adjusted q_1^*. A point on the upper bound for projected risk is selected (usually 1 in 10^5 or 1 in 10^6) and the dose corresponding to this risk is used as a limit on human exposure. This dose (generally expressed as a daily body burden in milligrams or micrograms per kilogram body weight per day) is used to determine the limits on human exposure based on estimates of average human behavior. For example, limits on chemical exposure via drinking water are typically based on an average daily ingestion of 2 L of water. Given a dose of 2 μg estimated to give an upper bound risk of 1 in 10^6, division of 2 μg by 2 L would place a limit for drinking water exposure of 1 μg/L. Similar considerations are typically used for inhalation and dietary exposure.

UNCERTAINTY IN THE DEFAULT RISK ASSESSMENT PROCESS

An upper bound on the uncertainty in the predictions of the default methodology is commonly ascribed to the 95% upper bound on risk calculated by the LMS model [1]. A closer analysis of the default methodology suggests that the uncertainty in the predicted risk from the model is underestimated. A list of inputs to the default methodology is provided in Table 34. Each of these values is incorporated as if it were a single absolutely accurate number (i.e., there is no way of incorporating either a measure of error or a description of a distribution of input values into the method). For example, in a typical chronic rodent feeding study, the weight and food consumption of each animal changes throughout the study, and each animal in each dose group has a different consumption record. Instead of describing this range of exposure with its accompanying interindividual variation, a single average value is calculated and provided as a program input (e.g., 10.0 mg/kg/day). At least 15 input parameters for the method are used in this way. The use of these precise input parameters is computationally expedient, but as a consequence, the confidence interval associated with the model output severely underestimates the overall uncertainty of the methodology.

TABLE 34. UNCERTAINTY FACTORS IN THE DEFAULT QUANTITATIVE RISK ASSESSMENT METHODOLOGY

1.	UF for Selection of Test Species (Default is the most sensitive species tested based in the carcinogenic dose–response.) Was the most sensitive test species tested? Is the test species more or less sensitive than humans? Is the test species the most appropriate model to evaluate human risk?
2.	UF for Description of Test Species (Default is to assume the average weight during the course of the chronic study as a single precise value with no error.) Should weight be expressed as a continuous function or range of values to reflect the change in weight with aging? If the average weight is used, should it be used with some indication of the range of observations for different animals?

(continued)

TABLE 34. Continued

3. UF for Measure of Dose
(Default is mg/kg/day.)

Is the most appropriate dose measure: a measure of concentration at the contact site, area-under-curve for blood concentration as a function of time, peak blood concentration, mg/kg/h, dose delivered to the target tissue, a measure of pharmacological dose, etc.?

4. UF for Accuracy of Dose Expression
(Default is the average dose for each dose group in mg/kg/day over the course of the rodent chronic study expressed as a precise value without an indication of error or variation during the course of the study or variability between animals.)

Is an average value with no indication of range or variability the most appropriate? Should a function describing the variation in dosing over the course of the animal's lifetime be introduced into the calculations? Should variability between animals be explicitly considered?

5. UF for Tumor Response in the Test Species
(Default is to assume that the pathology results are "solid" data that are reproducible within normal statistical variation.)

Pathologists often differ in their professional opinions relative to the diagnosis of specific lesions and these opinions often change over a period of time. What level of diagnostic uncertainty should be factored into the methodology?

6. UF for Relevance of Tumor Response in the Test Species
(Default is to assume that any increase in tumor response is relevant for carcinogenic risk assessment for humans. Conversely, the lack of an increased tumor response in a well-designed study is implicitly assumed to confer a measure of safety.)

Some rodent tumor responses may not be relevant for humans due to differences in physiology or anatomy. Conversely, the sensitivity of humans to some carcinogens may not be modeled effectively by the common test species.

7. UF for Selection of the Dose Response Model
(Default is the linearized multistage model.)

Is another dose–response model more appropriate (e.g., probit, Weibull, Moolgavkar, etc.?

8. UF for Calculation of Uncertainty of Dose-Response
(Default is upper 95% confidence interval of risk by the log-likelihood method commonly used with the linearized multistage model.)

Is this the most appropriate method to calculate the confidence interval or should another mathematical method be used? Should the 99% confidence interval be used? Or the 90%?

(continued)

TABLE 34. Continued

9. UF for Interspecies Extrapolation Method
(Default is to assume that humans have a carcinogenic sensitivity to the test compound after correction for the difference in body surface area, body weight, or an intermediate value based on a power of body weight.)

Should the interspecies extrapolation be conducted based on a single precise value or should a range of values be employed? What power of body weight is the best estimate and what is the range of acceptable values? Do all carcinogenic responses conform to allometric scaling?

10. UF for Expression of Human Dose
(Default is mg/kg/day.)

Is the most appropriate dose measure: a measure of concentration at the contact site, area-under-curve for blood concentration as a function of time, peak blood concentration, mg/kg/h, dose delivered to the target tissue, a measure of pharmacological dose, etc.?

11. UF for Description of Target Human
(Default is a generic 70 kg human.)

Is the most appropriate human model a child (nominally considered to be the most sensitive), a geriatric adult (highest incidence), a distribution of sizes, male or female, etc.? What variance should be allocated to differing genetic susceptibility?

12. UF for Measure of Exposure Dose
(Default is generally ppm or ppb inhaled, ingested, or dermally absorbed.)

Typically, the exposure dose measure assumes that a fixed proportion of the exposure concentration that contacts the body is absorbed. Is the relationship between external dose and delivered dose (or biologically effective dose) linear or nonlinear? Does it change with age, sex, race, or environmental factors?

13. UF for Route Extrapolation
(All routes of exposure are considered to carry equal risk. That is, given a risk per mg/kg/day body burden calculated by the linearized multistage model, any exposure scenario by any route that permits that amount of compound to enter the body would carry an equivalent risk.)

Are risks unequal by different exposure routes due to differences in metabolism at the portal of entry, first pass effects in the liver, differences in disposition, etc?

14. UF for Environmental Exposure
(Typically, a "worst case" exposure scenario is formulated and used as a basis for the risk assessment.)

Is the "worst case" really the worst case? Would it be more appropriate to use an average exposure scenario or a distribution of exposure scenarios?

THE DEVELOPMENT AND USE OF PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODELS

In an effort to provide more accurate estimates of carcinogenic risk, biologically based risk assessment models have been constructed for a variety of chemicals. Although these models are being continually refined and validated, they elucidate many of the sources of variability in the default methodology. Furthermore, they allow the incorporation of growth-dependent parameters and distributions for input parameters via Monte Carlo sampling. Sensitivity analysis and data from the validation of PBPK models can suggest further data collection and model refinement for still more accurate estimates of risk. Several recent analyses of the uncertainty associated with the predictions of the biologically based risk assessment models have indicated that the distribution of risk predicted by the models may be wide, but realistic based on the individual variability expressed in an outbred population [3-6].

It is worth noting that the variability in human response to xenobiotic exposure has probably been most thoroughly documented in the many human clinical studies that have been conducted with pharmaceutical products (xenobiotics dosed with therapeutic intent). This variability is often wide but narrow enough to allow dosing based upon general guidelines related to body weight. Refinement of dosing based on pharmacokinetic analysis of an internal dose measure generally further limits the variability between dose and pharmacological response [7]. The pharmacological data also provides valuable information describing the variability observed in extrapolating the dose–response for a pharmacological effect from rodents to humans (e.g., [8,9]). Again, although the variability is wide for this interspecies extrapolation, it is generally narrow enough to be useful for therapeutic purposes when the relevant pharmacokinetic data are considered.

EXPERIENCE FROM THE DEVELOPMENT OF A PBPK MODEL FOR ETHYL ACRYLATE

Ethyl acrylate (EA) is a representative of a large class of acrylate monomers used in the synthesis of a variety of polymers. Ethyl acrylate induces irritation at the site at which it initially contacts mammalian tissues at moderate concentrations, but EA has low systemic mammalian toxicity. It has been evaluated in four continuous dosing chronic toxicity studies. No dose-related increase in tumors was noted following chronic dermal dosing of mice, inhalation dosing of rats and mice, or oral (drinking water) dosing of rats [10-12]. However, an oral gavage study conducted by the National Toxicology Program (NTP) resulted in an increase in tumors at the dosing site, forestomach (nonglandular stomach), in both rats and mice [13]. No tumors or other signs of compound-induced toxicity were reported in the adjacent glandular stomach (histologically similar to the human stomach) or in tissues remote from the dosing site. National Toxicology Program scientists have noted that this study is an example of the association of chronic toxicity in the target organ with carcinogenicity [14]. Other NTP scientists have conducted a "stop-dose" chronic study that indicated that chronic toxicity is critical for the carcinogenic response [15]. Rats dosed at high levels for 13 weeks

exhibited severe epithelial hyperplasia that penetrated into the submucosa. When the animals were allowed to recover for 19 months, no tumors were observed and the forestomachs were found to have recovered.

The chronic studies suggest that the toxicity observed with EA may be dependent on the rate of absorption into an initial contact site tissue. The lack of toxic effects at remote organ sites suggests the presence of very efficient systemic detoxification. Metabolic studies indicate that EA is metabolized by the following reactions: ester hydrolysis by carboxylesterases to release ethanol and acrylic acid (e.g., [16-19]), glutathione conjugation [16,17,19-22], and excretion of the conjugates in the urine [22,23], and binding to protein thiols [19,23]. Acrylic acid is, in turn, detoxified by mitochondrial oxidation to carbon dioxide (CO_2) and acetyl CoA [24]. Each of these metabolic pathways represents detoxification at low dose levels, but severe glutathione depletion and extensive protein binding have been associated with cytotoxicity for other compounds (e.g., acetaminophen [43] and bromobenzene [44]).

Oral Pharmacokinetic Model

The oral pharmacokinetic model for EA and its accompanying validation has previously been described in detail [19]. A brief description of the oral model will be provided to support the following discussion. The model was generally based on previous models (e.g., [25,26]) that were modified to incorporate gavage dosing of an oil vehicle and to include metabolic parameters for 14 organs (Figure 44). The model was designed to explore the biochemical basis for the difference in toxic response between the forestomach and a variety of nontarget tissues. All of the known biochemical pathways were described for each organ, which include a Michaelis-Menten description of the enzymatic hydrolysis of the ester function, production and turnover of glutathione, nonenzymatic conjugation with glutathione, and binding to tissue protein (Figure 45). The metabolic rates were determined with *in vitro* studies, and all rate constants determined *in vitro* were used directly in the model.

Dermal PBPK Model

A compartmental dermal model for rat skin [27] was constructed based on a prior compartmental model design described by Guy et al. [28]. The model was based on a "compartment in series" approach similar to that used to describe the liver in the oral EA PBPK model (Figure 46). The model incorporated a nonmetabolizing stratum corneum layer with protein binding sites and a fully metabolically capable epidermis and dermis. Because EA is quite volatile, an evaporation term was also included for simulations of nonoccluded exposure scenarios. Metabolic rates were determined with *in vitro* studies with rat skin homogenates in the same manner as the oral model. Studies were conducted with EA dosed onto hairless rat skin in a Franz cell apparatus to provide transfer rates and to evaluate the model.

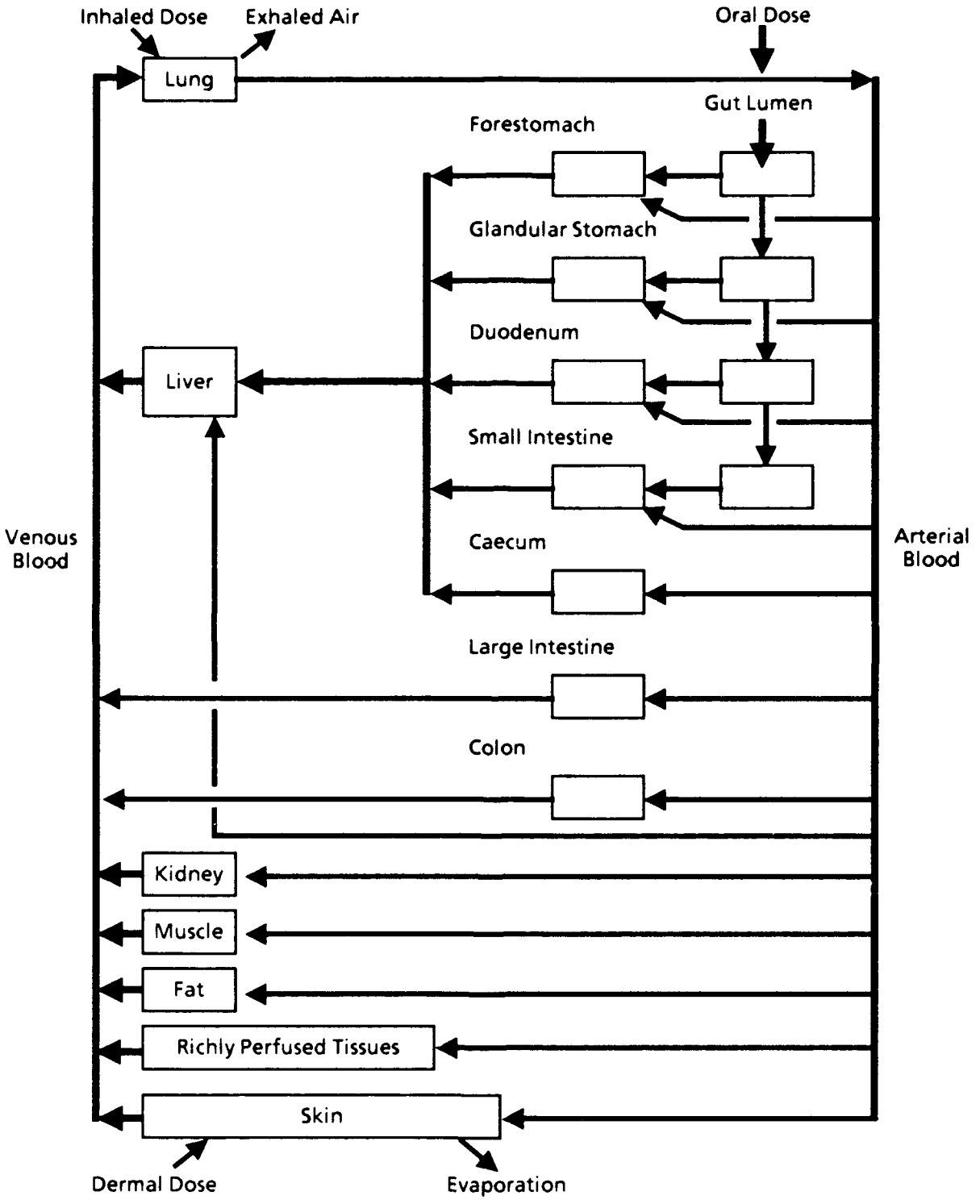

Figure 44. A Schematic Diagram of a PBPK Model for EA.

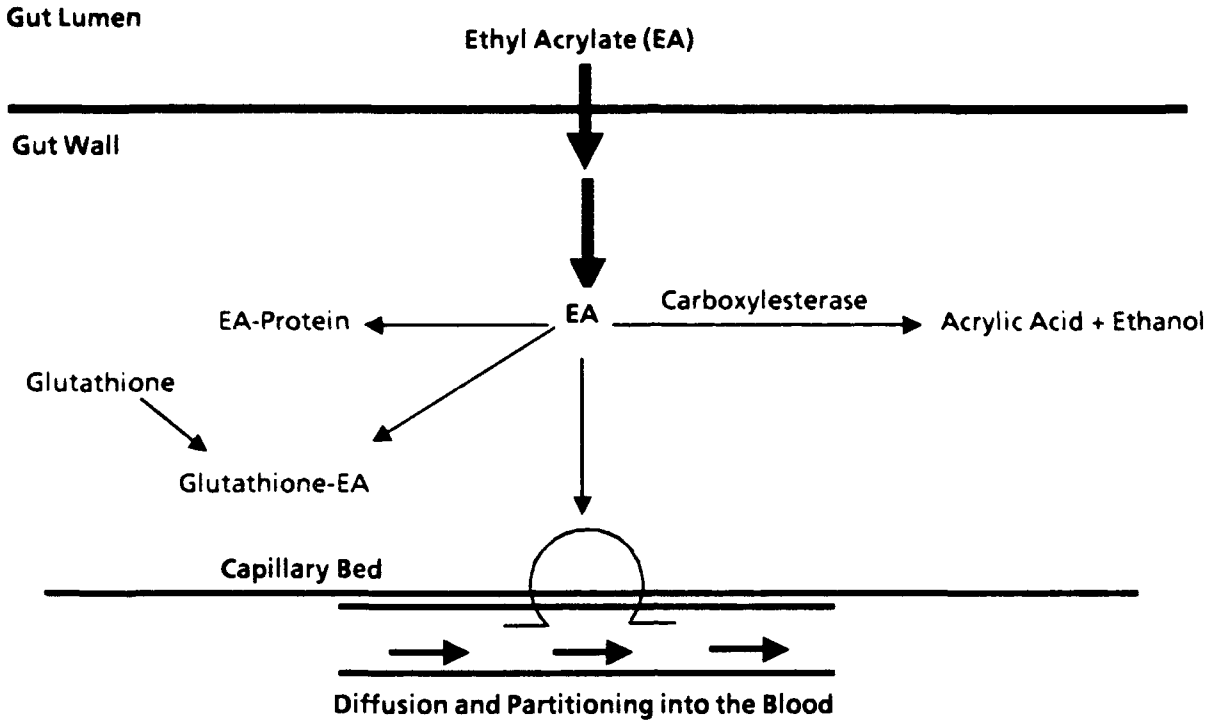

Figure 45. A Description of the Biochemical Processes Modeled for Absorption of EA at Initial Contact Sites in the Gut Following Oral Dosing.

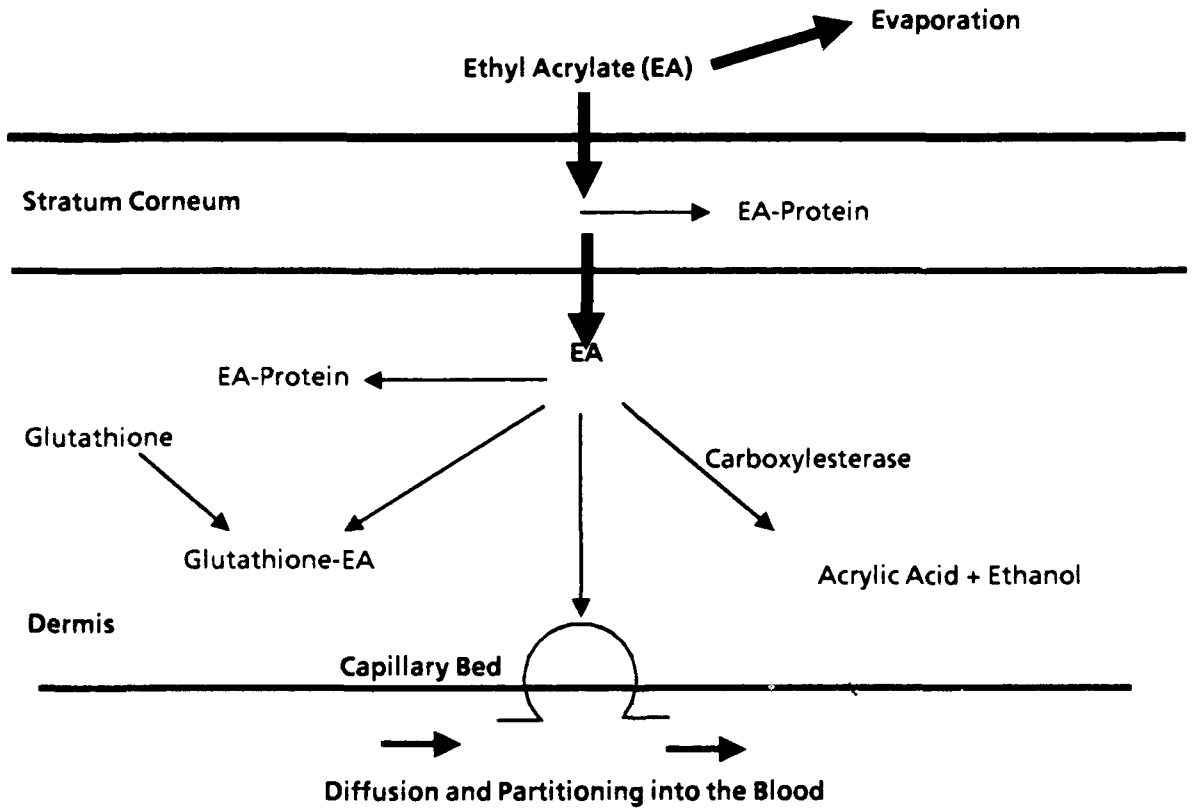

Figure 46. A Description of the Processes Modeled for Dermal Absorption of EA Following Nonoccluded Exposure.

Inhalation PBPK Model

A preliminary inhalation PBPK model for EA in rats (Figure 47) is currently being evaluated against the data from *in vivo* studies. The appropriate *in vitro* metabolic studies have been completed and the data indicate very rapid hydrolysis of EA in the rat upper respiratory tract (an estimated hydrolysis half- life of less than 0.2 sec based on V_{max}/K_m analysis).

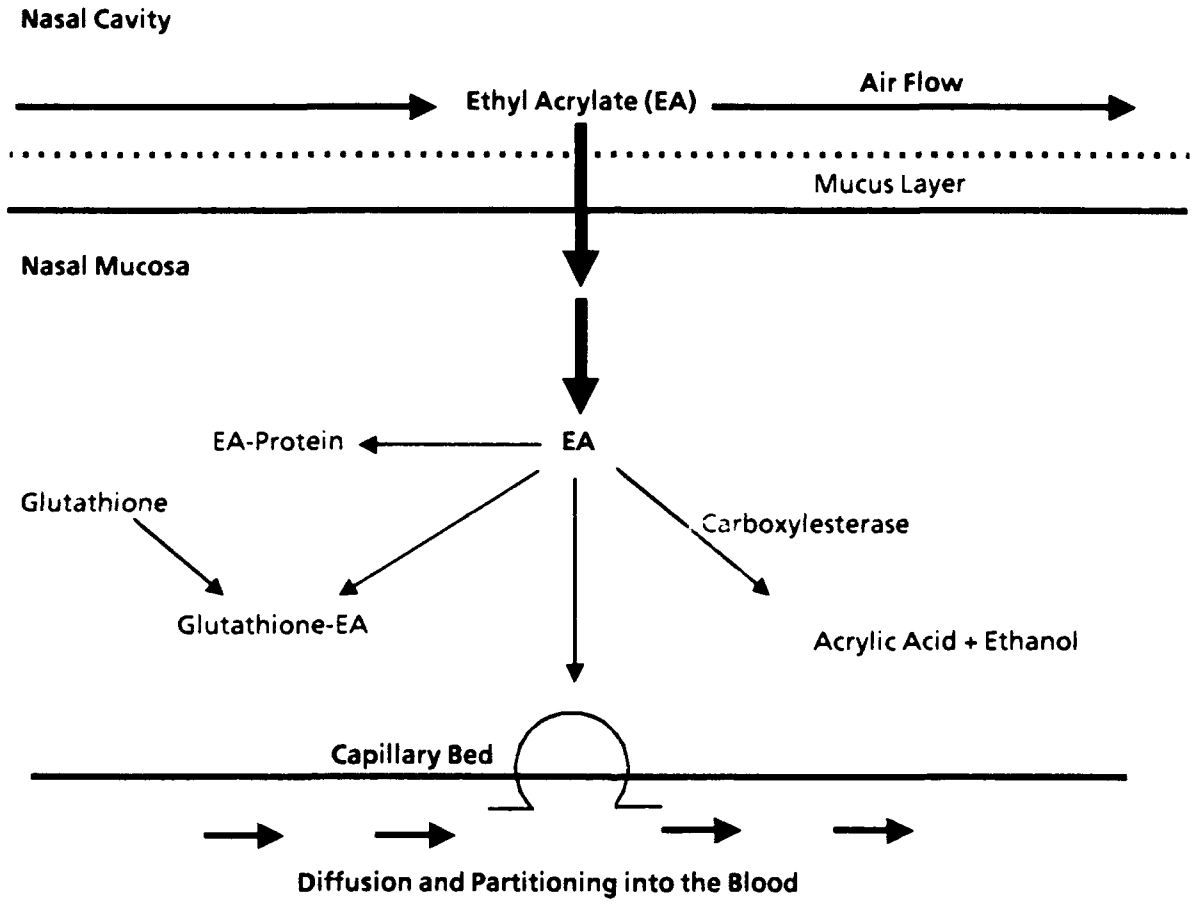

Figure 47. A Description of the Processes Modeled for Absorption of EA at Initial Contact Sites in the Upper Respiratory Tract Following Inhalation Exposure.

COMPARISONS OF MODEL PREDICTIONS TO EXPERIMENTAL DATA

Because of its very rapid metabolism, EA was not detected in tissues or blood following gavage dosing [29]. Therefore, it was necessary to use alternative means of model validation. In the case of oral dosing, effects of EA on glutathione concentrations in the target tissue and a variety of nontarget tissues were correlated with the toxic responses (or lack thereof) in the tissues [19]. At carcinogenic doses, severe glutathione depletion was predicted by the oral EA PBPK model and observed experimentally in the forestomach (target organ). Severe glutathione depletion was not

predicted or observed in a wide variety of nontarget tissues (e.g., liver, lungs, and kidneys). The predictions of the oral PBPK model were also compared to published data from other laboratories. For example, $^{14}CO_2$ excretion was predicted from a simulated gavage dose of ^{14}C-EA and compared to experimental data from a published study [23]. Monte Carlo sampling of normal distributions for the various input parameters suggested that the overall uncertainty in model output fell within a reasonable range for predicting this overall measure of xenobiotic metabolism.

Evaluation of the output of the dermal PBPK model and our preliminary inhalation PBPK model indicates that severe glutathione depletion is not predicted at the highest dose levels used in the chronic bioassays. This is consistent with the observation that no dose-related tumors were observed following chronic dermal or inhalation exposure. In the case of nonoccluded dermal exposure, this is probably because approximately 95% of the applied dose evaporates from the skin [27]. For inhalation exposure, the model and its validation are not complete, but the *in vitro* metabolic data may have provided some indication why EA is an irritant but not a carcinogen following inhalation exposure. Although there is a high rate of EA deposition in the upper respiratory tract following inhalation dosing [30], the high local EA concentration may be efficiently detoxified by the very rapid rate of ester hydrolysis in this region, which is much faster than any other tissue examined.

INTERSPECIES EXTRAPOLATION OF THE EA PBPK MODEL TO PRIMATES

A variety of studies are being pursued to evaluate the validity of the rodent EA PBPK model for human risk assessment. To facilitate *in vivo* studies for interspecies extrapolation, an effort is being made to establish a primate model for human metabolism of EA by evaluating the glutathione concentration and carboxylesterase activity of a variety of tissues from rhesus and cynomolgous monkeys. These data will be compared with the rodent data and data from freshly frozen human tissues. Interim data from the analysis of rhesus monkey tissues indicates that glutathione levels and carboxylesterase activity, when expressed per gram of tissue, appear to be quite comparable to the corresponding rat tissues. This would allow scaling of the rodent model based on measured differences in organ size between species. These interim data also suggest that primates are similar to rodents in their very efficient systemic detoxification of EA.

IMPLICATIONS OF THE BIOLOGICALLY BASED MODELING OF EA FOR RISK ASSESSMENT

One reasonable approach to risk assessment based upon the body of mechanistic data collected for EA would be the calculation of a reference dose (RfD) or reference concentration (RfC) [31,32]. This approach effectively assumes that any carcinogenic risk from EA is associated with toxicity (i.e., inflammation, hyperplasia, irritation). Exposures of EA that do not induce detectable signs of toxicity would be associated with a carcinogenic risk that is either zero or below the detection

limits or reliable prediction limits of current technology. The RfD/RfC would be calculated by applying uncertainty factors to account for uncertainty in interspecies extrapolation, population variation, or other issues of concern. A recent analysis of the chronic studies conducted by NTP provided some support for this approach [14]. The NTP gavage study with EA was noted as an example for which there was evidence for a relationship between target organ toxicity and an increase in tumor incidence. In a recent regulatory action that follows this philosophy, the European Economic Community has decided not to classify EA as a suspect carcinogen. Instead, it will be labeled as an irritant that should be used in a well ventilated area with protective clothing and gloves. A similar approach has been used for a related forestomach carcinogen, butylated hydroxyanisole (BHA), by the State of California under the risk assessment and labeling requirements of Proposition 65.

An alternative approach would be to use a measure of delivered dose to a target tissue in the linearized multistage model used in the default risk assessment methodology. One convenient delivered dose measure that has experimental data for support would be the area under the glutathione concentration-time (AUC) curve in the region of severe glutathione depletion, because severe glutathione depletion has been correlated with tissue toxicity in other studies with other compounds (e.g., [33-35]). This correlation does not necessarily imply that these biochemical effects are causally implicated in the cytotoxicity and reparative hyperplasia observed at high delivered doses, but may only reflect a further correlation with other cytotoxic biochemical effects (e.g., disruptions in ionic homeostasis, activated oxygen toxicity, etc.). Human delivered dose risk assessment requires tissue-to-tissue, route-to-route, and interspecies extrapolation because humans do not have a forestomach and oral exposure to EA is not a common route of human exposure. Use of a delivered dose measure can facilitate this process and minimize the uncertainty associated with the expression of the dose measure in the linearized multistage model.

The use of the linearized multistage model requires certain assumptions regarding the mechanism of carcinogenesis of the test compound. Essentially, the model assumes that the compound conforms to the multistage carcinogenesis model and that the compound has some initiating activity (i.e., some risk is assumed for any exposure to the test compound). However, the negative tumor results from a recent NTP "stop-dose" study [15] are not consistent with these biological assumptions and would argue against use of the LMS model. Furthermore, various genotoxicity studies have demonstrated that EA is not genotoxic with mammalian cells *in vitro* at concentrations that are not cytotoxic [36,37]. Kligerman et al. [38] have also evaluated the *in vivo* genotoxicity of EA and found that EA did not induce an increase in chromosomal aberrations or sister chromatid exchange in the spleen of mice at ip doses 5-fold higher than the highest dose used in the

NTP chronic study. These results suggest that the use of the linearized multistage model for EA may be inappropriate.

An alternative elegant approach to the risk assessment of EA would be based upon a complete biologically based risk assessment model (e.g., [39-41]). This approach could be used to couple the EA PBPK model to a cell dynamics model to fully describe the critical events associated with rodent forestomach carcinogenesis. The development of such a model would foster mechanistic understanding that would facilitate interspecies and interorgan extrapolation to provide the most accurate description of human risk that is possible, based upon the chronic rodent bioassay.

In summary, although several risk assessment procedures are viable, the range of risk assessment alternatives has become more limited. Many of the policy decisions used by the default risk assessment methodology can be replaced with data, and the overall variance in the output of the PBPK models is within reasonable bounds. The modeling effort has provided a reasonable and coherent explanation for the lack of systemic toxicity of EA and has focused the risk assessment methodology on risk related to contact site toxicity. In a rodent oral dosing model, severe depletion of tissue glutathione by carcinogenic doses of EA has been correlated with target tissue toxicity; nontarget tissues have not exhibited this effect. Lower EA doses have not significantly depleted glutathione in the target tissue or induced a toxic response [19,27,42]. Models for nonoccluded dermal exposure and inhalation exposure appear to be consistent with the lack of contact site carcinogenicity at other dosing sites. The interim primate biochemical data suggest that the rodent PBPK model can be extrapolated to other species. Simulation of a variety of exposure scenarios for humans may be predictive of the possibility of a toxic response from EA exposure, and may prove useful for risk assessment.

COMMUNICATION OF THE RESULTS OF A RISK ASSESSMENT MODEL WITH AN INDICATION OF THE RANGE OF RISKS PREDICTED BY THE MODEL FOR A POPULATION

As described above, a biologically based risk assessment model allows a quantitative estimate of the uncertainty associated with the predictions of the model. By explicitly incorporating a mean and an estimate of the range or distribution associated with each input value, a mean prediction of the model can be calculated as well as a distribution of model predictions reflecting the composite uncertainty of the model. Because some model parameters are correlated (e.g., organ size and body weight), the correlated variables may also be explicitly described. Given the outbred nature of the human population, the range of predicted metabolism should vary widely within the bounds generally observed in human clinical trials of pharmaceutical agents.

Based on many factors (such as behavioral patterns, diet, occupation, geographic location, and migration patterns, etc.), human exposure to chemicals in the environment also varies widely. To

provide the most accurate estimate of risk for a human population that is currently possible, a composite of individual metabolic variability, biological sensitivity, and exposure variability should be considered. This need may be addressed by conducting simulations on models of representative subpopulations.

For example, for a volatile chemical that might be of concern as an industrial emission (e.g., benzene, perchloroethylene, etc.), simulations could be conducted on three representative subpopulations. The first simulation might include a model for a highly sensitive subpopulation that spends an entire lifetime in close proximity to an industrial site that is constantly emitting high levels of the compound of interest. This type of simulation is currently the most common conducted by regulatory agencies, because it is considered to be the most conservative in estimating population risk (i.e., it incorporates a sensitive subpopulation with maximum exposure). The estimate of risk can be refined by incorporating estimates of the number of individuals in the population likely to fall into this category, by incorporating biologically based estimates of uncertainty into the risk assessment model, and by incorporating distributions of values to describe exposure.

A second simulation might describe a subpopulation that has some proximal exposure to the compound of interest but reflects the distribution of metabolism, biological sensitivity, and exposure typical of the general population. For example, this subpopulation would be described by the average metabolic capability of the general population and by the average characteristics associated with exposure. Again, the size of this subpopulation would be estimated, and it would be described by distributions of metabolic parameters and exposure parameters. A third simulation would then be conducted on the subpopulation with no proximal exposure, but incidental exposure through common consumer activities to the compound of interest (e.g., exposure to benzene while filling an automobile with gasoline at a service station). Again all of the parameters described for the other populations would be considered with appropriate distributions to describe the variability in the estimates. Additional simulations could be conducted similarly on other subpopulations of interest, but these three would appear to provide a minimum to describe overall population risk.

The results of these simulations could then be expressed to describe a composite estimate of population risk. Based on this information, the appropriate risk manager(s) could make an informed cost-benefit decision for society. The decision would be based on quantitative estimates of risk with corresponding estimates of uncertainty in the predictions. The results of this analysis could result in exposure values that are equal to, less than, or greater than the values derived from the current methodology. The advantages of this approach include a stronger scientific basis (and implicitly more accuracy in the risk estimates) and a more explicit and rigorous expression of the uncertainty in the risk estimates.

REFERENCES

1. **Anderson, E.L. and the Carcinogen Assessment Group of the U.S. Environmental Protection Agency.** 1983. Quantitative approaches in use to assess cancer risk. *Risk Anal.* 3:277-295.

2. **U.S. Environmental Protection Agency.** 1992. Draft Report: A cross-species scaling factor for carcinogen risk assessment based on equivalence of $mg/kg^{3/4}/day$. *Federal Register* 57:56 FR 24152.

3. **Bois, F.Y., L. Ziese, and T.N. Tozer.** 1990. Precision and sensitivity of pharmacokinetic models for cancer risk assessment: Tetrachloroethylene in mice, rats, and humans. *Toxicol. Appl. Pharmacol.* 102:300-315.

4. **Farrar, D., B. Allen, K. Krump, and A. Shipp.** 1989. Evaluation of uncertainty in input parameters to pharmacokinetic models and the resulting uncertainty in output. *Toxicol. Lett.* 49:371-385.

5. **Portier, C.J. and N.L. Kaplan.** 1989. Variability of safe dose estimates when using complicated models of the carcinogenic process. *Fundam. Appl. Toxicol.* 13:533-544.

6. **Portier, C.J.** 1990. Utilizing biologically based models to estimate carcinogenic risk. In: S. Moolgavkar, ed. *Scientific Issues in Quantitative Cancer Risk Assessment*, pp. 252-266. Boston: Birkhauser.

7. **Mungall, D.R.** 1983. Applied clinical pharmacokinetics. New York: Raven Press.

8. **Collins, J.M., D.S. Zaharko, R.L. Dedrick, and B.A. Chabner.** 1986. Potential roles for preclinical pharmacology in Phase I clinical trials. *Cancer Treat. Rep. 70:73-80.*

9. **Dedrick, R.L. and P.F. Morrison.** 1992. Carcinogenic potency of alkylating agents in rodents and humans. *Cancer Res.* 52:2464-2467.

10. **DePass, L.R., E.H. Fowler, D.R. Meckley, and C.S. Weil.** 1984. Dermal oncogenicity bioassays of acrylic acid, ethyl acrylate, and butyl acrylate. *J. Toxicol. Environ. Health* 14:115-120.

11. **Miller, R.R., J.T. Young, R.J. Kociba, D.G. Keyes, K.M. Bodner, L.L. Calhoun, and J.A. Ayres.** 1985. Chronic toxicity and oncogenicity bioassay of inhaled ethyl acrylate in Fischer 344 rats and B6C3F1 mice. *Drug. Chem. Toxicol.* 8:1-42.

12. ***Borzelleca, J.F., P.S. Larson, G.R. Hennigar, E.G. Huf, E.M. Crawford, and R.B. Smith.*** 1964. Studies on the chronic oral toxicity of monomeric ethyl acrylate and methyl methacrylate. *Toxicol. Appl. Pharmacol.* 6:29-36.

13. **National Toxicology Program.** 1986. Carcinogenesis Bioassay of Ethyl Acrylate. Technical Report Series 259, Publication NIH 82-2515. Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health.

14. **Hoel, D.G., J.K. Haseman, M.D. Hogan, J. Huff, and E. McConnell.** 1988. The impact of toxicity on carcinogenicity studies: Implications for risk assessment. *Carcinogenesis* 9:2045-2052.

15. **Ghanayem, B.I., H.B. Matthews, and R.R. Maronpot.** 1991. Sustainability of forestomach hyperplasia in rats treated with ethyl acrylate for 13 weeks and regression after cessation of dosing. *Toxicol. Pathol.* 19:273-279.

16. **Silver, E.H. and S.D. Murphy.** 1981. Potentiation of acrylate ester toxicity by prior treatment with the carboxylesterase inhibitor triorthotolyl phosphate (TOCP). *Toxicol. Appl. Pharmacol.* 57:208-219.

17. **Miller, R.R., J.A. Ayres, L.W. Rampy, and M.J. McKenna.** 1981. Metabolism of acrylate esters in rat tissue homogenates. *Fundam. Appl. Toxicol.* 1:410-414.

18. **Stott, W.T. and M.J. McKenna.** 1985. Hydrolysis of several glycol ether acetates and acrylate esters by nasal mucosal carboxylesterase *in vitro*. *Fundam. Appl. Toxicol.* 5:399-404.

19. **Frederick, C.B., D.W. Potter, M.I. Chang-Mateu, and M.E. Andersen.** 1992. A physiologically based pharmacokinetic and pharmacodynamic model to describe the oral dosing of rats with ethyl acrylate and its implications for risk assessment. *Toxicol. Appl. Pharmacol.* 114:246-260.

20. **Boyland, E. and L.F. Chasseaud.** 1967. Enzyme-catalysed conjugations of glutathione with unsaturated compounds. *Biochem. J.* 104:95-102.

21. **Boyland, E. and L.F. Chasseaud.** 1968. Enzymes catalysing conjugations of glutathione with alpha,beta-unsaturated carbonyl compounds. *Biochem. J.* 109:651-661.

22. **deBethizy, J.D., J.D. Udinsky, H.E. Scribner, and C.B. Frederick.** 1987. The disposition and metabolism of acrylic acid and ethyl acrylate in male Sprague-Dawley rats. *Fund. Appl. Toxicol.* 8:549-561.

23. **Ghanayem, B.I., L.T. Burka, and H.B. Matthews.** 1987. Ethyl acrylate distribution, macromolecular binding, excretion, and metabolism in male Fisher 344 rats. *Fundam. Appl. Toxicol.* 9:389-397.

24. **Finch, L. and C.B. Frederick.** In Press. Rate and route of oxidation of acrylic acid to carbon dioxide in rat liver. *Fundam. Appl. Toxicol.*

25. **Ramsey, J.C. and M.E. Andersen.** 1984. A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol. Appl. Pharmacol.* 73:159-175.

26. **Andersen, M.E., H.J. Clewell III, M.L. Gargas, F.A. Smith, and R.H. Reitz.** 1987. Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol. Appl. Pharmacol.* 87:185-205.

27. **Frederick, C.B. and I.M. Chang-Mateu.** 1990. Contact site carcinogenicity: Estimation of an upper limit for risk of dermal dosing site tumors based on oral dosing site carcinogenicity. In: T.R. Gerrity and C.J. Henry, eds. *Principles of Route-to-Route Extrapolation for Risk Assessment*, pp. 237-269. New York: Elsevier.

28. **Guy, R.H., J. Hadgraft, and D.A.W. Bucks.** 1987. Transdermal drug delivery and cutaneous metabolism. *Xenobiotica* 17:325-343.

29. **Udinsky, J.R. and C.B. Frederick.** 1990. Analysis of ethyl acrylate (EA) and acrylic acid (AA) residues from rat tissues following oral EA dosing. *Toxicologist* 10:240.

30. **Stott, W.T. and M.J. McKenna.** 1984. The comparative absorption and excretion of chemical vapors by the upper, lower, and intact respiratory tract of rats. *Fundam. Appl. Toxicol.* 4:594-602.

31. **Barnes, D.G. and M. Dourson.** 1988. Reference dose (RfD): Description and use in health risk assessments. *Regul. Toxicol. Pharmacol.* 8:471-486.

32. **Overton, J.H. and A.M. Jarabek.** 1989. Estimating equivalent human concentrations of no observed adverse effect levels: A comparison of several methods. *Exp. Pathol.* 37:89-94.

33. **Mitchell, J.R., D.J. Jollow, W.Z. Potter, J.R. Gillette, and B.B. Brodie.** 1973. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.* 187:211-217.

34. **Potter, W.Z., S.S. Thorgeirsson, D.J. Jollow, and B.B. Brodie.** 1974. Acetaminophen-induced hepatic necrosis. V. Correlation of hepatic necrosis, covalent binding and glutathione depletion in hamsters. *Pharmacology* 12:129-143.

35. **Wendel, Z., H. Jaeschke, and M. Gloger.** 1982. Drug-induced lipid peroxidation in mice. II. Protection against paracetamol-induced liver necrosis by intravenous liposomally entrapped glutathione. *Biochem. Pharmacol.* 31:3601-3605.

36. **Moore, M.M., A. Amtower, C.L. Doerr, K.H. Brock, and K.L. Dearfield.** 1988. Genotoxicity of acrylic acid, methyl acrylate, and ethyl methacrylate in L5178Y mouse lymphoma cells. *Environ. Mol. Mutagen.* 11:49-63.

37. **Moore, M.M. and C.L. Doerr.** 1990. Comparison of chromosome aberration frequency and small-colony TK-deficient mutant frequency in L5178Y/TK +/--3.7.2C mouse lymphoma cells. *Mutagenesis* 5:609-614.

38. **Kligerman, A.D., A.L. Atwater, M.F. Bryant, G.L. Erexson, P. Kwanyuen, and K.L. Dearfield.** 1991. Cytogenetic studies of ethyl acrylate using C57BL/6 mice. *Mutagenesis* 6:137-141.

39. **Moolgavker, S.H., A. Dewanji, and D.J. Venzon.** 1988. A stochastic two-stage model for cancer risk assessment. I. The hazard function and the probability of tumor. *Risk Anal.* 8:383-392.

40. **Cohen, S.M. and L.B. Ellwein.** 1990. Proliferative and genotoxic cellular effects in 2-acetylaminofluorene bladder and liver carcinogenesis: Biological modeling of the ED01 study. *Toxicol. Appl. Pharmacol.* 104:79-93.

41. **Conolly, R. B. and M.E. Andersen.** 1991. Biologically based pharmacodynamic models: Tools for toxicological research and risk assessment. *Annu. Rev. Pharmacol. Toxicol.* 31:503-523.

42. **Frederick, C.B., G.A. Hazelton, and J.D. Frantz.** 1990. The histopathological and biochemical response of male F344/N rats following two weeks of oral dosing with ethyl acrylate. *Toxicol. Pathol.* 18:247-256.

43. **Potter, W.Z., S.S. Thorgeirsson, D.J. Jollow, and B.B. Brodie.** 1974. Acetaminophen-induced hepatic necrosis. V. Correlation of hepatic necrosis, covalent binding and glutathione depletion in hamsters. *Pharmacology* 12:129-143.

44. **Cassini, A.F., M. Ferrali, A. Pompella, E. Maellaro, and M. Comporti.** 1986. Lipid peroxidation and cellular damage in extrahepatic tissues of bromobenzene-intoxicated mice. *Am. J. Pathol.* 123:520-531.

DIOXIN HEPATIC CARCINOGENESIS: BIOLOGICALLY MOTIVATED MODELING AND RISK ASSESSMENT

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SUMMARY

There are several key portions of the exposure–dose–response continuum with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) that have to be described quantitatively in developing a comprehensive mechanistically based dose–response model. These include: (1) the accumulation of TCDD in the target tissue, (2) formation of a complex between dioxin and the *Ah* receptor, (3) activation of transcription of growth regulatory genes by the TCDD-*Ah* receptor complex, (4) cellular responses to the altered expression of growth regulatory gene products, and (5) the effect of these cellular events on tumor initiation, promotion, and progression. Physiologically based pharmacokinetic (PBPK) models have been used as tools to integrate knowledge of the determinants of dioxin disposition, including specific binding to dioxin-inducible hepatic cytochromes, and to link TCDD tissue dosimetry with gene activation by pharmacodynamic (PD) models crafted to examine dioxin-regulated gene expression. Biological studies on growth factor regulation suggest hypotheses for the role of these gene products in transient cell proliferation, prolonged growth suppression, and hepatic tumor promotion. We have used these hypotheses as the basis for stochastic cell growth models of the promotional events with TCDD and to suggest experimental strategies for future research. The combination of PBPK, PBPD and stochastic cell growth models provides a seamless exposure–dose–response model for TCDD induction of liver tumors in rodents. This comprehensive exposure–dose–response model should prove useful for risk assessment, experimental design, and analysis of noncancer end points with this potent, ubiquitous environmental contaminant. This paper outlines progress in formulating and evaluating these models for TCDD.

INTRODUCTION

Dioxin causes a variety of toxic effects in experimental systems. At doses of 0.01 to 0.1 μg/kg/day it is a potent carcinogen in both rats and mice [1,2]. Its carcinogenicity is thought to be due to its action as a tumor promoter, as opposed to as an initiator [3]. The covalent binding of TCDD to rat liver DNA is at least 4 to 6 orders of magnitude less than observed for other chemical carcinogens (e.g., aflatoxin B1), and is equivalent to one molecule of TCDD bound to the DNA of a single cell in every 35 diploid cells [4]. At exposures lower than those which lead to tumors, TCDD

causes immunotoxic, reproductive, and teratogenic effects [5,6,7]. All of these toxic responses to TCDD are believed to be mediated through the binding of TCDD to a cytosolic cellular receptor [8]. This receptor also binds polycyclic aromatic hydrocarbons (e.g., 3-methylcholanthrene), and is known as the *Ah* receptor. The binding of TCDD to this receptor, in combination with at least two other proteins, (e.g., heat shock protein 90, and *Ah* receptor nuclear translocator (arnt)), forms a complex which, following as yet unknown molecular events, binds to the DNA and alters gene transcription [9,10]. The alteration of gene transcription is thought to be an important step in the carcinogenic process as some of the genes affected include transforming growth factors, cytokines, and modulators of the extracellular matrix [11,12]. It is because of the obligate role of the *Ah* receptor in the toxic effects of TCDD that it is called a receptor-mediated carcinogen. In this paper we describe progress in developing quantitative biologically based models for dioxin pharmacokinetics, dioxin-induced gene expression, and hepatic tumor promotion.

Pharmacokinetics/Protein Induction

Amongst the genes affected by TCDD are members of the cytochrome P-450 family, specifically P-4501A1, P-4501A2, and the recently identified P-4501B1 [13]. The induction of P-4501A1 has been used as a marker of TCDD exposure in many experimental systems, but the induction of P-4501A2 is of particular interest for studies into the disposition of TCDD. Even though TCDD is highly lipophilic, in animal studies and in humans exposed to TCDD environmentally, it is found in higher concentrations in the liver than in fat [14,15]. This is suggestive of specific TCDD binding sites within the liver, different from the *Ah* receptor. Cytochrome P-4501A2 readily binds to TCDD [16] and TCDD has been shown to be a tight binding inhibitor of the enzymatic activity of P-4501A2 [17]. The induction of this, and possibly other, protein(s) is hypothesized to be the cause of the selective sequestration of TCDD to the liver. A comprehensive description of TCDD disposition must therefore include the presence of inducible binding proteins mediated by the interaction of the TCDD-*Ah*-receptor complex with the DNA. A mechanistic understanding of this induction process is required to accurately predict the disposition characteristics of TCDD. The PBPK model initially developed by Leung and coworkers for rats and mice [18,19] has been extended (Figure 48) to include a preliminary mechanistic description of the induction processes for TCDD [20,21].

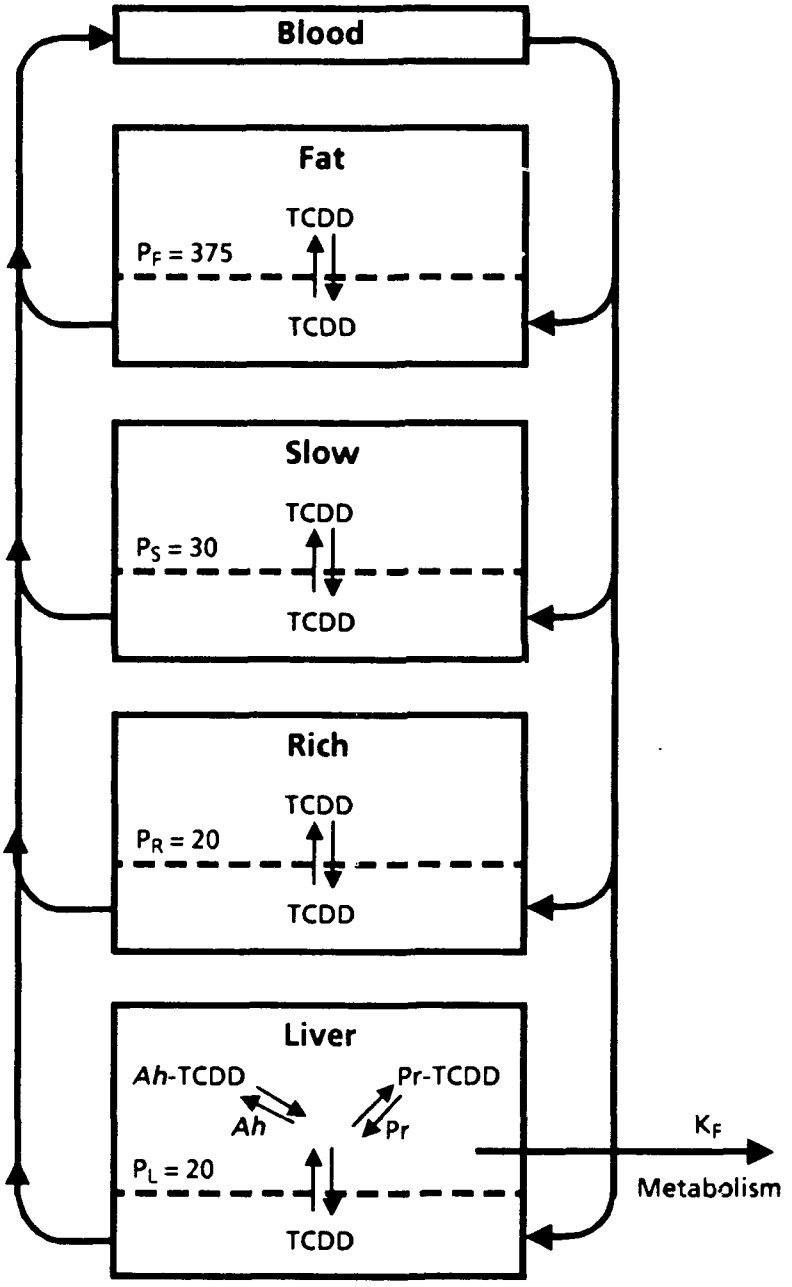
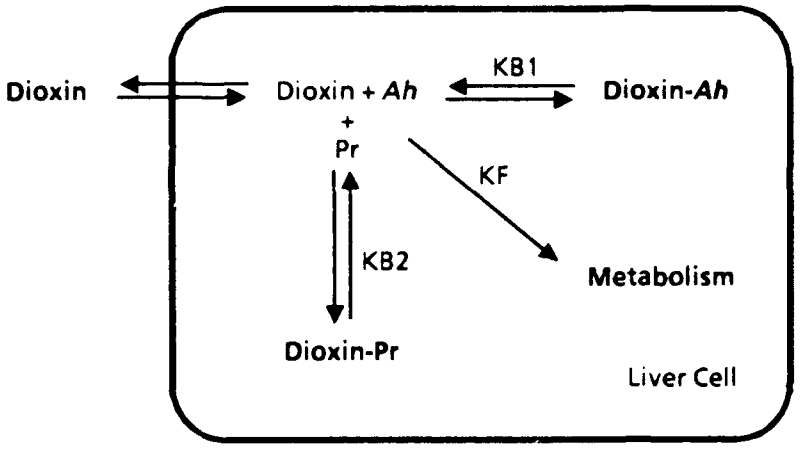


Figure 48. A Schematic of the Five-Compartment Diffusion Limited PBPK Model for TCDD Disposition. Symbol definitions are in the Appendix.

The PBPK model for TCDD describes the disposition of dioxin to five compartments based on the intrinsic solubility (i.e., the partition coefficient (P_t)) of TCDD within each compartment. Dioxin is described as entering the tissue via the tissue blood subcompartment. Within the liver, however, other factors are involved in the disposition. After diffusing into the tissue (Figure 49), TCDD partitions between three pools dependent on availability and affinity. The *Ah* receptor is a high affinity, but low capacity binding site; the inducible binding protein (Pr) has a lesser affinity but a higher capacity; and the remaining TCDD is partitioned according to intrinsic solubility. The relative amounts of TCDD in these pools are dependent on the tissue concentration and the shape of the dose–response curve for the induction of the binding protein (Pr).



Pr = Microsomal dioxin binding protein
Ah = Cytosolic dioxin receptor

Figure 49. The Cellular Disposition of TCDD. TCDD is distributed between three pools, *Ah* receptor, Binding protein, and partitioned TCDD, dependent on the TCDD concentration.

Regulation of Gene Expression

In this PBPK model [21] the induction of binding proteins (P-4501A2) and metabolizing enzymes (P-4501A1) is described by ternary interactions between the *Ah*-receptor-TCDD complex and binding sites on the DNA.

$$Ah + \text{TCDD} \rightleftharpoons Ah\text{-TCDD complex} \quad (1)$$

$$Ah\text{-TCDD} + \text{DNA} \rightleftharpoons Ah\text{-TCDD-DNA} \quad (2)$$

$$\frac{(Ah\text{-TCDD-DNA})^n}{(Ah\text{-TCDD-DNA})^n + Kd^n} \longrightarrow \text{Altered Gene Expression} \quad (3)$$

The altered gene expression is described by a Hill relationship (Equation 3), where Kd represents the dissociation constant and n is an indication of cooperativity occurring between binding sites on the DNA. This approach has been employed in several recent pharmacokinetic descriptions [22,23].

The disposition of TCDD to the liver and fat is highly dose-dependent (Figure 50) in the dose range 1 to 10,000 ng/kg body weight (bw) as seen in female Wistar rats 7 days after a single subcutaneous dose of radiolabelled TCDD [14]. The smooth curves were generated by the PBPK description using the parameters in the Appendix. The concentration of cytochrome P-4501A2 is increased about 9-fold on complete induction, from 10 nmoles/liver to 85 nmoles/liver, which is consistent with literature values from male Fischer 344 rats [24]. The binding constant, KB2, was estimated by simulations to fit the data in Figure 50. The n value (Equation 3) for cooperativity was 1.0, indicating little interaction among DNA binding sites for the TCDD-*Ah*-receptor complex. The dose–response curve for the induction of ethoxyresorufin 0-deethylase (EROD) activity, a P-4501A1 dependent process, was also modeled from the same data set, using this PBPK description (Figure 51). The induction of this protein required an n value of 2.3 indicating a greater degree of interaction among the DNA binding sites for the TCDD-*Ah*-receptor complex and the P-4501A1 upstream regulatory regions. This suggests that different molecular events may be involved in the induction processes for these two cytochromes.

Regional Regulation of Genes/Cell Proliferation

The time-course and dose–response characteristics of cytochromes P-4501A1 and P-4501A2, with regard to their regional distribution within the liver lobule, shows that the cells around the acinus, (i.e., the centrilobular region of the liver), respond to TCDD in a dose- and time-dependent manner [25,26]. At low doses, very few cells express P4501A2 protein, and these cells are clustered around the central veins. However, as the liver TCDD concentration increases, the number of cells expressing 1A2 increases radially outwards from the central vein, until at high doses the response is panlobular [26,27]. This pattern is different from that observed with TCDD-induced cell proliferation in very recent studies [28] using female Sprague-Dawley rats, (the species and sex used in the current risk assessment). Using a loading/maintenance dosing regimen with TCDD, designed to very quickly achieve a "pseudo-steady-state" liver concentration, the total cell labelling index, as measured by BrdU incorporation, was similar between treated and control animals however, the regional distribution was very different. An increase in proliferation was seen in the periportal region, *accompanied by a decrease in hepatic cell labelling in the centrilobular regions* [28]. This differing response to TCDD, both in terms of a biochemical process and a growth regulatory effect, demonstrates that differential responses can and do occur within the liver and should be reconciled in any description of the mechanism of TCDD carcinogenesis. Presently, the PBPK descriptions do not describe intrahepatic variabilities but have assumed a homogeneous liver compartment. For the

incorporation of heterogeneous liver compartments into PBPK models of the type described here, a mechanistic understanding of the differential responses to TCDD is needed. At the present time this understanding is lacking, however, the recent cloning of the genes for the *Ah* receptor [29] and arnt [30], and the use of immunolocalization to study response heterogeneity [25-28], will allow a more detailed study of the intricacies of the interactions of TCDD within the liver.

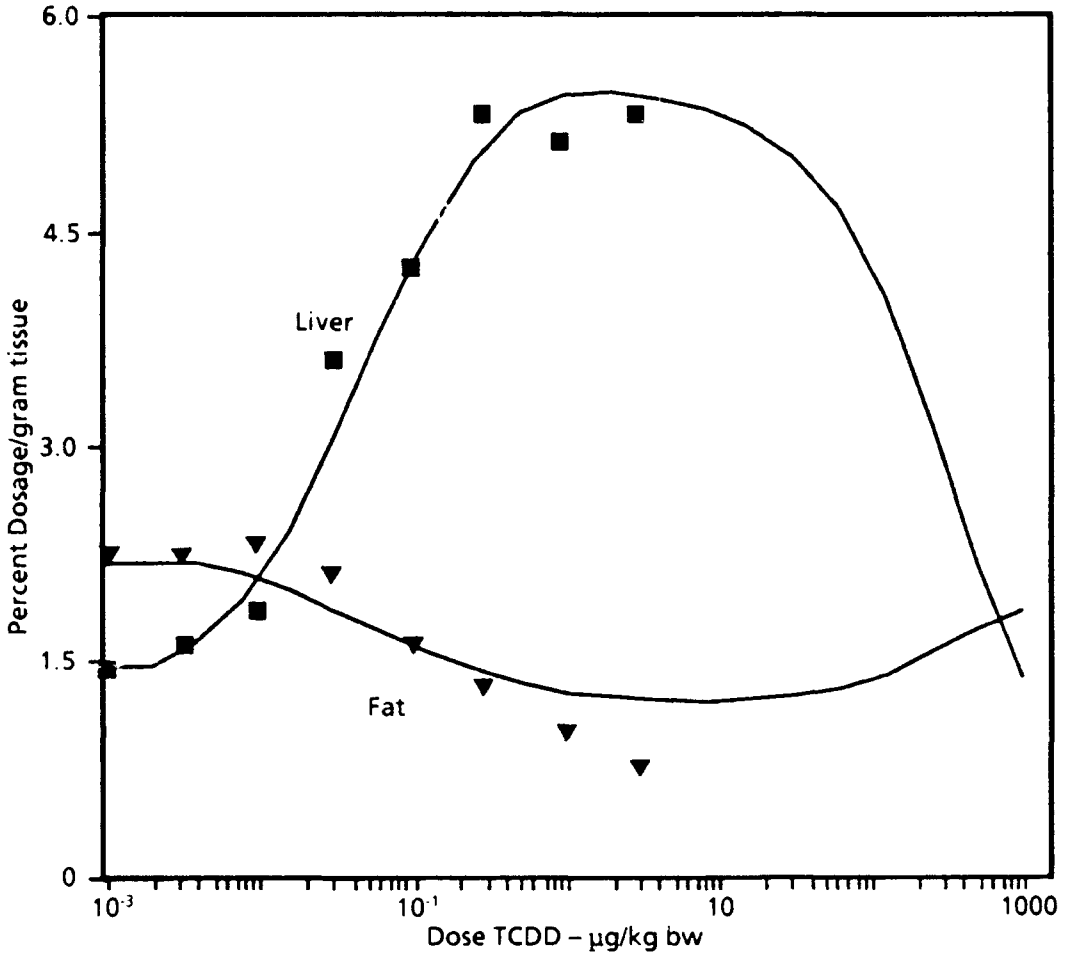


Figure 50. Dose-Dependent Dioxin Disposition in Liver and Fat. Data from female Wistar rats administered a single dose of radiolabelled TCDD s.c. [14]. Dioxin concentration is expressed as percent dose/gram tissue normalized to dose. The smooth curves represent the model simulations.

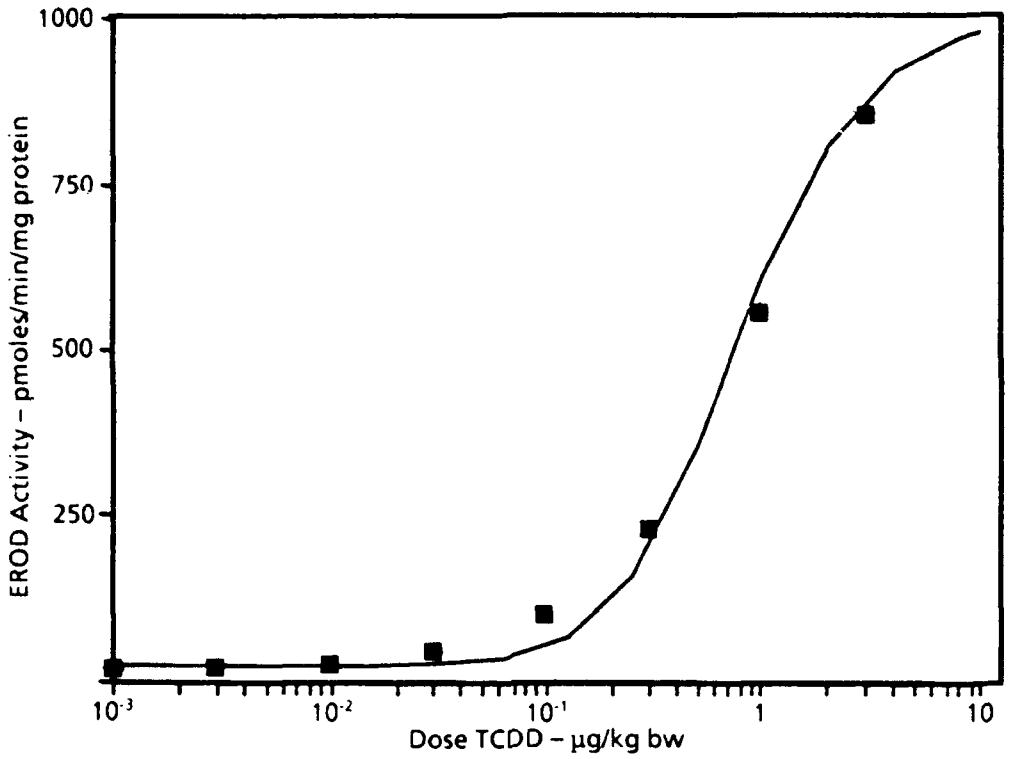


Figure 51. Dose-Dependent Induction of Cytochrome P4501A1. Data from female rats [14], acutely exposed to TCDD. The curve is the model simulation.

Growth Factors and Tumor Promotion

Transforming growth factors alpha and betas 1, 2, and 3 (TGF-α, TGF-β1, β2, β3) are potent cytokines which can have stimulatory and inhibitory effects, respectively, on cell growth. Disruption of the balance between these growth-controlling factors is believed to be important in the cleft palate lesion observed in mice exposed to TCDD prenatally [31]. Temporal changes in the expression of TGF-α, TGF-β1, and β2 are critical for fusion of the palatal shelves and dioxin has been shown to change the expression of these factors leading to cleft palate [31]. Dioxin has been shown to affect the regulation and expression of TGF-α and TGF-β2 in human keratinocyte cell-lines [11], associated with alterations in growth and differentiation characteristics.

These same growth-regulatory elements are important in the functional growth responses of the liver. Experiments investigating the processes involved in liver regeneration and following treatment by a tumor promoter, phenobarbital, have demonstrated that TGF-α and TGF-β1 are important in regulating hepatocyte growth [32]. Following partial hepatectomy, there is an initial proliferative stimulus provided by TGF-α, and others factors including hepatocyte growth factor (HGF), followed by an inhibition of that proliferation partially caused by increased TGF-β isoforms, particularly β1 [33]. In hepatic initiation-promotion studies with TCDD, Pitot and coworkers [34] found that the dose–response curve for altered hepatic foci, over the concentration range used in the

original Kociba bioassay [1], was U-shaped (Figure 52). Low doses of TCDD reduced the number of enzyme-altered foci, following diethylnitrosamine (DEN) initiation and partial hepatectomy. In more recent studies, dioxin caused an apparent inhibition of cell proliferation in areas of the liver [28] and a decrease in cell surface epidermal growth factor receptors [27], the former possibly due to increased TGF-β, and the latter hypothesized to be due to increased TGF-α [23].

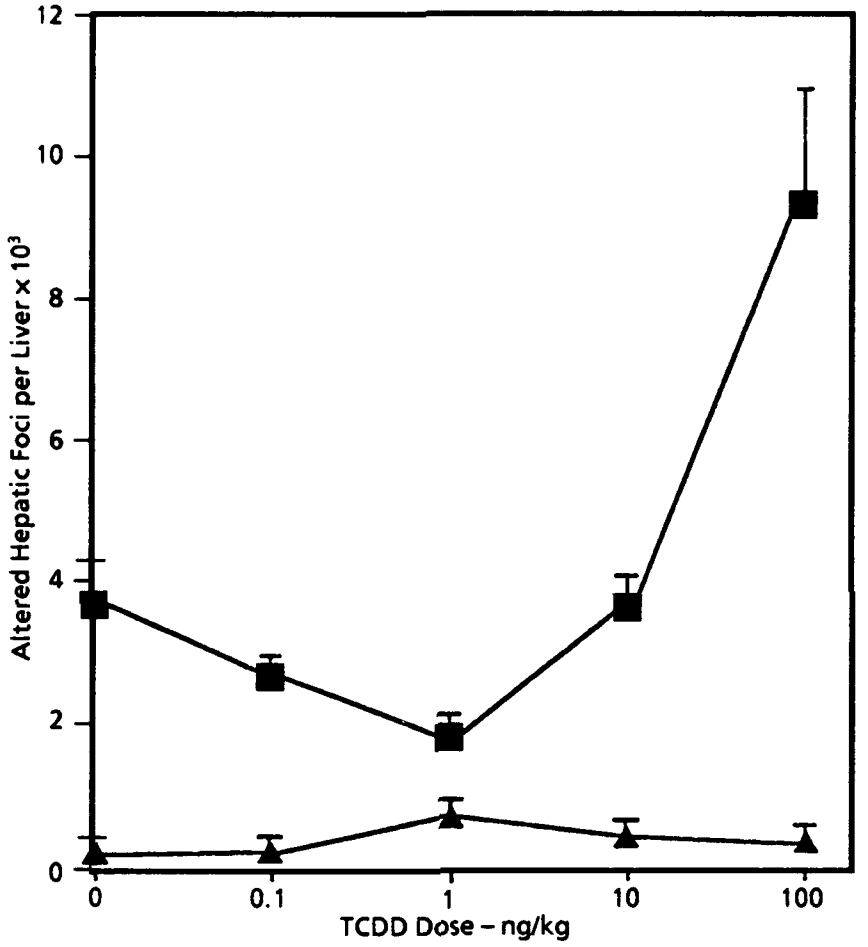


Figure 52. Dose–Response Curve for Altered Hepatic Foci in Female Fischer 344 Rat Livers. Rats were given an initiating dose of DEN (10 mg/kg) following a 70% hepatectomy. Two weeks later, TCDD was administered via intramuscular injection biweekly, for 6 months [31].

Drawing on the proposed mechanism of phenobarbital promotion [35,32], we have developed and are in the process of examining a stochastic model for promotion that proposes a biphasic activity of TCDD on cell proliferation [36]. In this hepatic promotion model, TCDD induces an initial proliferative stimulus which leads to a compensatory tissue response to suppress mitogenesis and establish a selection environment for cells with alterations in growth-regulatory pathways. In one formulation, this stochastic model supposes that there are two clonal populations (Figure 53). In DEN-treated rats not exposed to TCDD, the foci that grow out are derived from TGF-β-sensitive,

initiated cell populations. At low doses of TCDD, the growth of these clonal populations is inhibited, due to increased negative regulatory factors (i.e., TGF-β), leading to a reduced promotional response. Whereas at higher TCDD exposures, TGF-β-resistant initiated cells, which do not grow out to foci in control animals, are "stimulated" by the higher dioxin concentration (i.e., there is a negative selection system "promoting" the growth of these cells to foci). Although a great deal of work is needed to test this two precursor cell tumor promotion model, the hypothesis suggests a unifying mechanism for many mitogenic liver tumor promoters and is consistent with the U-shaped dose–response curve. It is not yet clear which factors regulated by TCDD would be involved in the initial stimulation of proliferation in the normal cells or the higher dose stimulation of the TGF-β-resistant initiated cells, but the general model, if correct, may have widespread applications in chemical risk assessments with TCDD and other liver tumor promoters.

CONCLUSION

Good progress is evident in establishing modeling strategies to produce a complete biologically based dose–response model for dioxin hepatic carcinogenesis. With PBPK models, the major determinants of disposition appear to be included, although there are some lingering questions about the nature of the binding proteins in the liver. These include: Are there proteins other than P-4501A2 that avidly bind TCDD? Is the novel cytochrome mRNA P-4501B1, induced by TCDD in skin and liver, translated into a protein and, if so, does it bind dioxin? In addition, induction of these proteins is described empirically by Hill relationships. More complete biological descriptions of gene regulation need to be articulated and described in the pharmacokinetic and pharmacodynamic models of gene regulation. Ongoing studies by a number of investigators are expected to greatly expand our understanding of the biological basis of TCDD regulation of gene expression. Among the individual steps that may need to be included are the initial TCDD-*Ah*-receptor interactions, hsp90 and arnt protein interactions with the liganded receptor, receptor phosphorylation/ dephosphorylation reactions, and the binding of the activated complex to the upstream regions of the DNA for specific growth regulatory genes.

Dioxin alters the expression of a variety of growth regulating factors as well as metabolizing enzymes. The exact mechanisms by which alterations in these factors cause growth regulatory responses in various tissues is unclear. This regulatory dysfunction has been hypothesized to be at the root of dioxin's untoward biological effects on the liver. For risk analysis modeling, it becomes critical to describe the biological basis of the suppression of proliferation in normal cells, and the basis of stimulation of growth in altered cells, in the presence of elevated levels of negative control factors (e.g., TGF-β). At this time, we can only determine the relative dose-dependencies of the compensatory mito-suppression and the high-dose stimulation of altered cells, and apply these relationships in the promotion models. These models can be linked to a cancer-incidence model

directly by assuming the equivalence of the altered cells in a promotion study to the intermediate cells in a two-stage clonal growth cancer model [37].

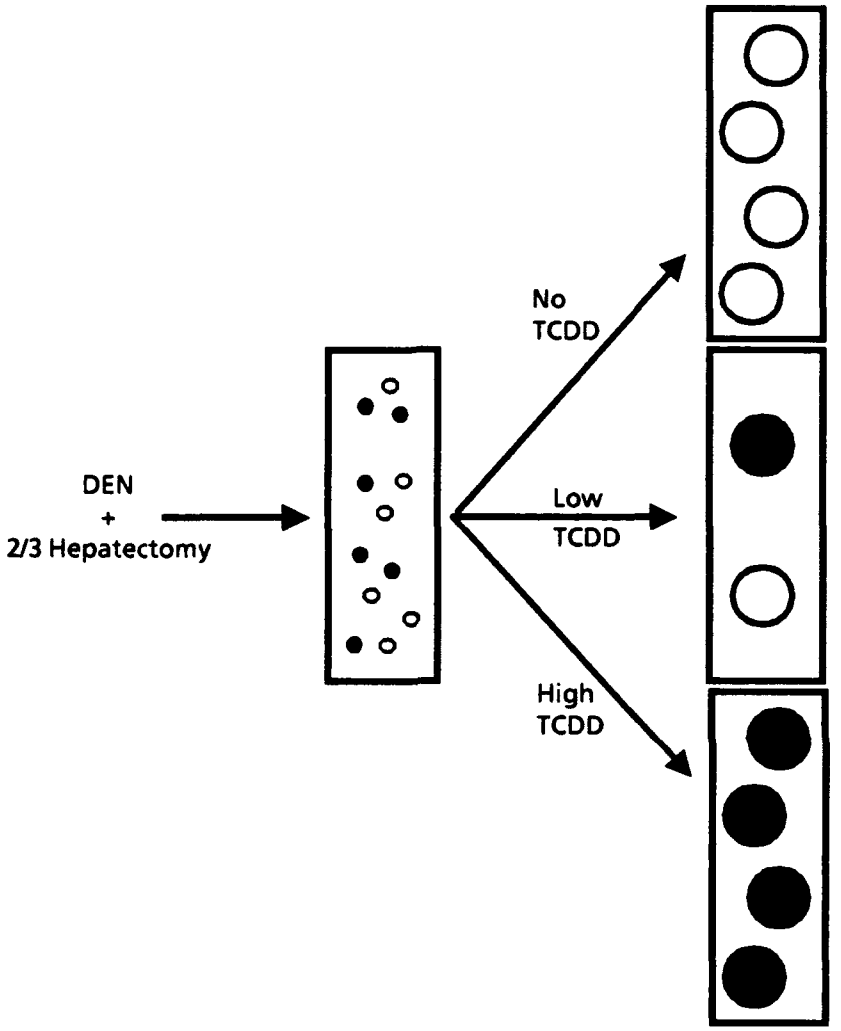


Figure 53. A Schematic of the Promotional Action of TCDD in Rat Liver. Following DEN and partial hepatectomy, the liver contains populations of initiated cells which are sensitive (O) or insensitive (●) to the effects of negative growth regulators. In animals without TCDD only the cells sensitive (O) to negative regulators (i.e., TGF-β) grow out to become observable foci. At low doses of TCDD, growth of these cells is diminished by increased in TGF-β leading to a reduced number of foci. While at higher TCDD exposures, giving rise to higher concentrations of negative growth regulators, the insensitive cells (●) have a growth advantage in relation to the rest of the liver and grow out to form foci.

Cancer is, however, not the only end point of interest with TCDD. Will this intense level of scrutiny on cancer provide any insight at all for other toxic end points? We believe that the evaluation of the mechanistic determinants for the alterations in growth characteristics in liver will provide dose–response insights for other tissues, and indirectly for other end points. Note that the suppression of liver cell mitogenesis, which may represent a primary response of normal cells to dioxin, occurs at doses almost two orders of magnitude below the doses causing frank increases in

hepatic tumor responses (Figure 52). The hepatic responses seen at low TCDD exposures may correlate with overt toxicity, or as a reduction in cancer, in other tissues. Ultimately, only completion of the development of the hepatic promotion model will tell of its utility for other end points. With hepatic promotion we are closest to having a response that can be dissected, studied, and modeled. A potential windfall, of course, is that this hepatic modeling work with TCDD may provide an improved strategy for risk assessment with a variety of hepatic promoters in rodents and have significance well beyond direct applications to TCDD alone, impacting the manner in which many rodent hepatic carcinogens become regulated.

REFERENCES

1. **Kociba, R., D. Keyes, J. Beyer, R. Carreon, C. Wade, D. Dittenber, R. Kalnins, L. Frauson, C. Park, S. Barnard, R. Hummel, and C. Humiston.** 1978. Results of a two year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats. *Toxicol. Appl. Pharmacol.* 46:279.

2. **National Institutes of Health.** 1982. Carcinogenesis bioassay of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in Osbourne-Mendel rats and B6C3f1 mice (gavage study). NTP Report Series No. 209.

3. **Pitot, H., T. Goldsworthy, H. Campbell, and A. Poland.** 1980. Quantitative evaluation of the promotion by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin of hepatocarcinogenesis from diethylnitrosamine. *Cancer Res.* 40:3616.

4. **Poland, A. and E. Glover.** 1979. An estimate of the maximum *in vivo* covalent binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to rat liver protein ribosomal RNA and DNA. *Cancer Res.* 39:3341.

5. **Buu-Hoi, N., P. Chanh, G. Sesque, M. Azum-Gelade, and G. Saint-Ruf.** 1972. Organs as targets of dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin intoxication. *Naturwissenschaften* 59:174.

6. **Murray, F., F. Smith, K. Nitschke, C. Humiston, R. Kociba, and B. Schwetz.** 1979. Three generation reproduction study of rats given 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the diet. *Toxicol. Appl. Pharmacol.* 50:241.

7. **Courtney, K. and J. Moore.** 1971. Teratology studies with 2,4,5-trichlorophenoxyacetic acid and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmacol.* 20: 396.

8. **Poland, A. and J.C. Knutson.** 1982. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanisms of toxicity. *Ann. Rev. Pharmacol.* 22:517.

9. **Perdew, G.H.** 1991. Comparison of the nuclear and cytosolic forms of the *Ah* receptor from Hepa 1c1c7 cells: Charge heterogeneity and ATP binding properties. *Arch. Biochem. Biophys.* 291:284.

10. **Wilhelmsson, A., S. Cuthill, M. Denis, A.C. Wikstrom, and J.A. Gustafsson.** 1991. The specific DNA-binding activity of the dioxin receptor is modulated by the 90 Kd heat shock protein. *EMBO J.* 9:69.

11. **Gaido, K.W., S.C. Maness, L.S. Leonard, and W.F. Greenlee.** 1991. TCDD-dependent regulation of transforming growth factors-alpha and beta-2 expression in a human

keratinocyte cell line involves both translational and post-translational control. *J. Biol. Chem.* 267:245.

12. **Sutter, T.R., K. Guzman, K.M. Dold, and W.F. Greenlee.** 1991. Targets for dioxin: Genes for plasminogen activator inhibitor-2 interleukin-1b. *Science* 254:415.

13. **Sutter, T.R. and W.F. Greenlee.** 1992. Identification of a human dioxin-responsive cDNA (clone 1) as a new member of the P450 super family. *Proceedings of Dioxin '92*, Volume 10:221.

14. **Abraham, K., R. Krowke, and D. Neubert.** 1988. Pharmacokinetics and biological activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 1. Dose-dependent tissue distribution and induction of hepatic ethoxy resorufin *O*-deethylase in rats following a single injection. *Arch. Toxicol.* 62:359.

15. **Carrier, G. and J. Brodeur.** 1991. Non-linear toxicokinetic behaviour of TCDD- like halogenated polcyclic aromatic hydrocarbons (H-PAH) in various species. *The Toxicologist* 11:895.

16. **Poland, A., P. Teitelbaum, and E. Glover.** 1989. [125I]2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin-binding species in mouse liver induced by agonists for the *Ah* receptor: Characterization and localization. *Mol. Pharmacol.* 36:113.

17. **Voorman, R. and S.D. Aust.** 1989. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is a tight binding inhibitor of cytochrome P450d. *J. Biochem. Toxicol.* 4:105.

18. **Leung, H., R. Ku, D. Paustenbach, and M. Andersen.** 1988. A physiologically based pharmacokinetic model for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in C57 Bl/6J and DBA/2J mice. *Toxicology Lett.* 42:15.

19. **Leung, H., D. Paustenbach, F. Murray, and M. Andersen.** 1990. A physiological pharmacokinetic dsecription of the tissue distribution and enzyme inducing properties of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat. *Toxicol. Appl. Pharmacol.* 103:399.

20. **Mills, J. and M. Andersen.** 1992. Toxicokinetics of dioxin and related compounds. In: *Proceedings of the Workshop on Risk Assessment and Risk Management Of Toxic Chemicals*, p. 102. *Natl. Inst. Environ. Stud.*

21. **Andersen, M.E., J.J. Mills, M.L. Gargas, L.B. Kedderis, L.S. Birnbaum, D. Neubert, and W.F. Greenlee.** 1992. Modelling receptor-mediated protein induction by dioxin: Implications for pharmacokinetics and risk assessment. *Risk Anal.* 13:25-34.

22. **Kedderis, L.B., J.J. Mills, M.E. Andersen, and L.S. Birnbaum.** In Press. A physiologically-based pharmacokinetic model of 2,3,7,8-tetrabromo-dibenzo-*p*-dioxin (TBDD) in the rat: Tissue distribution and CYP1A induction. *Toxicol. Appl. Pharmacol.*

23. **Kohn, M.C., G.W. Lucier, G.C. Clark, C. Sewall, A.M. Tritscher, and C.J. Portier.** Submitted. A mechanistic model of the effects of dioxin on gene expression in the rat liver.

24. **Kedderis, L., J. Diliberto, P. Linko, J. Goldstein, and L. Birnbaum.** 1991. Disposition of 2,3,7,8-tetrabromodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat: Biliary excretion and induction of cytochromes CYP1A1 and CYP1A2. *Toxicol. Appl. Pharmacol.* 111:163.

25. **Bars, R.G. and C.R. Elcombe.** 1991. Dose–dependent acinar induction of cytochromes in rat liver. *Biochem. J.* 277:577.

26. **Mills, J.J., J. Murphy, O. Lyght, T.L. Goldsworthy, and M.E. Andersen.** 1993. Time course and regional distribution of cytochrome P4501A in rat liver following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *The Toxicologist* 13:192.

27. **Trischer, A.M., J.A. Goldstein, C.J. Portier, Z. McCoy, G.C. Clark, and G.W. Lucier.** 1991. Dose–response relationships for chronic exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a rat promotion model: Quantification and immunolocalization of CYP1A1 and CYP1A2 in the liver. *Cancer Res.* 52:3436.

28. **Fox, T.R., L.L. Best, S.M. Goldsworthy, J.J. Mills, and T.L. Goldsworthy.** Submitted. Gene expression and cell proliferation in the rat liver after acute exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

29. **Ema, M., K. Sogawa, N. Watanabe, Y. Chujoh, N. Matsushita, O. Gotoh, Y. Funae, and Y. Fujii-Kuriyama.** 1992. cDNA cloning and structure of mouse putative *Ah* receptor. *Biochem. Biophys. Res. Comm.* 184:246.

30. **Hoffman, E.C., H. Reyes, F.F. Chu, F. Sander, L.H. Conley, B.A. Brooks, and O. Hankinson.** 1991. Cloning of a factor required for activity of the *Ah* (dioxin) receptor. *Science* 252:954.

31. **Abbott, B.D., M.W. Harris, and L.S. Birnbaum.** 1992. Comparisons of the effects of TCDD and hydrocortisone on growth factor expression provide insight into their interaction in the embryonic mouse palate. *Teratology* 45:35.

32. **Jirtle, R.L., S.A. Meyer, and J.S. Brockenbrough.** 1991. Liver tumor promoter phenobarbital: A biphasic modulator of hepatocyte proliferation. In: *Chemically Induced Cell Proliferation: Implications for Risk Assessment*, p. 209. Wiley-Lies Inc.

33. **Jakowlew, S.B., J.E. Mead, D. Danielpour, J. Wu, A.B. Roberts, and N. Fausto.** 1991. Transforming growth factor-beta (TGF-β) isoforms in rat liver regeneration: Messenger RNA expression and activation of latent TGF-β. *Cell Reg.* 2:535.

34. **Pitot, H.C., T.L. Goldsworthy, S. Moran, W. Kennan, H.P. Glauert, R.R. Maronpot, and H.A. Campbell.** 1987. A method to quantitate the relative initiating and promoting potencies of hepatocarcinogenic agents in their dose-response relationships to altered hepatic foci. *Carcinogenesis* 8:1491.

35. **Schulte-Hermann, R., W. Parzefall, and W. Bursch.** 1987. Role of liver growth in hepatocarcinogenesis. In: Butterworth and Slaga, eds. *Non-Genotoxic Mechanisms of Carcinogenesis.* Banbury Report 25:91, Cold Spring Harbour.

36. **Andersen, M.E., J.J. Mills, L.S. Birnbaum, and R. Conolly.** 1993. Stochastic dose response modelling of the hepatic promotion by dioxin. *The Toxicologist* 13:196.

37. **Moolgavkar, S.H.** 1989. A two stage carcinogenesis model for risk assessment. *Cell Biol. Toxicol.* 5:445.

APPENDIX

PARAMETERS IN THE PBPK MODEL FOR DIOXIN

Model Parameters	Abbreviations	Wistar Rat
Body weight (kg)	bw	0.215
Volumes (L)		
Liver	V_l	0.0375 × bw
Fat	V_f	0.07 × bw
Richly perfused (Viscera)	V_r	0.0525 × bw
Slowly perfused (Muscle/skin)	V_s	0.75 × bw
Blood	V_b	0.05 × bw
Tissue blood volumes		
Liver	V_{lb}	0.01 × V_f
Fat	V_{fb}	0.05 × V_f
Richly Perfused	V_{rb}	0.01 × V_r
Slowly perfused	V_{sb}	0.05 × V_s
Cardiac output (L/h)	Q_c	4.4
Tissue blood flow (% Qc)		
Liver (portal and arterial)	Q_l	25
Richly perfused	Q_r	51
Fat	Q_f	9
Slowly perfused	Q_s	15
Diffusional tissue clearance (L/h)		
Liver	PA_l	0.5 × Q_l
Fat	PA_f	0.2 × Q_f
Richly perfused	PA_r	0.5 × Q_r
Slowly perfused	PA_s	0.5 × Q_s
Metabolic Constants		
Metabolism (h^{-1})	kfc	1.65
Induction (fold over basal rate)	fold	1.00
Partition Coefficients		
Liver/blood	P_l	20
Fat/blood	P_f	375
Richly perfused/blood	P_r	20
Slowly perfused/blood	P_s	30

Model Parameters	Abbreviations	Wistar Rat
Protein Binding		
Ah maximum (pmoles/liver)	BM_1	3.75
Ah affinity (pM)	KB_1	35
1A2 basal level (nmoles/liver)	$BM2_0$	10
1A2 maximum (nmoles/liver)	$BM2_1$	85
1A2 affinity (nM)	KB_2	6.5
1A2 - Hill term	n	1.0
1A2 - Hill binding constant (pM)	Kd	50
1A1 - Hill term	n_1	2.3
1A1 - Hill binding constant (pM)	Kd_1	180
1A1 - degradation rate constant (h^{-1})	k_1	0.035
1A1 synthesis-basal rate (units/h)	K_0	0.7
1A1 maximum induction (fold)	K_{0max}	50

Tissue volumes are given as liters and are referenced to tissue as a proportion of the total body weight, assuming unit density (1 kg = 1 L).

SESSION IV
SELECTED MECHANISMS IN CARCINOGENICITY

PEROXISOME PROLIFERATORS: PARADIGMS AND PROSPECTS

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SUMMARY

Peroxisome proliferators are a structurally diverse group of chemicals. They include fibrate hypolipidaemic drugs, phthalate ester plasticisers, phenoxy acid herbicides, azole antifungal drugs, and perfluorinated fatty acids. This presentation will focus on the common pleotropic responses produced by these compounds including hepatomegaly (hyperplasia and hypertrophy), activation of cell cycle *S*-phase ploidy changes, cytochrome P450 4A1 induction, morphometric/biochemical analysis of peroxisome proliferation and stimulation of growth factors, and oncogene activation. Consideration will also be given to the role of recently described Peroxisome Proliferator Activated Receptor in these diverse hepatic responses.

Peroxisome proliferators are uniformly negative in a wide range of genotoxicity tests, but nevertheless are complete carcinogens, particularly in rodent liver. Mechanisms of nonmutagenic carcinogenesis will be discussed including the active oxygen hypothesis involving 8-hydroxydeoxyguanosine adducts and the possibility of peroxisome proliferators promoting preexisting lesions by clonal expansion, eventually resulting in frank tumorigenesis. Finally, a consideration of the risk assessment of peroxisome proliferation to humans will be discussed.

INTRODUCTION

The peroxisome proliferators are a structurally diverse group of chemicals and include the fibrate hypolipidemic drugs (clofibrate, ciprofibrate), the phthalate ester plasticisers (di-(2-ethylhexyl)phthalate [DEHP] and mono-ethylhexyl phthalate [MEHP], certain polychlorinated biphenyl isomers, chlorophenoxy acid herbicides, dehydroepiandrosterone (a naturally occurring C_{19}steroid), certain azole antifungal drugs (bifonazole), perfluoro fatty acids, high fat diets, and certain pathophysiological states such as diabetes [1-3]. The major toxicological interest in peroxisome proliferators arises because of the potential risk of carcinogenesis. Quite clearly the exposure element is a predominant factor in the risk assessment equation but the human hazard associated with these compounds is less easy to assess. Accordingly, it is the purpose of this paper to describe current concepts on the mechanisms of peroxisome proliferation, the mechanism(s) of hepatocarcinogenicity, and the risk assessment to humans.

Genetic versus Nonmutagenic Carcinogens

The classical definition of chemically induced carcinogenesis includes the stages of initiation, promotion, and progression and one of the key characteristics of genetic carcinogens being that they cause eitner direct genetic damage through DNA mutations, strand breaks, or rearrangements, or indirect damage mediated by a metabolite after activation [4]. However, the nonmutagenic carcinogens do not fall easily into this category, because as their name implies, they are uniformly negative in a battery of *in vitro* and *in vivo* tests designed to identify genetic toxicity and are therefore classified on this basis as nonmutagenic carcinogens. The existence of this latter group of carcinogens, therefore, represents three major problems. First, if they are nonmutagenic, how can we test for them? Quite clearly, they will turn up positive in a cancer bioassay in experimental animals but this is usually a late event, occurring approximately 9 months after initial exposure at the very earliest. However, both the pharmaceutical and chemical industries are generally dissatisfied with this "delayed toxicity" and the existence of some form of testing and/or early identification system would be desirable.

The second problem that emerges is that if these compounds are not directly genotoxic (nor their metabolites), what is the molecular mechanism of action of these nonmutagenic carcinogens?

The third problem that emerges is that if we are not fully sure of the molecular mechanisms of action, how can we, in general terms, classify nonmutagenic carcinogens? This is indeed a difficult question in the absence of mechanistic information but it is possible to classify them on the organelle they affect most. For example, most nonmutagenic hepatocarcinogens cause liver enlargement which may result from either endoplasmic reticulum induction, increases in peroxisomes, or increases in mitochondria. In addition, there are some nonmutagenic hepatocarcinogens that produce chronic liver damage and hence possible toxicity by the ensuing reparative hyperplasia by as yet undefined mechanisms. It is very likely that the above groups of nonmutagenic hepatocarcinogens will not cover all the examples, but is a useful starting point for discussion.

Biological Effects of Peroxisome Proliferators

Of the nonmutagenic carcinogens identified to date, the peroxisome proliferators are by far the most extensively studied [3]. These compounds cause morphological and/or biochemical changes in several tissues including the testis, thyroid, kidney, intestine, adrenals, brown adipose tissue, heart, and liver [1-3]. The liver effects of peroxisome proliferators have been most extensively investigated and the four major hepatic responses are

- hepatomegaly,
- induction of drug metabolizing enzymes,

- peroxisome proliferation, and
- nonmutagenic hepatocarcinogenesis.

The hepatomegaly leads to an approximate doubling of the liver size, usually as a result of both hypertrophy and hyperplasia, concomitant with induction of drug metabolizing enzymes such as glucuronyl transferase [3], epoxide hydrolase and the cytochrome P4504A subfamily of fatty acid w-hydroxylases [5], the latter comprising approximately nine separate genes. Much work has been carried out on the latter w-hydroxylases and it has been suggested that the induction of cytochrome P450s and the phenomenon of peroxisome proliferation are mechanistically interrelated [2,6].

Peroxisome proliferation itself is characterized both by substantial increases in peroxisome numbers per cell and by very marked increases in some, but not all, peroxisome enzyme activities. For example, the peroxisomal fatty acid β-oxidation system can be induced up to 50-fold by these compounds, whereas catalase is marginally induced, and cytosolic glutathione peroxidase enzyme activity is actually inhibited/switched off by the peroxisome proliferators [3]. The peroxisomal fatty acid β-oxidation system is broadly similar to that in the mitochondrion, with the major exception that the first enzyme in the peroxisomal β-oxidation spiral produces hydrogen peroxide as a side product, which has been suggested to contribute to the hepatocarcinogenicity of this class of compounds [3] and see below). Lower rodents such as rats and mice appear to be particularly susceptible to peroxisome proliferation whereas the balance of evidence tends to suggest that higher species such as the guinea pig and the marmoset appear much less susceptible to peroxisome proliferation [3]. In this context it should also be pointed out that much controversy exists as to species differences in susceptibility to the hepatocarcinogenicity of peroxisome proliferators, with obvious implications for human risk assessment (see below).

With respect to the timeframe of biological responses to the peroxisome proliferators, it appears that, at least for fenofibrate (Richard Hinton, personal communication), the following sequence of events occurs: alteration in fatty acid metabolism (minutes), DNA synthesis and enzyme induction (hours/days), lipofuscin accumulation and foci development (months) and finally tumor formation (months/years).

The Peroxisome Proliferator Activated Receptor (PPAR)

The fact that many functionally diverse genes are regulated by peroxisome proliferators and the above ordered sequence of events would indicate the existence of a receptor or receptor-related events that may be closely related to the phenomenon of peroxisome proliferation. This is very likely the case as a PPAR has recently been isolated, cloned, and sequenced by Stephen Green and his colleagues [7,8]. This receptor belongs to the steroid hormone receptor superfamily of receptors and contains both DNA-binding and ligand (enzyme inducer)-binding domains. The DNA-binding domain

shows a substantial degree of sequence homology (approximately 50%) to the equivalent region in several steroid hormone receptors and may well rationalize why so many functionally diverse enzymes are up-regulated by the peroxisome proliferators. As shown in Figure 54, the common recognition of motifs in the 5' flanking regions of inducible genes by the ligand-PPAR complex provides at least a conceptual basis to rationalize other phenomena such as growth, differentiation, and species differences in response, among others [8].

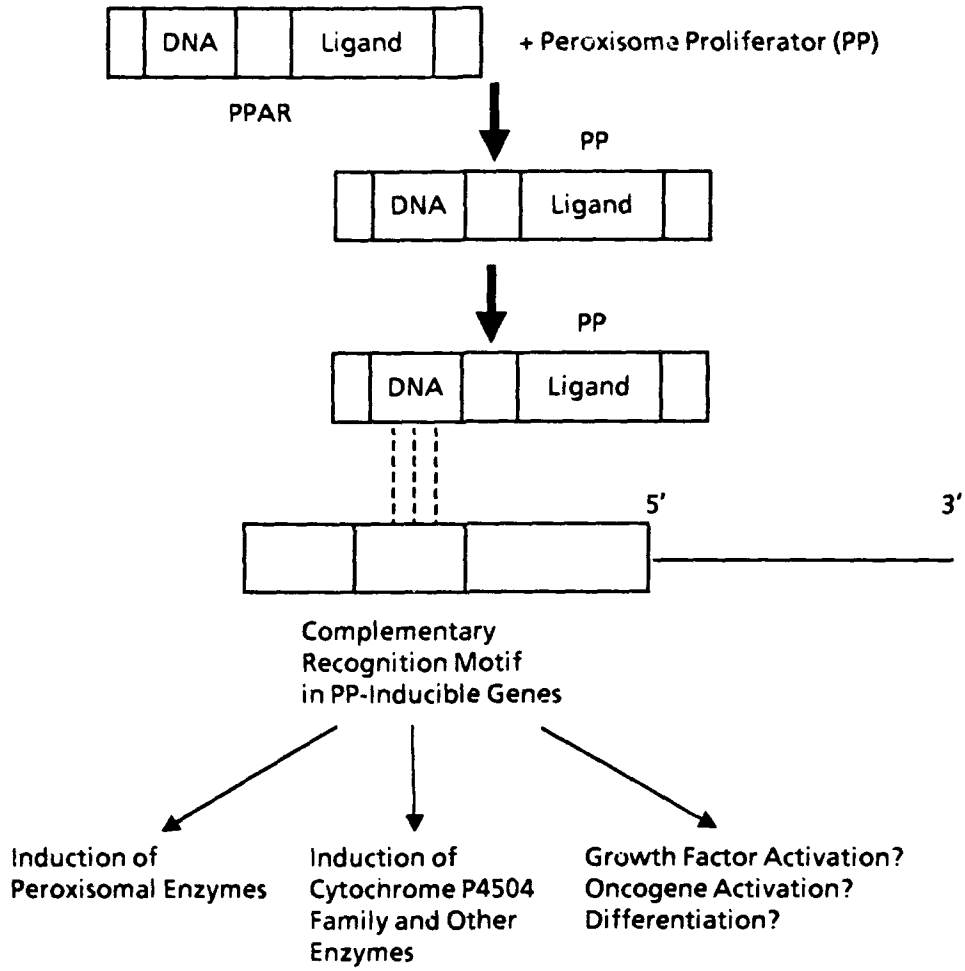

Figure 54. The Role of Peroxisome Proliferator Activated Receptor (PPAR) in Hepatic Pleotropic Responses to Peroxisome Proliferators [23].

Any consideration of the biological effects of peroxisome proliferators must take into account the structural diversity of the chemicals. Although the majority of peroxisome proliferators contain a carboxyl group (or are metabolized to one or contain a chemical grouping which is bioisosteric to the carboxyl group), there are several which do not. Therefore, is the ligand binding site of the PPAR very accommodating or do multiple PPAR receptors with different ligand-binding properties exist? Unfortunately the receptor protein has not been isolated to date, and we currently have no firm

details regarding the geometry of the ligand binding site. However, the ligand binding domain has been modeled using a knowledge of the primary amino acid sequence deduced from the cDNA sequence [7] and standard protein folding software packages (D. Lewis, University of Surrey, personal communication). This model identifies tyrosine residues in the helix which possibly ring overlap with the ring systems in inducers such as clofibrate. Of even more importance, the epsilon-amino group of a lysine residue in the ligand binding domain may play a crucial role in binding the carboxyl function of peroxisome proliferators containing this chemical group. However, it must be emphasized that these modeling studies are preliminary in nature and must await physical characterization of the receptor protein itself. With respect to the existence of multiple PPAR receptors, there is a small amount of preliminary information that this may indeed be the case but again this awaits further progress in this field.

Mechanisms of Hepatocarcinogenicity of the Peroxisome Proliferators

It should be clearly recognized from the outset that no general agreement has been reached on the precise molecular mechanism(s) of action of the hepatocarcinogenicity of the peroxisome proliferators, and it remains an area of active debate and ongoing investigation. The "active oxygen" hypothesis put forward by Jan Reddy and his colleagues [1] was the first, and has been extensively addressed by several research groups. As shown in Figure 55, this oxidative stress hypothesis originates in the observation that, after chemical challenge with a peroxisome proliferator, there is a several-fold induction of the peroxisomal fatty acid β-oxidation spiral with the associated increase in the peroxisomal hydrogen peroxide H_2O_2 synthesis. Normally the cell is protected from the hepatotoxicity of H_2O_2 (or more likely its metabolites such as the hydroxide radical) by peroxisomal catalase and cytosolic glutathione peroxidase enzymes. According to this theory the cell is now compromised because the excessive H_2O_2 cannot be adequately detoxified by glutathione peroxidase (the enzyme is inhibited by peroxisome proliferators) and is also compromised because the catalase induction (1- to 2-fold) does not match the substantial increase in peroxide production. Subsequently (Figure 2) the hydroxide radical is thought to cause several responses including lipid peroxidation, membrane damage, and lipofuscin accumulation, the latter being a characteristic hepatic response to all peroxisome proliferators. The ultimate DNA damage by peroxisome proliferators is thought to arise by the interaction of peroxide-derived hydroxide radicals with deoxyguanosine yielding the 8-hydroxy deoxyguanosine adduct [9]. This adduct is formed by several peroxisome proliferators and the level of adduct formation is approximately 1.5- to 2.0-fold above control values in long-term feeding studies [9]. If this oxidative stress hypothesis is correct then the level of this adduct formation must be sufficient to account for the tumorigenicity. Whether or not this small increase in adduct formation is sufficient to initiate genetic damage which is further "promoted" by the ongoing hyperplasia is not clear at present and requires further examination.

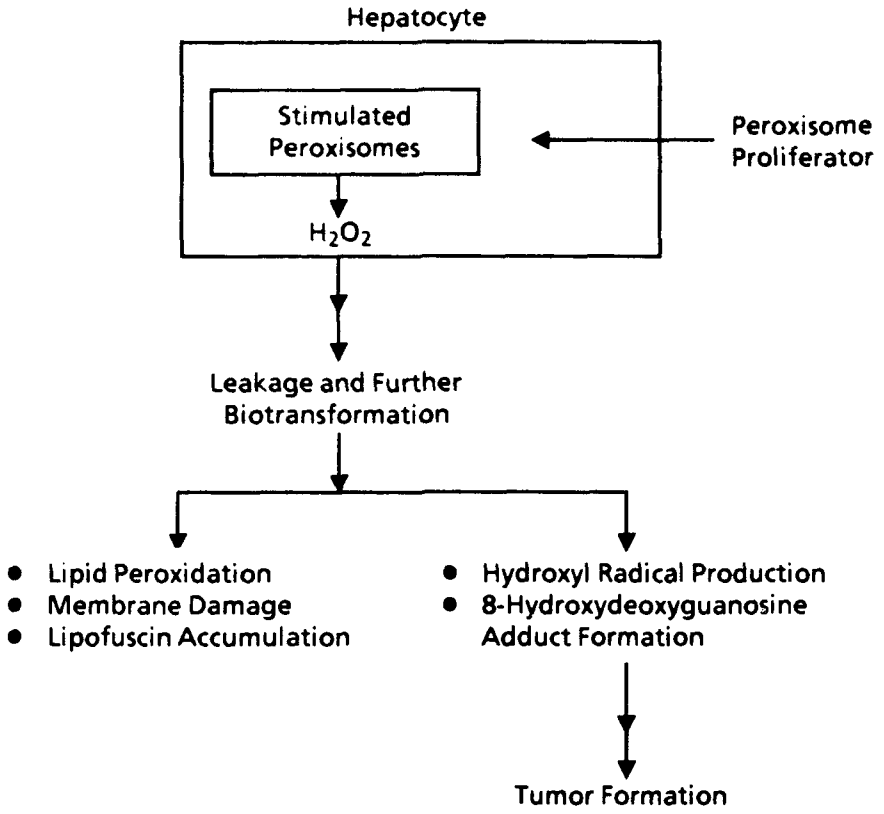

Figure 55. The Oxidative Stress Hypothesis for Peroxisome Proliferator-Induced Hepatocarcinogenicity.

It may well be that the hyperplastic response is a much better indicator of eventual nonmutagenic hepatocarcinogenesis than peroxisome proliferation per se. Specifically, doubt has been cast on the correlation between peroxisome proliferation and eventual neoplasia [10]. In these latter experiments, rats were fed either Wy-14,643 or DEHP at dose levels that produced a 100% incidence and 10% incidence of hepatocellular tumors respectively, despite little difference in the magnitude of peroxisome enzyme induction between the two compounds. Accordingly, this study has opened up a debate on the relevance of peroxisome proliferation (exclusively) contributing to the hepatocarcinogenicity of these compounds and has indicated that other factors and/or mechanisms also need to be considered [19].

Peroxisome proliferators do not appear to act as tumor initiators in that several chemicals (Nafenopin, DEHP, Wy-14,643 and clofibrate) are uniformly negative as initiators in several promotion systems [11,12]. Some evidence exists to classify peroxisome proliferators as tumor promoters in several experimental protocols [13,14] and that the eventual neoplasia is a result of selection and clonal expansion of these preinitiated foci in older rodents and their greater susceptibility to peroxisome proliferator-induced hepatocarcinogenesis [15]. Although this remains

an attractive hypothesis, it is quite clear that further experimentation is required to clearly identify a precise role(s) for peroxisome proliferators in the complex biology of tumor promotion.

Prospects in Peroxisome Proliferation

In addition to the areas identified above, there are also several areas that warrant experimental emphasis in the future. For example, the ploidy status and peroxisome proliferation has been investigated by Jerry Styles and colleagues at ICI, Macclesfield, U.K. Acute peroxisome proliferator-induced hyperplasia occurs principally in a fraction of the binucleated ($2 \times 2n$) hepatocytes in the rat (a susceptible species). By contrast, the guinea pig (a much less responsive, if not nonresponsive species) is largely devoid of these apparently susceptible $2 \times 2n$ hepatocytes. In the rat, there are ploidy changes, which leads to two daughter mononucleated tetraploid cells, (i.e., an increase in ploidy and decreased binucleated population of cells). What the biological significance of binucleation and polyploidisation is and their relevance to hyperplasia and peroxisome proliferation remains to be further clarified and the interested reader is referred to reference 16 for a more extensive discussion of this topic.

Signal transduction pathways [17] related to protein kinase C activation [17] and the role of growth factor modulation and oncogene activation [18] remain active areas of research interest and must await a more robust integration into conceptual frameworks of toxicity for this class of compounds.

Risk Assessment for Peroxisome Proliferators in Humans

Although most investigators agree that lower rodents such as rats and mice are susceptible to the hepatocarcinogenicity of peroxisome proliferators, there is much less agreement on the hazard posed to humans. Certainly humans are extensively exposed, sometimes at high dose levels to peroxisome proliferators such as the clinically used hypolipidaemic drugs such as clofibrate, fenofibrate and the newly introduced ciprofibrate and other environmentally derived chemicals. But what is the hazard and the risk? It is the view of several (but not all) investigators in this area that the balance of evidence infers that humans are not a susceptible species. The evidence for this is derived from several sources including lack of the characteristic responses in otherwise viable human hepatocytes [19] and the available epidemiological evidence [20, 21]. In addition, it appears that the human PPAR is expressed at very low levels and perhaps even in mutated or truncated forms (Stephen Green and Eric Johnson, personal communications).

ACKNOWLEDGEMENTS

The author would like to thank the U.S. Air Force Office of Scientific Research for support.

REFERENCES

1. **Reddy, J.K. and N.D. Lalwani.** 1983. Carcinogenesis by peroxisome proliferators: Evaluation of the risk of hypolipidaemic drugs and industrial plasticisers to humans. *CRC Crit. Rev. Toxicol.* 12:1-58.

2. **Lock, E.A., A.M. Mitchell, and C.R. Elcombe.** 1989. Biochemical mechanisms of induction of hepatic peroxisome proliferation. *Ann. Rev. Pharmacol. Toxicol.* 29:145-163.

3. **Moody, D.E., J.K. Reddy, B.G. Lake, J.A. Popp, and D.H. Reese.** 1991. Peroxisome proliferation and nongenotoxic carcinogenesis: Commentary on a symposium. *Fundam. Appl. Toxicol.* 16:233-248.

4. **Williams, G.M. and J.H. Weisburger.** 1991. Chemical carcinogenesis. In M.O. Amdur, J. Doull and C.D. Klassen, eds. *Casarett and Doull's Toxicology*, Fourth Edition, pp. 127-200. New York: Pergamon Press.

5. **Gibson, G.G., T.C. Orton, and P.P. Tamburini.** 1982. Cytochrome P450 induction by clofibrate. Purification and properties of a hepatic cytochrome P450 relatively specific for the 12- and 11-hydroxylation of dodecanoic acid (lauric acid). *Biochem. J.* 203:161-168.

6. **Sharma, R., B.G. Lake, J. Foster, and G.G. Gibson.** 1988. Microsomal cytochrome P452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver: A mechanistic inter-relationship. *Biochem. Pharmacol.* 37:1193-1201.

7. **Isseman, I. and S. Green.** 1990. Activation of a member of the steriod hormone receptor superfamily by peroxisome proliferators. *Nature* 347:645-650.

8. **Green, S.** 1992. Commentary: receptor-mediated mechanisms of peroxisome proliferators. *Biochem. Pharmacol.* 43:393-401.

9. **Kasai, H., Y. Okada, S. Nishimura, M.S. Rao, and J.K. Reddy.** 1989. Formation of 8-hydroxydeoxyguanosine in liver DNA of rats following long-term exposure to a peroxisome proliferator. *Cancer Res.* 49:2603-2605.

10. **Marsman, D.S., R.C. Cattley, J.G. Conway, and J.A. Popp.** 1988. Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di-(2-ethylhexyl) phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (WY-14,643) in rats. *Cancer Res.* 48:6739-6744.

11. **Cattley, R.C., D.S. Marsman, and J.A. Popp.** 1989. Failure of the peroxisome proliferator WY-14,643 to initiate growth-selectable focii in rat liver. *Toxicology* 56:1-7.

12. **Glauert, H.P. and T.D. Clark.** 1989. Lack of initiating activity of the peroxisome proliferator ciprofibrate in two stage hepatocarcinogenesis. *Cancer Lett.* 43:95-100.

13. **Cattley, R.C., and J.A. Popp.** 1989. Difference between the promoting activities of the peroxisome proliferator Wy-14,643 and phenobarbital in rat liver. *Cancer Res.* 49:3246-3251.

14. **Kraup-Grasl, B., W. Huber, B. Putz, V. Gerbracht, and R. Schulte-Hermann.** 1990. Tumor promotion by the peroxisome proliferator nafenopin involving a specific subtype of altered focii in rat liver. *Cancer Res.* 50:3701-3708.

15. **Kraup-Grasl, B., W. Huber, H. Taper, and R. Schulte-Hermann.** 1991. Increased susceptibility of aged rats to hepatocarcinogenesis by the peroxisome proliferator nafenopin and the possible involvement of altered liver focii occurring spontaneously. *Cancer Res.* 51:666-671.

16. **Styles, J.A.** In Press. Hyperplastic responses to peroxisome proliferators. In: G.G. Gibson and B.G. Lake, eds. *Peroxisome Proliferation: Mechanisms and Toxicity.* London: Taylor and Francis.

17. **Bronfman, M., A. Orellano, M.N. Morales, F. Bieri, F. Waechter, W. Staubli, and P. Bentley.** 1989. Potentiation of diacyglycerol-activated protein kinase C by acyl-CoA thiosters of hypolipidaemic drugs. *Biochem. Biophys. Res. Commun.* 159:1026-1031.

18. **Cherkaoui-Malki, M., Y.C. Lane, M. Corral-Dbriniski, and N. Latruffe.** 1990. Differential proto-oncogene mRNA induction from rats treated with peroxisome proliferators. *Biochem. Biophys. Res. Commun.* 173:855-861.

19. **Elcombe, C.R. and A.M. Mitchell.** 1986. Peroxisome proliferation due to di-(2- ethythexyl) phthalate (DEHP). Species differences and possible mechanisms. *Environ. Health Perspec.* 70:211-219.

20. **Blumcke, S., W. Schwartzkopff, H. Lobeck, N.A. Edmonson, D.E. Prentice, and G.F. Blane.** 1983. Influence of fenofibrate on cellular and subcellular liver structure in hyperlipidaemic patients. *Athero.* 46:105-116.

21. **Hanefield, M., C. Kemmer, and E. Kadner.** 1983. Relationship between morphological changes and lipid-lowering action of p-chlorphenoxy-isobutyric acid (CPIB) on hepatic mitochondria and peroxisomes in man. *Athero.* 46:239-246.

THE ROLE OF DICHLOROACETATE IN THE HEPATOCARCINOGENICITY OF TRICHLOROETHYLENE

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SUMMARY

The induction of hepatic tumors in B6C3F1 mice treated with trichloroethylene (TRI) has been attributed to its metabolism to trichloroacetate (TCA). Trichloroacetate is an effective peroxisome proliferator in mice at blood concentrations that are readily achieved with carcinogenic doses of TRI. Recent data has demonstrated that both TCA and dichloroacetate (DCA) are capable of inducing liver tumors in B6C3F1 mice. Although long recognized as a metabolite of TRI, little attention has focussed on the role DCA might play in the hepatocarcinogenic effects of TRI. There are significant differences in the effects of DCA and TCA on the liver of B6C3F1 mice. Trichloroacetate treatment induces peroxisome proliferation, increases lipid deposition, and results in a marked accumulation of lipofuscin in the liver with long-term exposures. Dichloroacetate induces a markedly enlarged liver associated with a cytomegaly and large accumulations of glycogen. The cytomegaly is associated with the development of focal areas of recurrent liver necrosis which in turn lead to high levels of cell proliferation in the area surrounding these lesions. Induction of peroxisomes with DCA is transitory and the accumulation of lipofuscin is much less evident than with TCA treatment. Studies of TRI metabolism demonstrate that blood levels of DCA produced are sufficient to account for the hepatocarcinogenic effects of TRI. The rather low concentrations of DCA found in the urine of mice treated with TRI relative to TCA concentrations are due to the much more rapid and complete metabolism of DCA. These data do not support the conclusion that the hepatocarcinogenic effects of TRI are simply related to peroxisome proliferation.

INTRODUCTION

A considerable amount of data [1,2,3] has been developed to support the hypothesis that the greater sensitivity of B6C3F1 mice to the hepatocarcinogenic effects of trichloroethylene (TRI) is the result of its much more rapid metabolism to trichloroacetate (TCA). The recent demonstration that TCA is itself capable of inducing hepatic tumors in B6C3F1 mice [4,5] adds considerable support to this hypothesis. This issue is very important to the assessment of the cancer risks that are posed by human exposure to this chemical, because TCA is well established as a peroxisome proliferator [6,7]. The apparent inability of peroxisome proliferators to induce this pleiotropic response in humans or

human hepatocytes *in vitro* [8], has resulted in the suggestion that humans are unlikely to be sensitive to the carcinogenic effects of this class of chemicals.

On the other hand, two other metabolites of TRI, dichloroacetate (DCA) [4,5,9] and trichloroacetaldehdye (TCAA) [10], have been shown capable of producing hepatic tumors in B6C3F1 mice. Previous work has demonstrated measurable amounts of DCA in the blood of mice treated with TRI [11,12], but there is little evidence of this metabolite in the blood of rats or dogs administered equivalent doses. As a consequence, the potential contribution of this metabolite to the hepatocarcinogenic responses to TRI require more quantitative evaluation.

Mechanisms of Hepatocarcinogenic Effects of TCA and DCA

If TCA and DCA were to act by the same mechanism, there would be little reason to be concerned about the relative impact of the two metabolites on the hepatocarcinogenic response. The mechanisms by which DCA or TCA act have not been definitively established. However, there are significant differences in the nontumor pathology induced by these chemicals, the biochemical effects they produce, and the properties of the tumors that they induce.

DCA induces a very severe cytomegaly which is associated with large accumulations of glycogen. These effects progress to a point where repeated focal areas of necrosis are observed after about 2 weeks of treatment [5, 14]. The production of these lesions is accompanied by a very intense local cell proliferation [14], which essentially elevates the overall rate of cell division in the liver of treated mice by a factor of two.

By way of contrast, TCA induces a very marginal hepatomegaly, and relatively minor increases in cell size. Chronic treatment with TCA results in significant accumulations of lipid and lipofuscin in the liver of mice [5]. Trichloroacetate treatment results in a peroxisome proliferation that is maintained as long as treatment is continued [7]. Although prior data had indicated that both DCA and TCA were effective as peroxisome proliferators, more recent information indicates that the ability of DCA to induce peroxisomes is very limited at doses that induce hepatic tumors [7].

Some differences have been noted in the tumors produced by TCA and DCA. Although much fewer in number and smaller in size, tumors induced by TCA are much more likely to progress to malignancies than tumors produced with DCA [5]. This observation is associated with a significantly greater level of expression of the proto- oncogene, c-myc, in the TCA-induced tumors [15]. On the other hand, the expression of c-H-ras was increased by very similar amounts in tumors induced by both TCA and DCA. It is not clear whether the increased expression of c-myc in TCA-induced tumors is causally responsible for the differences in progression compared to DCA tumors or if the higher levels of c-myc expression simply reflect the more malignant character of the TCA-induced tumors.

These different manifestations of TCA's and DCA's effects do not definitively identify the actual mechanisms involved. The data do show that there are activities that are clearly not held in common by the two chemicals. Therefore, clarification of the relative contribution of these two metabolites to the induction of the liver tumors induced in mice by TRI may be very important in determining the hazards that might be involved in human exposures to this chemical.

Comparative Metabolism of TRI to TCA and DCA

The comparative metabolism of TRI at the doses that have been utilized in the carcinogenesis bioassays differs very substantially by species. This was first noted as a substantial difference in the production of the metabolites, trichloroethanol (TCE) and TCA [1,2,3]. Hathway [11], however, noted that mice also produced measurable quantities of DCA from TRI. Unfortunately, these authors examined a single dose of TRI in mice and made no formal comparisons of the amounts of this metabolite that were formed in other species. Other investigators make mention of DCA [1,2], but no attempts were made to study its relative production between species.

Recently, our laboratory [12,13] examined the relative amount of TCA and DCA produced in the blood of mice and rats from the administration of TRI. In Figure 56, the peak concentrations of TCA reached at varying doses of TRI in mice can be compared with the concentrations that are achieved in rats. It can be seen that as doses of TRI exceed 1.5 mmol/kg (200 mg/kg), the peak concentrations of TCA achieved in the rat become much less dose-dependent. In mice, this behavior is not observed until doses of TRI exceed 4.5 mmol/kg (600 mg/kg). As a consequence, the peak concentrations of TCA in mouse blood exceed 600 nmol/mL (100 μg/mL) at a dose of 4.5 mmol/kg TRI, but levels in rat blood are only about 20% of this value. Although greater doses of TRI continue to increase the peak concentrations of TCA, the increase with dose above the 4.5 mmol/kg dose becomes sharply attenuated in both species.

In Figure 57, two fitted lines describe the equivalency between oral doses of TRI and TCA in terms of the peak blood concentrations achieved. Because of the nonlinear metabolism of TRI at high doses, a log-log scale had to be utilized to approximate a linear relationship. If the concentrations of TCA produced in blood from metabolized TRI are carcinogenic, they should approximate those observed when doses of TCA are administered that approach the level capable of inducing hepatic cancer. The slightly subcarcinogenic dose of 0.6 mmol (100 mg/kg) TCA produces a blood level of TCA of approximately 800 nmol/mL (120 μg/mL) of blood in mice. A dose of 600 mg/kg TRI produces blood levels of TCA in excess of 600 nmol/mL (90 μg/mL). Therefore, it appears that peak concentrations of TCA achieved with a carcinogenic dose of TRI approach the levels required for a carcinogenic response.

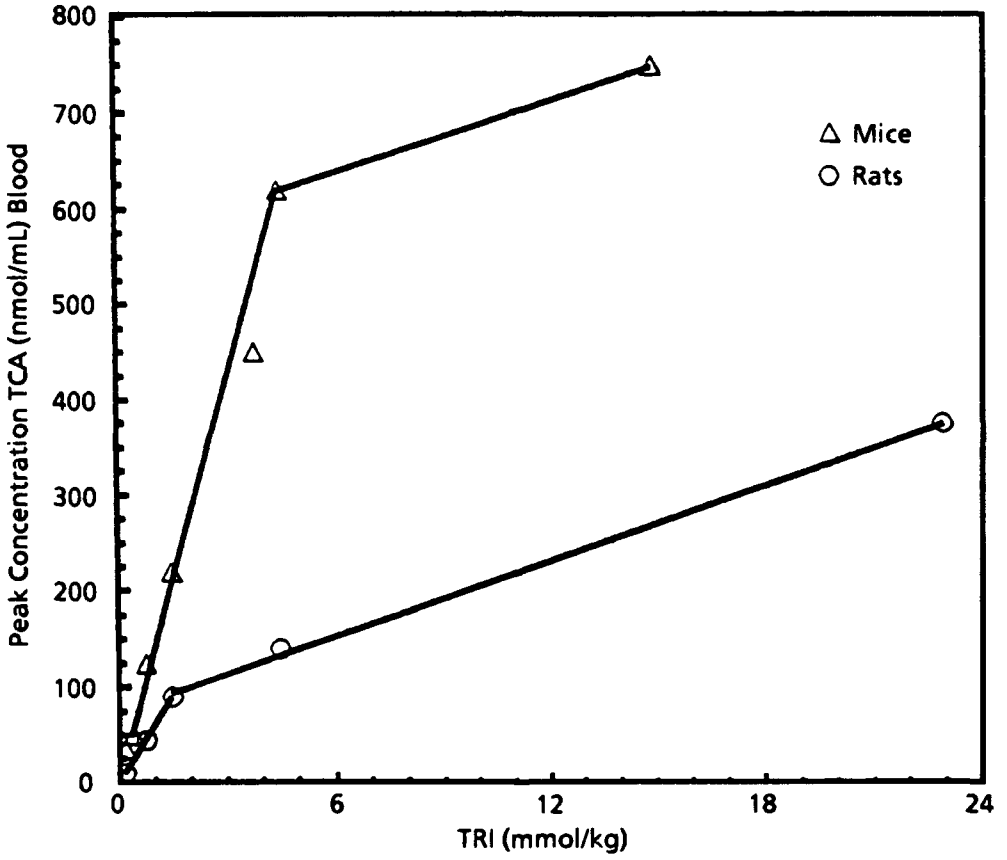

Figure 56. Peak Blood Concentrations of Trichloroacetate (TCA) in Mice and Rats Administered Varying Doses of Trichloroethylene (TRI). Trichloroethylene was administered in an emulsion with 1% Tween in water. Blood concentrations of TCA were monitored over a 72-h period. Each point represents the mean values for four animals [12].

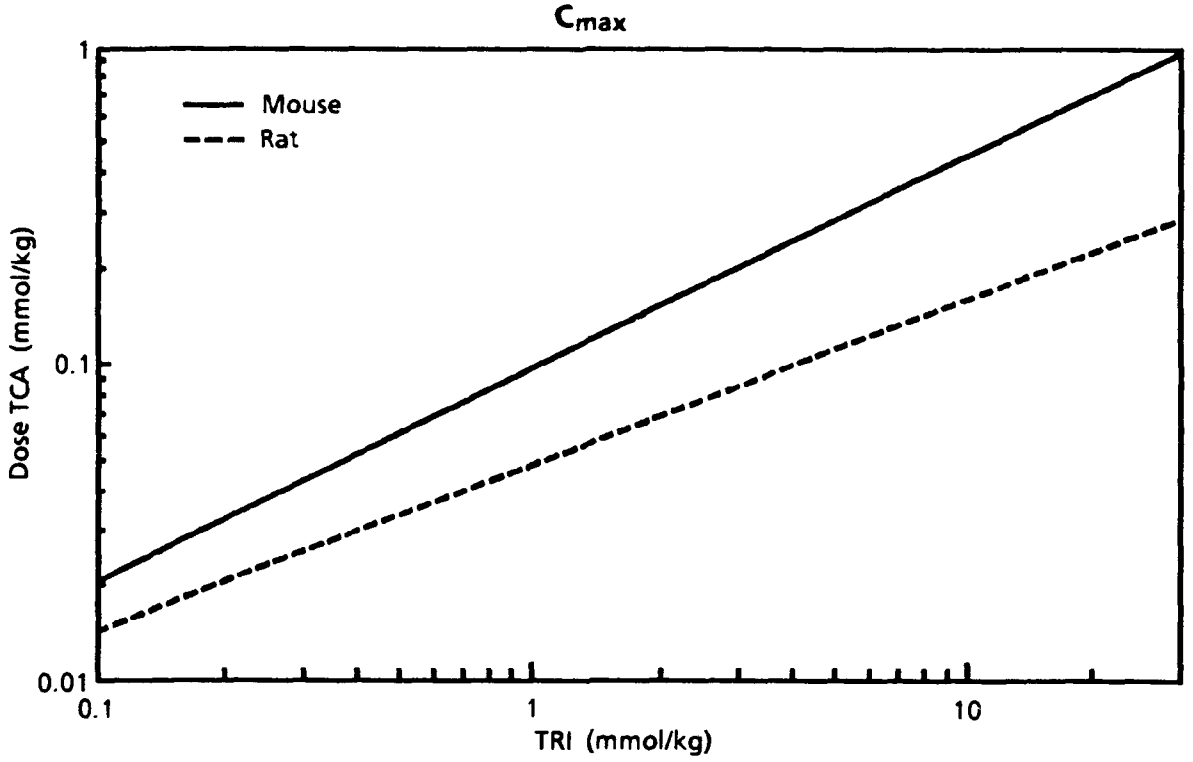

Figure 57. Equivalency of Trichloroethylene (TRI) Doses to Trichloroacetate (TCA) Doses in Terms of the Peak Concentrations of TCA Achieved in the Blood of Rats and Mice. Trichloroacetate was administered by gavage in an aqueous vehicle [13].

Similar results are obtained if the area under the curve (AUC) of the TCA concentrations versus time in blood following TRI or TCA are compared (Figure 58). Figure 59 provides a graph showing the relationship between oral doses of TRI and TCA in terms of the AUC of TCA that is achieved. Again the AUC for TCA in rats administered TRI is consistently below that observed in mice. This measure of dose strongly suggests that sufficient TCA is produced from TRI to induce hepatic tumors in mice. Therefore, this measure again supports the hypothesis that the metabolism of TRI to TCA could be rate-limiting in rats, accounting for the insensitivity of this species to the hepatocarcinogenic effects of TRI.

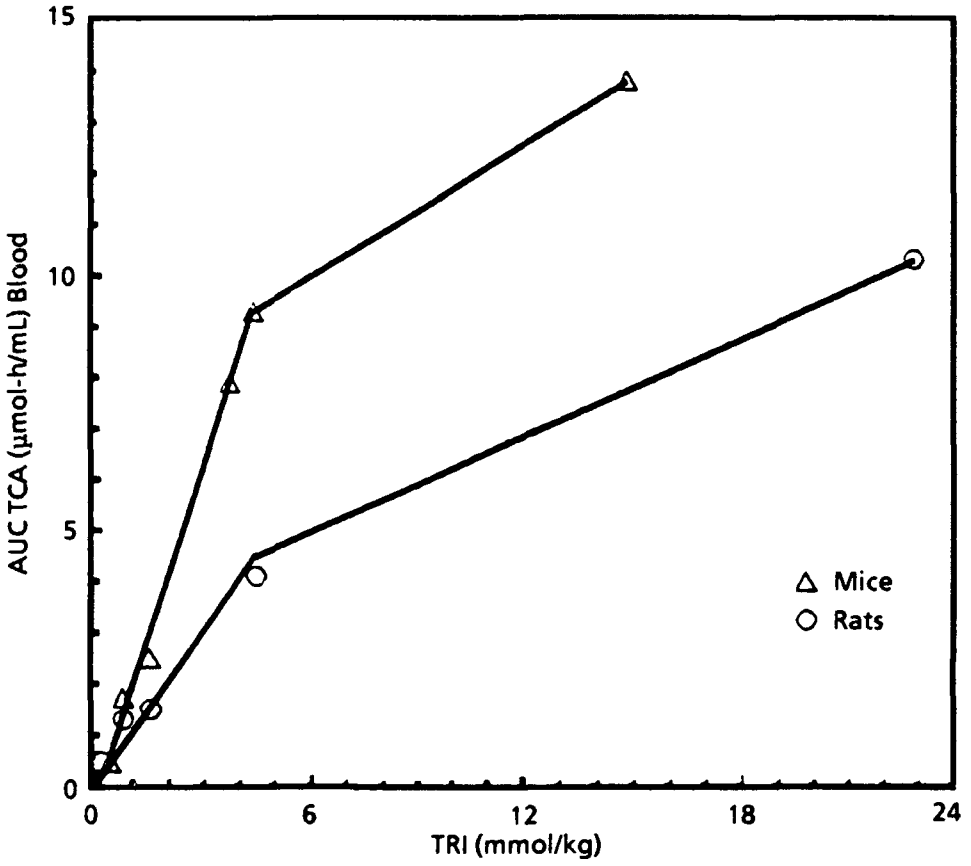

Figure 58. The Area Under the Trichloroacetate (TCA) Blood Concentration vs. Time Curve with Varying Doses of Trichloroethylene (TRI) to Rats and Mice. Experimental details the same as outlined in Figure 56.

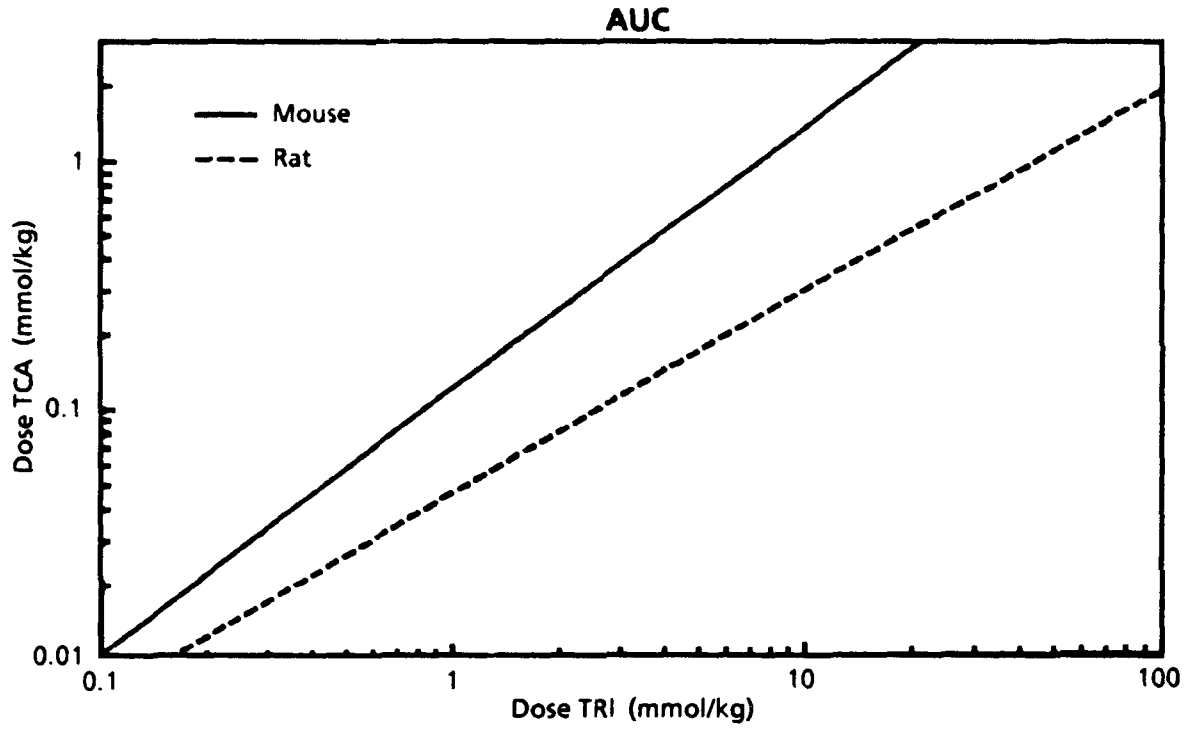

Figure 59. The Equivalency of Oral Trichloroethylene (TRI) and Trichloroacetate (TCA) Doses in Terms of the Area Under Blood Concentrations vs. Time Curve for TCA. Experimental details the same as outlined in Figure 57.

The formation of significant amounts of DCA from TRI appears to occur only in mice [12]. In Table 35 the peak concentrations of DCA detected in the blood of mice and rats administered TRI, TCA, and DCA are compared. In mice, the concentration of DCA achieved in blood at 15 mmol/kg (2000 mg/kg) of TRI exceeds that produced by a dose of 0.78 mmol/kg (100 mg/kg) DCA. Subsequent data [Templin et al., manuscript in preparation] has shown that peak concentrations of DCA in this same range are observed with doses as low as 0.75 mmol/kg TRI. At 0.38 mmol/kg, DCA is observed, but is not measurable. Although there is little indication of increasing peak concentrations of DCA in the dose range of 0.78 to 15 mmol/kg, the AUC of the blood concentration versus time curve for DCA does increase significantly with dose.

TABLE 35. PEAK BLOOD CONCENTRATIONS OF DCA FOLLOWING ADMINISTRATION OF TRICHLOROETHYLENE (TRI), TRICHLOROACETATE (TCA), OR DICHLOROACETATE (DCA) TO MICE AND RATS.

Treatment (mmol/kg)		nmol DCA/mL Blood	
		Mice	Rats
TRI	15	65	ND
	23	ND	<4
TCA	0.12	2	2
	0.60	5	31
DCA	0.16	4	15
	0.78	20	378

ND = not done because it is a lethal dose to mice.

The amount of DCA generated from a 0.6 mmol/kg (100 mg/kg) dose of TCA was considerably less, amounting to about 25% of that observed when DCA was administered. In Sprague-Dawley rats, the levels of DCA were not detectable at a dose of 23 mmol/kg (3000 mg/kg) of TRI.

Equivalent concentrations of DCA were observed in the blood of rats administered TCA at a dose of 0.12 mmol/kg (20 mg/kg); however, at 0.6 mmol/kg the peak concentrations of DCA were markedly elevated in rats versus mice. Similar effects are observed when DCA is administered. The peak blood concentrations observed in rats are markedly greater than in mice. The exaggerated size of this response in DCA concentrations following TRI (a 15-fold increase with an increase in dose of only 5-fold) and evidence of a similar response when DCA is administered suggests a much more limited capacity for systemic clearance of DCA in rats in relation to mice. Therefore, the reasons why significant quantities of DCA were not produced in the blood of rats administered TRI is unclear at this time.

DISCUSSION

The recent finding that TCA in drinking water is incapable of inducing hepatic tumors in rats (DeAngelo, personal communication) and that it is a potent hepatocarcinogen in mice [4,5] makes it necessary to consider alternate explanations for the species difference in response to TRI. Indications that TCA given in drinking water [7] rather than by corn oil gavage [6] is also a poor inducer of peroxisome synthesis in the rat although it is a potent inducer in mice [7] adds further impetus to the consideration of different explanations.

Our studies of the metabolism of TRI indicate that at the high doses utilized in the bioassays of its carcinogenic activity there are probably a myriad of interactions. Prior studies have treated this problem as if the nonlinearities observed at the high doses simply reflected saturation of the metabolism of TRI, itself. Our data suggest that a much more complicated pattern arises in the

B6C3F1 mouse and this is reflected most clearly in the behavior of DCA. The fact that DCA is produced in sufficient quantities to account for the hepatocarcinogenic responses of mice to TRI should raise the concern over the possibility that TRI may be carcinogenic in humans, because DCA is clearly not an effective peroxisome proliferator. On the other hand, the fact that this behavior occurs only in mice should be of considerable concern when utilizing the induction of liver tumors as a basis for extrapolating risks to humans.

There are no data to specifically account for the extreme nonlinear behavior of DCA in the blood of mice treated with TRI. It seems clear that the DCA formed cannot be arising simply from the further metabolism of TCA. Hathway [11] previously suggested that at high doses in mice there was a spill-over of trichloroethylene oxide to dichloroacetyl chloride as an alternative to rearrangement to chloral. This reactive intermediate will decompose to DCA. The behavior of DCA at lower doses of TRI, however, is not entirely consistent with a "spill-over" model because peak concentrations do not change with increasing doses of TRI between 0.75 and 15 mmol/kg. As a consequence, we look to the possibility of other pathways of TRI metabolism to DCA with some interest. These pathways must include the possibility of reductive dehalogenation of two other metabolites of TRI, trichloroacetaldehyde and trichloroethanol (Figure 60). This problem is currently being addressed in our laboratory.

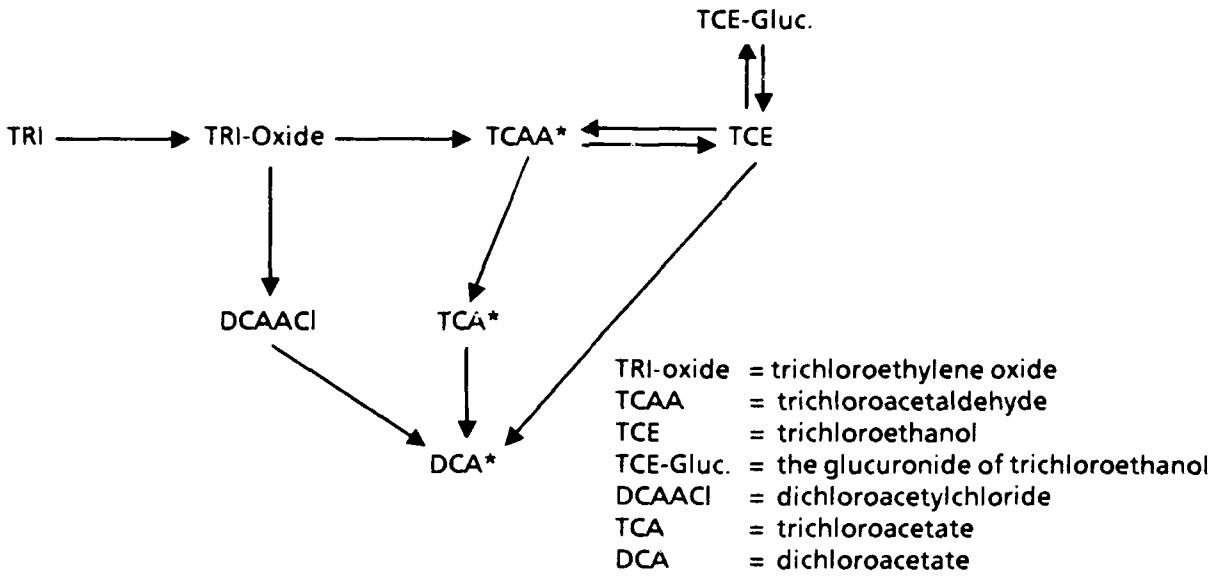

Figure 60. Abbreviated Metabolic Scheme of Trichloroethylene (TRI) Showing its Metabolism to Metabolites Potentially Involved in its Hepatocarcinogenic Effects. Metabolites that have been demonstrated to produce hepatic tumors are marked with an asterisk (*).

ACKNOWLEDGEMENTS

This grant was supported by a Cooperative Agreement with the U.S. Environmental Protection Agency CR-815216-01.

REFERENCES

1. **Prout, M.S., W.M. Provan, and T. Green.** 1985. Species differences in response to trichloroethylene. I. Pharmacokinetics in rats and mice. *Toxicol. Appl. Pharmacol.* 79:389-400.

2. **Green, T. and M.S. Prout.** 1985. Species differences in response to trichloroethylene. II. Biotransformation in rats and mice. *Toxicol. Appl. Pharmacol.* 79:401-411.

3. **Elcombe, C.R.** 1985. Species differences in carcinogenicity and peroxisome proliferation due to trichloroethylene: A biochemical human hazard assessment. *Arch. Toxicol.* 8, 6-17.

4. **Herren-Freund, S.L., M.A. Pereira, M.D. Khoury, and G. Olson.** 1987. The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid, in mouse liver. *Toxicol. Appl. Pharmacol.* 90:183-189.

5. **Bull, R.J., I.M. Sanchez, M.A. Nelson, J.L. Larson, and A.J Lansing.** 1990. Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacetate. *Toxicology* 63:341-359.

6. **Goldsworthy, T.L. and J.A. Popp.** 1987. Chlorinated hydrocarbon-induced peroxisomal enzyme activity in relation to species and organ carcinogenicity. *Toxicol. Appl. Pharmacol.* 88:225-233.

7. **DeAngelo, A.B., F.B. Daniel, L. McMillan, P. Wernsing, and R.E. Savage, Jr.** 1989. Species and strain sensitivity to the induction of peroxisome proliferation by chloroacetic acids. *Toxicol. Appl. Pharmacol.* 101:285-298.

8. **Conway, J.G., R.C. Cattley, J.A. Popp, and B.E. Butterworth.** 1989. Possible mechanisms in hepatocarcinogenesis by the peroxisome proliferator d-(2-ethylhexyl)phthalate. *Drug Metabolism Reviews* 21:65-102.

9. **DeAngelo, A.B., F.B. Daniel, J.A. Stober, and G.R. Olson.** 1991. The carcinogenicity of dichloroacetate in the male B6C3F1 mouse. *Fundam. Appl. Toxicol.* 16:337-347.

10. **Daniel, F.B., A.B. DeAngelo, J.A. Stober, G.R. Olson, and N.R. Page.** In Press. Hepatocarcinogenicity of chlorinated aldehydes: A two-year bioassay of chloral hydrate and 2-chloroacetaldehyde in the male B6C3F1 mouse. *Fundam. Appl. Toxicol.*

11. **Hathway, D.E.** 1980. Consideration of the evidence for mechanisms of 1,1,2-trichloroethylene metabolism including new identification of its dichloroacetic acid and trichloroacetic acid metabolites in mice. *Cancer Lett.* 8:263-265.

12. **Larson, J.L. and R.J. Bull.** 1992a. Species differences in the metabolism of trichloroethylene to the carcinogenic metabolites, trichloroacetate and dichloroacetate. *Toxicol. Appl. Pharmacol.* 115:278-285.

13. **Larson, J.L. and R.J. Bull.** 1992b. Metabolism and lipoperoxidative activity of trichloroacetate and dichloroacetate in rats and mice. *Toxicol. Appl. Pharmacol.* 115:268-277.

14. **Sanchez, I.M. and R.J. Bull.** 1990. Early induction of reparative hyperplasia in the liver of B6C3F1 mice treated with dichloroacetate and trichloroacetate. *Toxicology* 64:33-46.

15. **Nelson, M.A., I.M. Sanchez, R.J. Bull, and S.R. Sylvester.** 1990. Increased expression of c-myc and c-H-ras in dichloroacetate and trichloroacetate-induced liver tumors in B6C3F1 mice. *Toxicology* 64:47-57.

APPLICATION OF KINETIC MODELS TO ESTIMATE TRANSIT TIME THROUGH CELL CYCLE COMPARTMENTS

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SUMMARY

Models of the carcinogenesis process emphasize the importance of understanding cell cycle-specific effects of a chemical exposure. Development of mathematical models describing the kinetics of individual cell movements within the growth cycle are applied to a cultured cell system. Treatment with 100 µg/mL trichloroacetic acid is shown to retard transit through the synthesis phase of the cycle. The models are compared with standard relative movement calculations and are found to be more sensitive. In addition, the DNA compartments are modeled over time to detect possible development of aneuploidy during treatment.

INTRODUCTION

Many recent studies of the mechanism of carcinogenesis have focused on the central role of cell proliferation in chemically induced tumors. Direct correlation between cell proliferation and carcinogenesis has been demonstrated *in vivo* [1]. Moolgavkar [2], among others, has proposed a two-stage model incorporating cell proliferation rates and other parameters of cell growth to be used in predicting cancer risk resulting from chemical exposure.

The purpose of this study is to demonstrate the application of kinetic models to estimate transit times through cell cycle compartments using an *in vitro* cell culture system. Recent models proposed by White et al. [3] are exercised for cultures treated with trichloroacetic acid (TCA), a proven rodent carcinogen [4]. The effect of treatment with TCA concentrations similar to human tissue levels following inhalation exposure to the parent compound trichloroethylene at occupationally relevant concentrations [5] is assessed for impact upon cell growth parameters.

MATERIALS AND METHODS

Cell Culture

The WB344 cell line was used throughout this study (a gift from Dr. T.J. Kavanagh, Department of Environmental Health, University of Washington, Seattle). It is a nontumorigenic, diploid, epithelial cell line derived from primary isolation of hepatocytes from a male Fisher 344 rat [6]. All cell culture media and buffers were obtained from Gibco BRL, Gaithersburg, MD. Assay reagents, unless otherwise noted, were obtained from Sigma Chemicals, St, Louis, MO. Cells stored in early passage

(<20 platings) were grown in minimum essential media (Gibco #320-1120AG) supplemented with vitamins, essential and nonessential amino acids, and sodium pyruvate.

Routinely, 5% defined calf serum (Hyclone Labs, Logan, UT), penicillin/streptomycin (100 U/mL and 100 µg/mL respectively) and 2.29 g/L bicarbonate/ 10 mM hepes buffer, pH 7.1, was added to form the complete media (DMEM). Treatment medium (DMEM-TX) consisted of DMEM containing 100 µg/mL TCA adjusted to pH 7.1. Culture was under standard conditions of 5% carbon dioxide in a humidified incubator held at 37 °C.

Growth Curves

Standard growth curves were obtained by plating duplicate plates in either DMEM or DMEM-TX at low density. Every 24 h, paired control and treated plates were harvested and cells were counted using a hemocytometer. These data were plotted to calculate a potential doubling time, T_{pot}, which is analogous to the total cell cycle time, T_C, in cell culture.

Cell Cycle/ Pulse Chase

Confluent plates of WB344 cells were trypsinized and split into 100 cm^2 tissue culture dishes (± 120,000 cells/plate) containing DMEM and allowed to incubate for 2 to 3 h to attach. Seeding density was chosen to prevent the cultures from becoming confluent over the 2-day course of the experiment. The medium was then replaced in control plates with DMEM and treatment plates with DMEM-TX. All cultures were returned to the incubator.

After 36 h all plates were washed with warm Dulbecco's phosphate buffered saline (DPBS) and flooded with DMEM containing 10 µM bromodeoxyuridine (BrDU) (Boehringer Mannheim, Indianapolis, IN). Cells were returned to the incubator for 1 h to allow DNA labeling by the incorporation of BrDU into nascent DNA during synthesis. Because this renders the cellular DNA photolabile, all subsequent manipulations were performed under photographic "safe light" illumination.

For harvest at each time point the appropriate plates were washed and trypsinized to release the cells. Following centrifugation at 200 x *g* for 20 min at 4 °C the supernatant was aspirated and the cell pellet resuspended in 200 µL of ice cold DPBS. Cells were then fixed in 70% ethanol and held at -20 °C pending staining and analysis. Bivariate staining followed standard protocols [7]. Briefly, each sample was adjusted to 1.5×10^6 cells per mL. Cellular DNA was denatured by 4N hydrochloric aicd (HCl) acid treatment, washed, and then labeled with anti-BrDU monoclonal antibody tagged with fluorescein isothiocyanate (anti-BrDU/FITC, Boehringer Mannheim, Indianapolis, IN). Counter staining for 10 min with 2.5 µg/mL propidium iodide (Boehringer Mannheim, Indianapolis, IN) followed. The samples were kept on ice and protected from light pending analysis on a FACScan laser flow cytometer (Becton Dickinson, San Jose, CA) using the 488nm excitation wavelength of the argon laser. Light was collected at 530 nm for the FITC signal (FL1) and at 585 nm for propidium iodide (FL2). Doublet cells were excluded from analysis by gating on the FL2 signal pulse integral (FL2 area, FL2-A).

Data was collected in list mode for a minimum of 30,000 cells from each sample. Data analysis was accomplished using Lysis™ software (Becton Dickinson, San Jose, CA). Bivariate contour plots of FL2-A vs. FL1 allowed cell populations to be separated visually based upon the degree of anti-BrDU/FITC incorporation. Cell compartments were designated and analyzed as described by White et al. (Figure 61). Populations were designated as follows:

F^{u}_{G2M} = Fraction of cells unlabeled in G2M compartment

F^{lu} = Fraction that are labeled but have not divided

F^{ld} = Fraction that are labeled and have divided

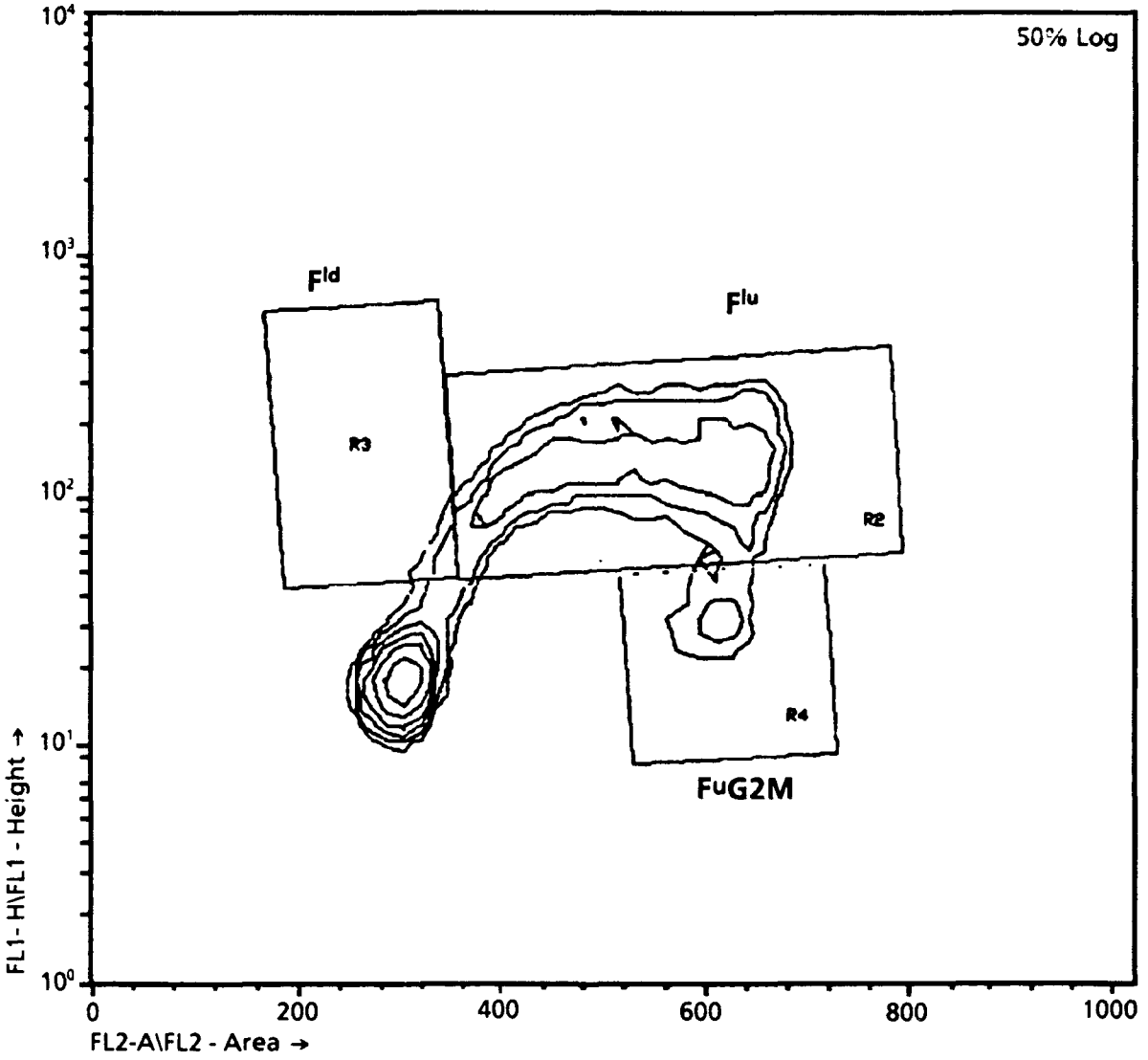

Figure 61. Bivariate Plot of Total DNA (X Axis, Propidium Iodide Signal, FL2-A) vs. BrDU-Labeled DNA (Y Axis, FITC Signal, FL1-H). Contours outline the total cell population of n = 30,000. Boxed regions depict the location of fractional subpopulations based upon uptake of BrDU label. As the cycle progresses the F^{U}_{G2M} compartment will disappear, as will the F^{lu} subset.

Nonlinear fitting was performed using Sigma Plot v5.0 (Jandel Scientific, San Rafael, CA) and parameter estimates are reported. For each model, the assumption of a cell population in log phase growth was fulfilled by ensuring that the cultures did not reach confluence before they were harvested.

Data from three independent experiments was normalized by the average labeling index (LI) for each run. From plots of $F^{lu} + F^{ld}/2$ vs. time, LI was calculated by fitting the equation:

$$F^{lu}(t) + F^{ld}(t)/2 = E^{ot}LI$$

where c is a numerical constant, t is time in hours, and e is natural log. The fractional compartments F^{lu} and F^{ld} are as previously defined. Convergence tolerance for this, and all subsequent model fits was set at 10^{-6}. Specific fractional compartments plotted with time allowed estimation of the transition times through each phase (i.e., T_{G1}, T_S, and T_{G2M} represent the transit times for the $G1$, S and $G2M$ phases of the cell cycle respectively). For T_{G2M}, the equation:

$$F^{u}{}_{G2M}(t) = e^{(T_{G2M} - t)} - 1$$

was fit to all three independent data sets using the constraint: $t \leq T_{G2M}$, and 0 for $T_{G2M} < t \leq T_S$.

The parameter estimates for T_{G2M} and T_S were calculated from two models. The first uses the fractional population of labeled undivided cells (F^{lu}) described by the equations:

$$e^{-ct} e^{c(T_{G2M} + T_5)} - 1$$

for $T_{G2M} \leq t \leq T_{G2M} + T_S$.

and,

$$e^{-ct} e^{cT_{G2M}} (e^{cT_s} - 1)$$

for $t \leq T_{G2M}$. A second model based on the labeled divided cell population (F^{ld}) applies the equations:

$$2 - 2e^{-c(t - T_{G2M})}$$

for $T_{G2M} \leq t \leq T_S + T_{G2M}$. For the case $T_S + T_{G2M} \leq t \leq T_{pot} + T_{G2M}$, the following equation was used:

$$2e^{-ct} e^{cT_{G2M}} (e^{cT_s} - 1)$$

Relative movement (RM) estimates of the S phase transit were calculated from the fractional mean of the total DNA fluorescence (the "red" or propidium iodide signal, FL-2A) of the BrDu labeled cells plotted with time. The equation:

$$RM = \frac{F1 - F_{G1}}{F_{G2M} - F_{G1}}$$

where F^l is the mean red fluorescence of the BrDu labeled cells, F_{G1} the mean red signal from the $G1$ population and F_{G2M} is the mean red signal from the $G2M$ population.

DNA Compartment Analysis

For DNA compartment analysis, high resolution histograms of FL2-A vs. cell number were displayed using Multicycle™ (Phoenix Systems, Los Angeles, CA) and curves fitted to the various cell population profiles. Compartment statistics and goodness-of-fit was reported for each sample as Chi square, with values greater than 5 rejected. The percent of total DNA in each compartment was plotted against time for treated cells expressed as a percent of controls.

Statistics

Treated and control means were compared using the independent t-test routine of SYSTAT (Systat, Evanston, IL). Significance was reported at the $p<0.05$ level. Cell transit times were reported as parameter estimates with goodness-of-fit standard error and coefficients of variation.

RESULTS

Cell Cycle Kinetics

The fractional populations of each sample were plotted against time, and equations describing subpopulation transition through each compartment-generated parameter estimates are summarized in Table 36. Estimates based on the F^{lu} and Fu_{G2M} fractional populations had high coefficients of variation ranging from 8.8 to 21%. Best-fit estimates were obtained from the F^{ld} data (Figure 62). The estimated transit time through S and G2M was similar for both treated and control cells. However, T_S was significantly delayed in those cells which had been treated with TCA. Total cycle time for both controls and TCA cells is 11.9 h (Figure 63), the G1 transition calculated by subtraction was 5.12 and 4.9 h for controls and TCA cells, respectively.

TABLE 36. KINETIC CELL CYCLE PARAMETERS

The best fit parameter estimates were obtained from model fits to the F^{ld} compartment. T_{pot} was obtained as previously described. Numbers in parentheses are the standard error for the estimate. Each parameter was based on 24 data points representing the mean fractional population of n = 30,000 cells. All times are in hours.

	Control	TCA 100 µg/mL
T_{pot}	11.9	11.9
T_{G2M}	1.76 (0.1)	1.80 (0.1)
T_S	5.02 (.18)	5.20 (.19)[a]
T_{G1}	5.12[b]	4.9[a]

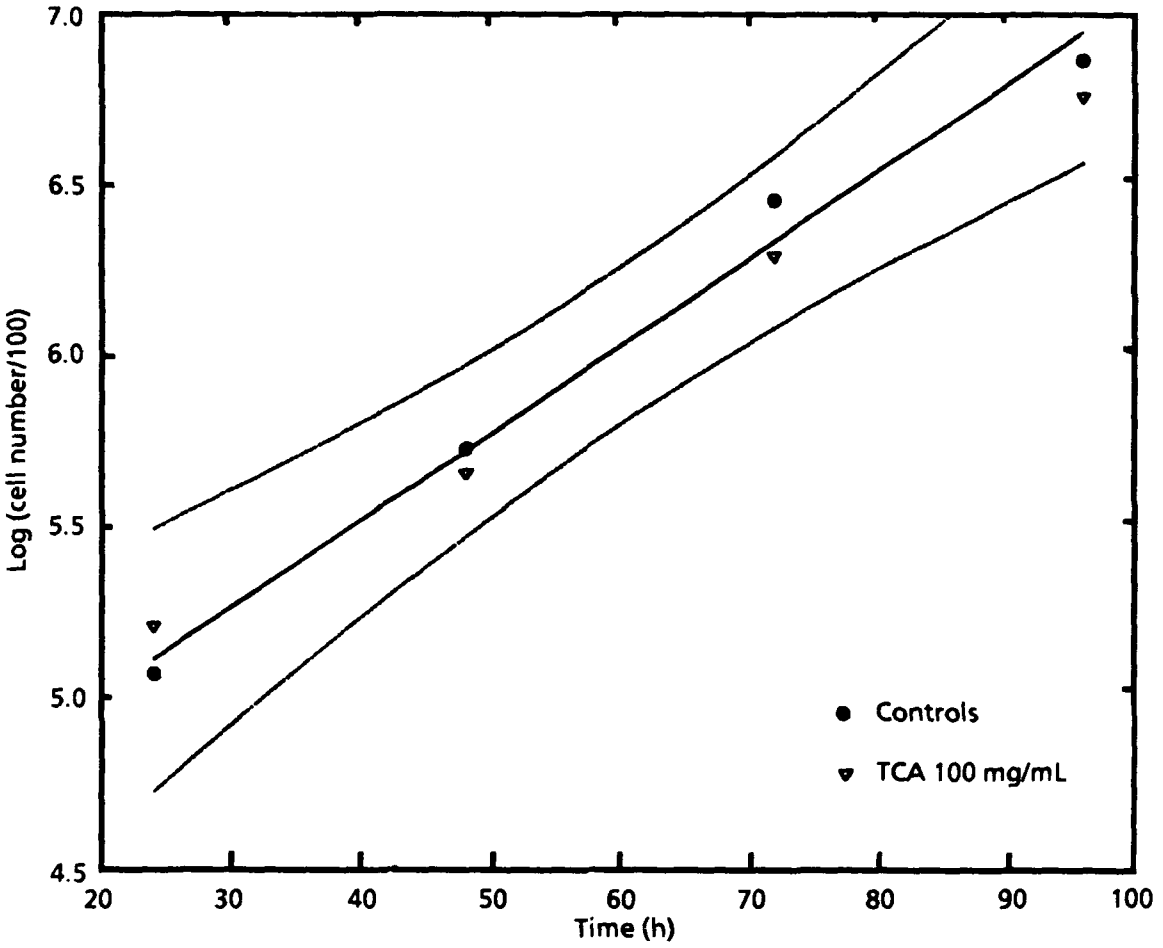

Figure 62. Growth Curve for WB344 Cells. Continuous treatment with 100 µg/mL TCA did not alter the doubling time of WB344 cells in culture. Dotted lines represent the 95% confidence interval for the control data.

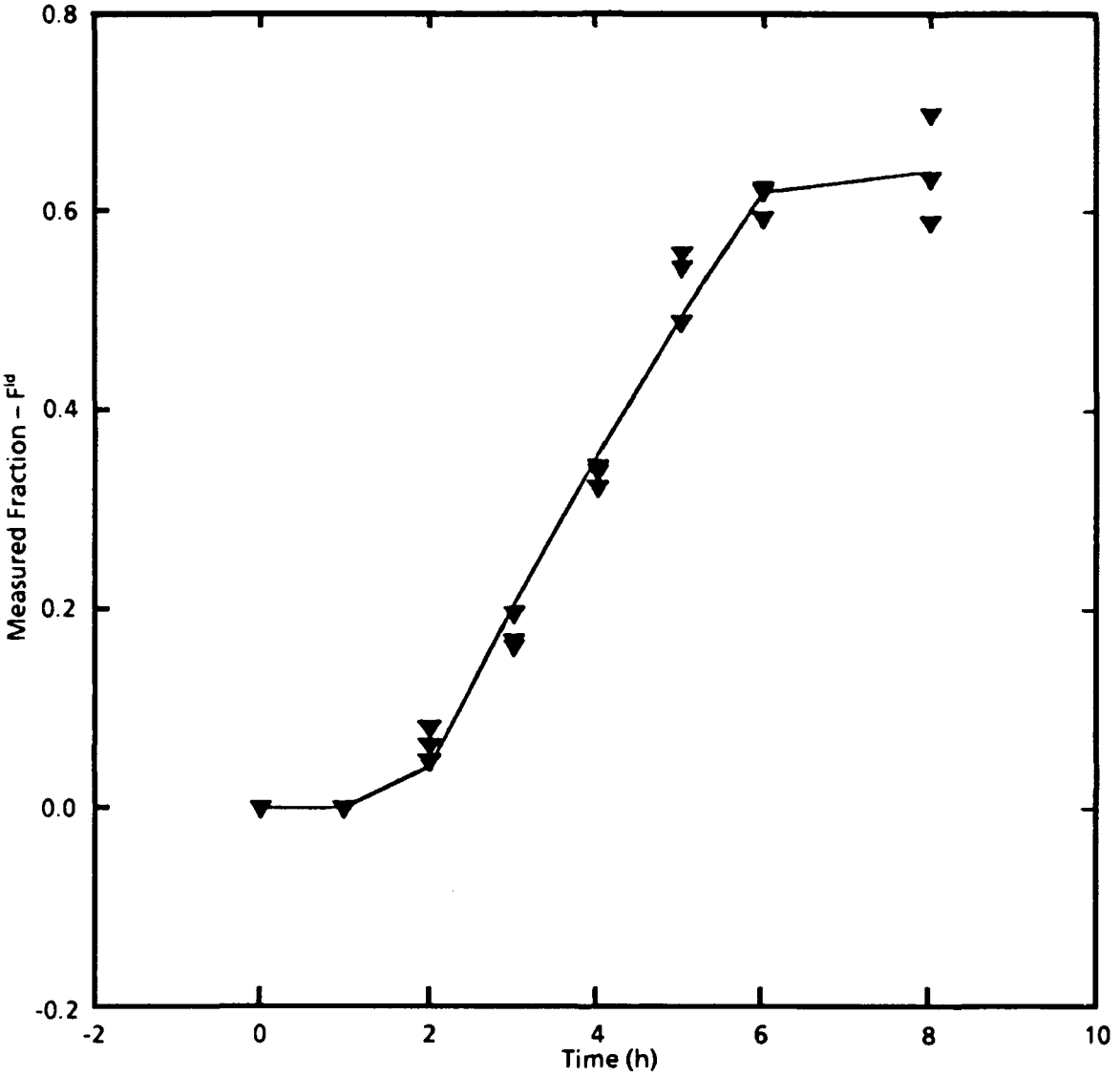

Figure 63. Model Fit for F^{Id} Subpopulation. The line represents the fit to the data from three independent experiments. Triangles are the fractional mean of at least 30,000 cells for each timepoint. Parameter estimates of transit times are included in Table 36.

Relative movement plots were identical for both control and treated cultures (Figure 64). This may indicate the insensitivity of this method for estimating the S phase transit time when treatment effects are small. In both cases the FL-2A signal at t = 0 was about midway between the G1 and G2M population signals, an effect to be expected if the S phase cells were evenly distributed within the S compartment. With time the labeled population moves toward G2M, reflected in the eventual rise to unity as FI approaches F_{G2M}. Treatment effects would appear as differences in slope of the regression lines describing these data [8].

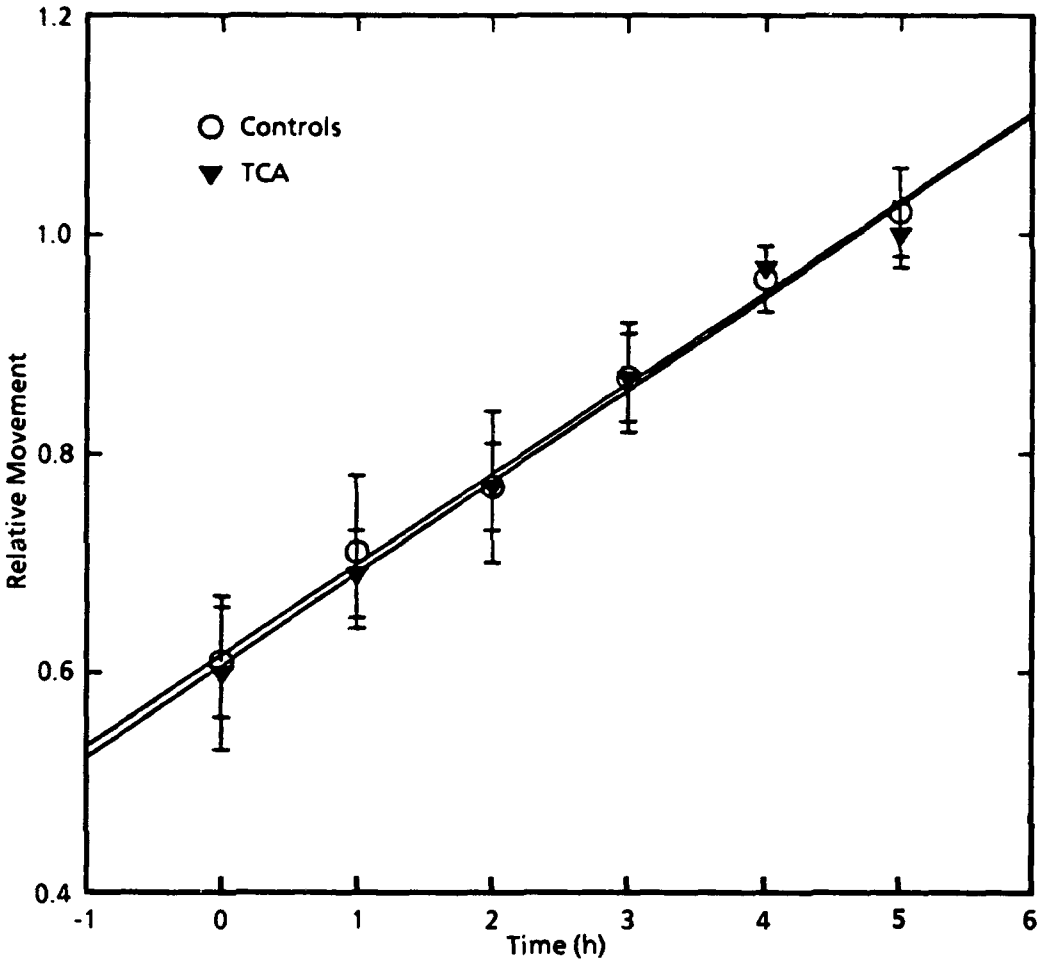

Figure 64. Relative Movement of BrDU-Labelled Cells. The slopes of each regression line are virtually identical, indicating no difference in the S-phase transit between control and treated cells. Note that the RM at t = 0 is an approximation of the LI. Each point is the mean and standard error for three independent experiments.

DNA compartments

When released from 36 h of continuous treatment with 100 µg/mL TCA, cells have a higher percentage of the cell population in the S compartment than do controls (Figure 65). This supports the model estimate for a prolonged T_S, however, the effect appears to be temporary because the DNA compartments equilibrate with control levels by 7 h after release into DMEM. No evidence of aneuploidy was observed in treated or control cultures (data not shown).

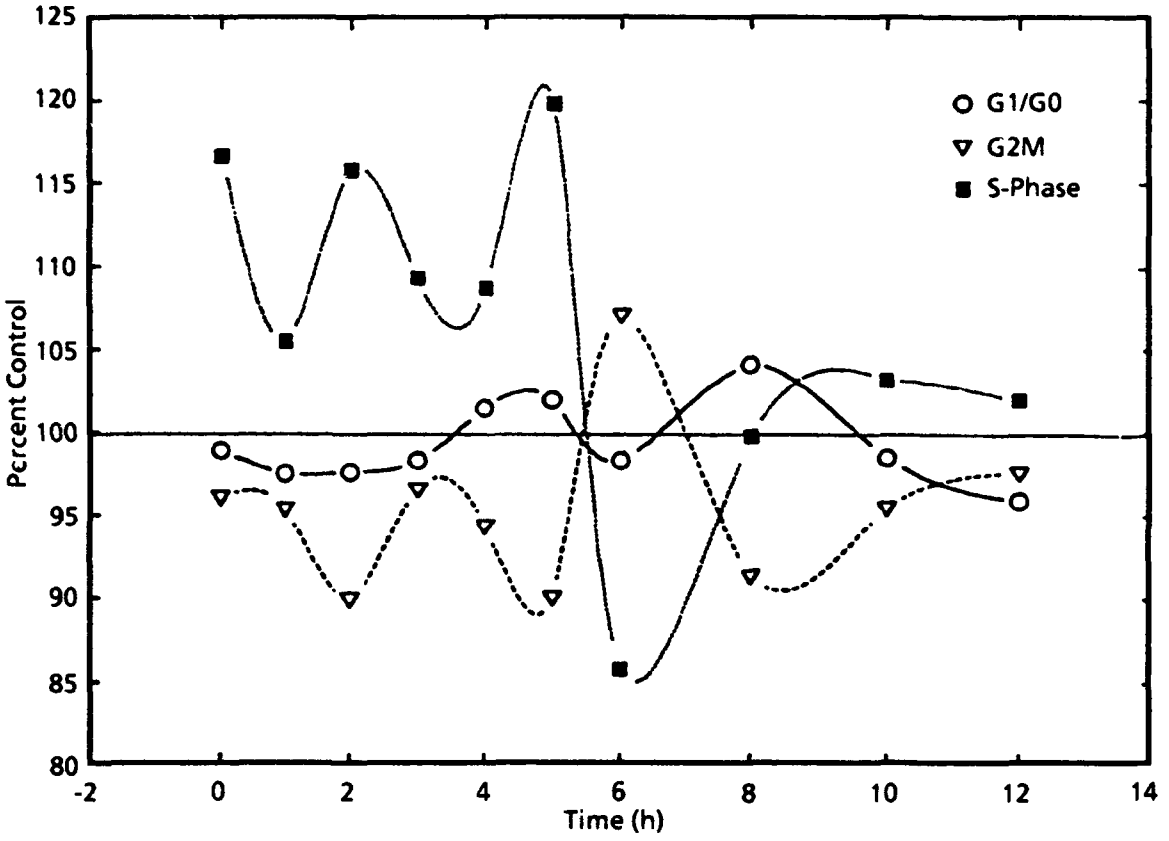

Figure 65. DNA Compartment Changes with Release from TCA Treatment. Initially S-phase is elevated for up to 6 h after release into normal media. However, by 7 h the treated cells equilibrate to control values, indicating that the S-phase arrest is not a permanent effect.

DISCUSSION

We have demonstrated the utility of this procedure to obtain estimates of cell cycle transit times. Nonlinear models provide a sensitive tool which is superior to standard relative movement calculations in this case.

The importance of DNA compartment modeling should be emphasized. Kinetic models return values for the rate of movement of individual cells through each phase of the normal growth cycle. However, if the mitotic process is disrupted and unequal apportionment of DNA occurs, cells could pass through G2M at a normal rate only to end up as aneuploid G1 subpopulations. By analyzing both the kinetic parameters and the absolute DNA compartments, each aspect of cellular growth can be described.

Studies of this type should be useful for dissecting chemical-specific effects upon cellular growth. Because each phase transit time is estimated, the implication of a chemical effect can be specified to a particular cell cycle event. For example, a chemical which shortened T_{G2M} could carry

more serious risk than one similar to TCA, which only slightly prolongs T_S. In the first case, acceleration through the critical stage of mitosis may increase the chance of chromosomal mismatch and rearrangement. In the latter instance, delay may be a consequence of sublethal cytotoxicity for which there is a threshold. For both instances, the model estimates suggest follow-on studies to specify the mechanisms of chemical alterations in the cell cycle.

REFERENCES

1. **Cunningham, M.L. and H.B. Matthews.** 1991. Relationship of hepatocarcinogenicity and hepatocellular proliferation induced by mutagenic noncarcinogens vs. carcinogens. *Toxicol. Appl. Pharmacol.* 110:505-513.

2. **Moolgavkar, S.H.** 1990. Two mutation model for carcinogenesis: Relative roles of somatic mutations and cell proliferation in determining risk. Scientific Issues in quantitative cancer risk assessment, pp. 136-152. Birkhauser Boston.

3. **White, R.A., N.H.A. Terry, and M.L. Meistrich.** 1990. New methods for calculating kinetic properties of cells *in vitro* using pulse labeling with bromodeoxyuridine. *Cell Tissue Kinet.* 23:561-573.

4. **Bull, R.J., I.M. Sanchez, M.A. Nelson, J.L. Larson, and A.J. Lansing.** 1990. Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacteate. *Toxicology* 64:341-359.

5. **Muller, G., M. Spassovski, and D. Henschler.** 1974. Metabolism of trichloroethylene in man II. Pharmacokinetics of metabolites. *Arch. Toxikol.* 32:283.

6. **Tsao, M.S., J.D. Smith, K.G. Nelson, and J.W. Grisham.** 1984. A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells. *Exp. Cell Res.* 154:38-52.

7. **Becton Dickinson.** 1988. Two Color Cell Cycle Analysis Using Anti-BrDU and Propidium Iodide. Monoclonal Source Book, sec. 3.80.1, *Becton Dickinson Immunocytometry Systems.* San Jose, CA.

8. **Begg, A.C., N.J. McNally, D.C. Shrieve, and H. Karcher.** 1985. A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. *Cytometry* 6:620-626.

SESSION V

ADVANCES IN THE ASSESSMENT OF TOXICOLOGICAL END POINTS

DEVELOPMENTAL TOXICITY, REPRODUCTIVE TOXICITY, AND NEUROTOXICITY AS REGULATORY END POINTS

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SUMMARY

For decades, cancer has been the primary toxicological end point used in the assessment of hazard and risk. Regulatory decisions related to the manufacture, transport, and use of a chemical are often based solely on cancer data. Federal policy is now shifting toward more frequent evaluation and application of alternative end points of toxicity. Among the end points of particular current interest are developmental toxicity, reproductive toxicity, and neurotoxicity. Significant progress has been made in the development of standardized guidelines for testing chemicals for their potential effects on these end points. Corresponding guidelines for the assessment of risk on the basis of data on these end points are in various stages of development.

INTRODUCTION

Changes in public policy occur at a pace that often seems agonizingly slow to the scientific community. But, they do occur. We are currently experiencing such a shift within the regulatory community in the United States. A comparable change is taking place internationally. After many years of discussion and debate, the longstanding emphasis on cancer as the regulatory end point for human health is giving way. In its place is an increased acknowledgement of other effects on human health that can follow environmental exposure to chemicals, and the responsibility to protect the public from such exposures. Although the benefits of this change are just beginning to draw public attention, they are the result of a decade or more of work on the part of scientists working within the regulatory community.

Three areas of increasing regulatory activity are reproductive toxicity, developmental toxicity, and neurotoxicity. This represents a major policy shift from three perspectives. First, these are end points for which an exposure threshold is assumed. That is, an acceptable level of exposure, below which no toxicity is predicted, can be calculated. This means that exposure can actually be regulated rather than simply banned. Second, it is possible for these types of toxic effects to develop following a single exposure to a chemical. Previously, the standard practice was to consider only lethality in assessing hazard due to acute exposure to a chemical. Risk assessments for carcinogens have almost always been based on lifetime exposures. Third, the economic impact of these effects can be calculated on the basis of an outcome other than death.

Because toxicologists have known for decades, if not centuries, that chemicals can have effects on many end points of human health, the regulatory response seems long overdue. One explanation for the delay is that the regulatory process itself is painfully cumbersome. Given the complexity of simply following the procedures in place, it is not surprising that effecting a change in those procedures takes a long time. The regulations promulgated by the Environmental Protection Agency (EPA) this year are the result of many years of work both within and outside the Agency.

DISCUSSION

The policy change underlying current regulations based on noncancer end points of toxicity is the result of deliberation and communication at many levels. Following any sort of scientific discovery or breakthrough resulting in a new awareness, including those related to health hazards, there is always a period of confirmatory information gathering. When this is completed, a period of time is required for general acceptance of a new concept within the scientific community. Then, the information must be disseminated from the scientific community to the general public. Each of these events can take several years. As public awareness of a potential health hazard grows, pressure on the government to act comes into play. Generally, it is in response to such pressure that necessary legislation and funding are obtained. When a regulatory agency such as the EPA receives the appropriate authority and resources to act, another complex process begins.

Federal authority to regulate exposure to chemicals is derived from a number of statutes. These include those listed in Table 37. The EPA has the authority to set standards on the basis of the Clean Air Act, Clean Water Act, Safe Drinking Water Act, Comprehensive Environmental Response, Compensation and Liability Act and Resource Conservation and Recovery Act. Authority to require testing is provided to EPA only by the Toxic Substances Control Act (TSCA) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). It is FIFRA which allows the EPA to require submission of data prior to registration of a product when significant relevant exposure can reasonably be expected. Requirements can include data on developmental toxicity, reproductive toxicity, neurotoxicity and developmental neurotoxicity. When TSCA was implemented, an inventory of chemicals existing in commerce at that time was compiled. These are regulated under Section 4 of the statute. New chemicals are regulated in accordance with Section 5. Both sections require that EPA demonstrate that a chemical may present an unreasonable risk of injury to health or the environment and that insufficient data to assess potential risk are available in order to require testing. A need for data on individual end points of toxicity must be demonstrated.

TABLE 37. MAJOR FEDERAL LAWS CONTROLLING EXPOSURE TO CHEMICALS

Law (Effective Date)	Promulgating Agency
Toxic Substances Control Act (1976)	EPA
Federal Insecticide, Fungicide, and Rodenticide Act (1947)	EPA
Federal Food, Drug, and Cosmetic Act (1938)	FDA
Occupational Safety and Health Act (1970)	OSHA
Comprehensive Environmental Response, Compensation, and Liability Act (1980)	EPA
Clean Air Act (1955)	EPA
Federal Water Pollution Control Act and Clean Water Act (1977)	EPA
Safe Drinking Water Act (1974)	EPA
Resource Conservation and Recovery Act (1980)	EPA
Consumer Product Safety Act (1972)	CPSC
Federal Hazardous Substances Act (1960)	CPSC
Controlled Substances Act (1972)	FDA
Federal Mine Safety and Health Act (1969)	MSHA
Marine Protection, Research, and Sanctuaries Act (1972)	EPA
Lead-Based Paint Poisoning Prevention Act (1973)	CPSC
Lead Contamination Control Act	HHS
Poison Prevention Packaging Act (1970)	CPSC

Note: Effective dates do not reflect dates of amendments.

Before EPA can propose to regulate exposure to a chemical, reliable evidence on its toxic effects is required. Legal challenges to regulatory action are the rule, not the exception. In most cases, data that form the basis of risk management decisions are generated by the chemical's manufacturer. In order to require the collection of such data, EPA must provide scientifically valid test protocols, in the form of test guidelines, to the chemical manufacturer, and allow a reasonable time for data collection, analysis, and submission. After data are provided to EPA, the entire data base must be reviewed and a risk assessment must be prepared for use in the development of a risk management strategy. Test guidelines, risk assessment guidelines, risk assessments, and risk management decisions are subject to both internal and external scientific review. Formal rulemaking under statutes such as TSCA and FIFRA is also subject to review by the Office of Management and Budget (OMB). For rulemaking under some statutes, including TSCA and FIFRA, Executive Order 12291 requires filing of a Regulatory Impact Statement (RIA), which weighs the costs and benefits of the regulation. Development of the economic analysis is the responsibility of the regulatory agency. Each of these steps, test and risk assessment guideline development, data collection and evaluation by both the manufacturer and the regulatory agency can take years. Preparation of the economic

analysis and OMB review require several additional months. Only when these are completed and approved can a Proposed Rule be published. This formal proposal initiates a public comment period of at least 30 days. At the end of the public comment period, all comments received must be evaluated and a response incorporated into the Final Rule.

When the decision to consider the areas of reproductive toxicity, developmental toxicity, and neurotoxicity as relevant end points in hazard assessment and risk characterization was made, it became necessary to develop test guidelines and risk assessment guidelines for each end point. As part of this process, EPA scientists worked with colleagues from other organizations, including the World Health Organization and the Organization of Economic Cooperation and Development. Now that these are available or in the final stages of development, EPA can systematically evaluate chemicals that are suspected to produce reproductive toxicity, developmental toxicity, or neurotoxicity. Regulation on the basis of these end points is now possible and several actions are in various stages of development and execution at this time.

The current status of EPA's preparation to evaluate chemicals on the basis of their potential to produce developmental toxicity, reproductive toxicity, or neurotoxicity can be summarized as follows.

Reproductive Toxicity

Reproductive toxicity may be expressed as alterations in the female or male reproductive organs or the endocrine system. End points include, but are not limited to, adverse effects on the onset of puberty, gamete production and transport, cyclicity, sexual behavior, fertility, gestation, parturition, lactation, pregnancy outcome, and premature reproductive senescence. Risk assessment guidelines for reproductive toxicity were proposed by EPA in 1988 as separate guidelines for male [1] and female [2] reproductive risk. These have since been revised and combined; publication is expected in 1992.

Developmental Toxicity

Developmental toxicity refers to the occurrence of adverse effects on the developing organism that result from exposure prior to conception (either parent), during prenatal development, or postnatally up to the time of sexual maturation. Adverse effects initiated during development may be detected at any point in the life span of the organism. The major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency. The EPA first issued developmental toxicity test guidelines in 1982. Guidelines for developmental toxicity risk assessment were issued in 1986 [3] and revised in 1991 [4].

Neurotoxicity

Neurotoxicity is defined as any adverse change in the structure, chemistry, or function of the central and/or peripheral nervous system following exposure to a chemical, physical, or biological agent. Test guidelines for neurotoxicity were first issued by EPA in 1985 [5]. They were revised in 1991 [6]. Along with general requirements for evaluation of motor activity and neuropathology and a functional observational battery, they also provide guidance for testing schedule controlled operant behavior, peripheral nerve function, and glial fibrillary acidic protein. Risk assessment guidelines for neurotoxicity are in the final review stages prior to publication.

Developmental Neurotoxicity

Test guidelines for developmental neurotoxicity have been prepared by EPA for use in the testing of specific chemicals. Test rules requiring developmental neurotoxicity testing were promulgated in 1988 (diethylene ethers [7]) and 1989 (triethylene glycol monoethyl ethers [8]). Generic guidance for developmental neurotoxicity testing and risk assessment is contained in the developmental and neurotoxicity guidelines. Specific factors used to establish the need for evaluation of a chemical's potential to produce developmental neurotoxicity have been identified. They include existing information that a chemical produces central nervous system malformations or adult neurotoxicity, is structurally related to a chemical producing such effects, is hormonally active, or is a peptide or an amino acid.

CONCLUSION

The risk assessment process followed by EPA is based on the National Research Council recommendations of 1983 [9]. The four components of the risk assessment are hazard identification, dose-response assessment, exposure assessment, and risk characterization. A reference dose (RfD) is calculated by dividing a no observed adverse effect level (NOAEL) by an uncertainty factor. The uncertainty factor is used to account for interspecies and intraspecies variations in response to a chemical. Typically, an uncertainty factor of 10 is used to allow for the potential higher sensitivity in humans than animals and another uncertainty factor of 10 to allow for variability in sensitivity among humans. These are multiplied to yield an uncertainty factor of 100. In this case, the RfD is equal to the NOAEL divided by 100. Values other than 10 and different combinations of uncertainty factors are used when supported by an evaluation of the data base for a particular chemical. The maximum uncertainty factor possible is 10,000. Selection of uncertainty factors and their values thus places the calculation of the RfD in the grey area between the science-based hazard assessment and the policy-influenced risk management decision process.

The EPA's risk management decision process employs a risk balancing approach that involves the consideration of the hazard assessment information derived from "science" in light of technical,

socioeconomic, and other "policy" factors. With the risk management decision, the process comes full circle; the socioeconomic and political factors that so often initiate regulatory activity can strongly influence the outcome of that activity. Perhaps the best explanation for the length of time that can elapse between scientific awareness of a problem and a public policy response to that problem is related to the fact that we live in a democratic society. In an attempt to ensure public participation and oversight of federal activity, a very complex process was developed. As long as we adhere to the process, changes in policy will be considered carefully and implemented slowly.

REFERENCES

1. **U.S. Environmental Protection Agency.** 1988. Proposed guidelines for the assessment of male reproductive risk. *Federal Register* 53:24850-24869.

2. **U.S. Environmental Protection Agency.** 1988. Proposed guidelines for the assessment of female reproductive risk. *Federal Register* 53:24834-24837.

3. **U.S. Environmental Protection Agency.** 1986. Guidelines for the health assessment of suspect developmental toxicants. *Federal Register* 51:34028- 34040.

4. **U.S. Environmental Protection Agency.** 1991. Guidelines for developmental toxicity risk assessment: Notice. *Federal Register* 56:63798-63826.

5. **U.S. Environmental Protection Agency.** 1985. Toxic substances control act test guidelines. *Federal Register* 50:39426-39434.

6. **U.S. Environmental Protection Agency.** Office of pesticides and toxic substances neurotoxicity test guidelines. *NTIS* PB91-154617.

7. **U.S. Environmental Protection Agency.** Diethylene glycol butyl ether and diethyl glycol ether acetate: Final test rule. *Federal Register* 53:5932-5953.

8. **U.S. Environmental Protection Agency.** Triethylene glycol monomethyl ether: Final test rule. *Federal Register* 54:13472-13477.

9. **National Research Council.** 1983. Risk Assessment in the Federal Government: Managing the Process. Washington, DC: National Academy Press.

NEUROBEHAVIORAL METHODS USED IN NEUROTOXICOLOGICAL RESEARCH

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SUMMARY

Exposure to chemicals in the environment and workplace can have adverse effects on the nervous system. Behavioral end points are being used with greater frequency in the hazard identification phase of neurotoxicology risk assessment. One reason behavioral procedures are used in animal neurotoxicology studies is that they evaluate neurobiological functions known to be affected in humans exposed to neurotoxic agents, including alterations in sensory, motor, autonomic, and cognitive function. In hazard identification, behavioral tests are used in a tiered-testing context. Tests in the first tier are designed to determine the presence of neurotoxicity. Examples of first-tier behavioral tests include functional observational batteries and motor activity. Second-tier tests are used to characterize neurotoxicant-induced effects on sensory, motor, and cognitive function. Second-tier tests are usually more complex and costly to perform. Reliance on behavioral end points in neurotoxicology risk assessment will likely increase in the future.

INTRODUCTION

Neurotoxicity has historically been defined in terms of chemical-induced changes in the structural integrity of the nervous system [1]. Neurotoxicity has recently been defined as any adverse effect on the structure or function of the central and/or peripheral nervous system produced by chemical exposure [2]. As summarized in Table 38, exposure in humans to neurotoxicants can result in sensory, motor, and cognitive dysfunction [3,4]. Thus, behavioral techniques derived from experimental psychology and neurology have been used to detect and characterize such changes in experimental animals [5,6,7,8,9].

Behavior is regarded as the net output of the sensory, motor, and cognitive functioning in the nervous system, making it a potentially sensitive end point of chemical-induced neurotoxicity [10]. It has also been suggested that, in some cases, behavioral changes may be more sensitive than other indicators of neurotoxicity and may be observed early during exposure [5]. Because of their potential sensitivity to neurotoxic agents, neurobehavioral tests have been recommended in the hazard identification step of the risk assessment process [10]. Neurobehavioral tests are used in countries other than the United States to assess chemicals for developmental neurotoxicity [3,11], whereas in the

United States, regulatory agencies utilize neurobehavioral data in the premarket approval of several chemicals [2,4,12].

TABLE 38. EXAMPLES OF EFFECTS SEEN IN HUMANS EXPOSED TO NEUROTOXIC CHEMICALS

Neurofunction	Effect
Autonomic	Body temperature change, "cholinergic crisis"
Cognitive	Learning, memory dysfunction
	Weakness, decreased strength, tremor
	Incoordination or ataxia
	Abnormal movements
	Hyper- or hypomotor activity
Sensorium	Hallucinations, delusions, apathy, stupor, coma
Sensory	Alterations in smell, vision, taste, hearing, balance, proprioception, feeling, pain

In the hazard evaluation of chemicals for neurotoxicity, behavioral techniques are generally used in a tiered-testing scheme [10]. In tiered-testing, each stage of evaluation includes decision points as to whether or not available information is sufficient for concluding whether or not a chemical is neurotoxic. At the first-tier, simple, rapid, inexpensive methods are typically used to detect the presence of neurotoxicity. Examples of such tests include simple measures of locomotor activity, sensorimotor reflexes, and neurological signs. Studies concerning biological mechanism(s) of action or those intended to determine a no observable adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) typically employ more sensitive tests. Examples of second-tier tests include discriminated conditioned response methodologies to assess specific sensory or motor dysfunction and procedures to measure chemical-induced alterations in cognitive function.

FIRST-TIER TESTS IN NEUROTOXICOLOGY

Behavioral tests employed in hazard identification studies are capable of evaluating large numbers of animals and are not usually regarded to be as sensitive as second-tier procedures. First-tier testing usually involves some form of a neurological screen (i.e., a battery of tests designed to assess a wide range of sensorimotor functions). Functional observational batteries [FOBs] for rodents [13,14,15,16,17], dogs [18] and nonhuman primates [19] have been described. As summarized in Table 39, FOBs assess several neurobiological domains including neuromuscular (i.e., weakness, incoordination, gait, and tremor), sensory (i.e., audition, vision, and somatosensory) and autonomic (i.e., pupil response, salivation) functions. Assessments using the FOB can yield important information

concerning dose–response characteristics and data on the onset, duration, and persistence of chemical-induced behavioral effects [20,21].

TABLE 39. FUNCTIONAL DOMAINS MEASURED IN THE FOB

Autonomic	Neuromuscular
Lacrimation	Gait score
Salivation	Mobility score
Pupil response	Landing foot splay
Palpebral closure	Forelimb grip strength
Defecation	Hindlimb grip strength
Urination	Righting reflex
CNS Activity	Physiological
Home cage posture	Body weight
Rearing	Body temperature
Motor activity	
CNS Excitability	Sensorimotor
Ease of removal	Tail pinch response
Handling reactivity	Click response
Clonic movements	Touch response
Tonic movements	Approach response
Arousal	
Vocalizations	

Another first-tier test used in neurotoxicology hazard identification is motor activity, which includes a broad class of behaviors reflecting the net integrated output of the nervous system [22]. Motor activity is usually quantified as the frequency of movements over time and chemical-induced changes are typically reported as cumulative counts or as a percentage of a vehicle control group or preexposure baseline. Automated motor activity devices have been used extensively to determine the effects of psychoactive and neurotoxic agents [22,23]. It is generally accepted that motor activity measurements are sensitive, reliable, and efficient [23]. A recent interlaboratory comparison [24] found a high degree of comparability in the data generated by different apparatuses and experimental protocols, including within-laboratory-control variability, within-laboratory-replicability of control data, between-laboratory variability in the effects of prototypic chemicals and between-laboratory comparison in the control rates of habituation.

Motor activity measurements alone, however, may lack specificity and may not differentiate psychoactive and other chemicals from neurotoxicants [25]. Motor activity measurements, however, are typically used in conjunction with other first-tier tests such as the FOB. One major conclusion from motor activity is that the acute effect of many chemicals is a depression of motor activity. Some neurotoxicants (i.e., trimethyltin, triadimefon, triadimenol, toluene, and *p*-xylene) increase motor activity, which may be evidence of a direct effect on the nervous system.

SECOND-TIER TESTS IN NEUROTOXICOLOGY

Motor Dysfunction

In his review of the 588 chemicals for which the American Conference of Governmental Industrial Hygienists (ACGIH) recommended threshold limit values (TLV), Anger [4] indicated that 167 (28%) had a TLV based totally or in part on neurological or behavioral effects. Anger [4] also found that 20% of the neurotoxic effects that were reported could be classified as motor effects. In his review on neurotoxicant-induced motor dysfunction, Newland [26] indicated that these effects could be classified into four categories, including tremor, convulsions and spasms, weakness, and incoordination. Table 40 lists examples of behavioral procedures used to quantify chemical-induced alterations in motor function [7,8]. Examples of chemicals known to produce motor dysfunction are acrylamide, 2,5-hexanedione, carbaryl, chlordecone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and manganese.

TABLE 40. BEHAVIORAL PROCEDURES THAT MEASURE CHEMICAL-INDUCED MOTOR DYSFUNCTION

Motor Function	Procedure	Reference
Convulsions	Rating scale	Racine [27]
Incoordination and Abnormal Movements or Posture	Rotorod performance	Bogo et al. [28]
	Rope climbing	Carlini et al. [29]
	Inclined screen	Graham et al. [30]
	Hindlimb splay	Edwards and Parker [31]
	Automated hindlimb test	Tanger et al. [32]
	Gait analysis	Lee and Peters [33]
	Negative geotaxis	Pryor et al. [34]
Tremor	Rating scale	Hong et al. [35]
	Spectral analysis	Newland [26]
		Gerhart et al. [36]
Weakness	Grip strength	Pryor et al. [34]
	Suspension from rod	Molinengo and Orsetti [37]
	Swimming endurance	Bhagat and Wheeler [38]
	Response force titration	Elsner et al. [39]
	Nautilus	Newland [26]

Sensory Function

Exposure to chemicals can produce alterations in sensory function. Of the 69 neurotoxic effects reported in the ACGIH documentation of TLVs, about 14% concerned sensory effects [4]. Anger [40] subsequently reported that some sensory effects may be more prevalent than others. Examples of chemicals having effects on sensory systems include toluene (auditory), methyl mercury (visual), acrylamide (somatosensory), parathion (pain) and 3-methylindole (olfaction).

Several tests have been developed to assess sensory dysfunction and these have been classified into three categories, classical conditioning, operant behavior, and reflex modification (Table 41) [41]. Behavioral methods used to assess sensory function are based on psychophysical principles, which study the relationship between a physical dimension of a stimulus and the response that is generated [41]. Psychophysical assessments involve the presentation of a stimulus along some physical dimension (wavelength, brightness) and require that a response be made or withheld in the presence or absence of that stimulus. Psychophysical methods have been recently described by Maurissen [41] and include the method of limits, constant stimuli, adjustment, and tracking.

TABLE 41. BEHAVIORAL METHODS THAT ASSESS SENSORY DYSFUNCTION

Classification	Procedure	Reference
Classical Conditioning	Conditioned avoidance	Pryor et al. [42]
Operant Behavior	Conditioned suppression	Fox et al. [43]
	Tracking procedure	Maurissen et al. [44]
	Flicker sensitivity	Merigan [45]
	Constant stimulus	Stebbins and Moody [46]
	Discrimination response	Hastings [47]
	Duration discrimination	Daniel and Evans [48]
Reflex Modification	Prepulse inhibition	Eastman et al. [49]
		Crofton et al. [50]
		Fechter and Carlisle [51]

The prepulse inhibition procedure is being used with increased frequency in neurotoxicological studies [49,50,51]. In this procedure, a weak stimulus presented prior to a second eliciting stimulus can suppress a reflex response to the eliciting stimulus. Because the degree of suppression is dependent upon the intensity of the inhibiting stimulus, sensory functioning can be assessed by determining the intensity of the inhibiting stimulus required to produce significant inhibition of the reflex (i.e., a sensory threshold).

Cognitive Dysfunction

Of the effects reported by humans exposed to neurotoxicants only about 6% were classified as cognitive [4]. Examples of chemicals that affect memory in humans include carbon disulfide, styrene, organophosphates, lead, and mercury [40]. The concern about cognitive dysfunction is greater in the area of developmental neurotoxicology where there is a frequent association between mental retardation and congenital anomalies [52,53]. Experimental evaluation of learning and memory in animals is frequently confounded by the fact that cognitive function must be inferred from a change in behavior following exposure. Learning is the process of adaptation to changed contingencies, whereas memory is a construct used to describe the influence of previous events on behavior. Furthermore, alterations in learning and memory must be separated from other changes in

performance that depend on intact sensory and motor functions and motivational states. Toxicant-induced changes in learning and memory should be demonstrated over a range of doses and neurobehavioral procedures. Behavioral techniques to assess chemical effects on learning and memory have been reviewed by Peele and Vincent [54] and Heise [55]. In general, tests for learning and memory can be categorized into measures of nonassociative learning, classical conditioning, and instrumental procedures [55]. Table 42 summarizes a number of techniques that have been used to assess neurotoxicant-induced changes in learning and memory in animals.

TABLE 42. BEHAVIORAL END POINTS THAT HAVE BEEN USED TO ASSESS LEARNING AND MEMORY

Classification	Method	Reference
Classical Conditioning	Eye-blink reflex	Yokel [56]
	Flavor aversion	Riley and Tuck [57]
		Peele et al. [58]
Habituation	Startle reflex	Overstreet [59]
Instrumental Conditioning	Passive avoidance	Walsh et al. [60]
	Active avoidance	Tilson et al. [61]
	Radial arm maze	Walsh et al. [62]
	Biel water maze	Vergieva and Zaikov [63]
	Morris water maze	Tilson et al. [64]
	Repeated acquisition	Paule and McMillan [65]
	Delayed acquisition	Bushnell [66]
		Rice and Karpinski [67]
	Matched to position	Bushnell et al. [68]
	Reversal learning	Rice [69]

Schedule-Controlled Behavior

Schedule-controlled behavior refers to a special application of operant behavior, which can be defined as movement that operates on or alters the environment. Operant responses become conditioned or learned when their frequency is modified by the consequences of the response through a process known as reinforcement. Schedule-controlled behavior is based on the principle that reinforcement is usually applied intermittently and behavior can be predicted on the basis of the programming or scheduling of reinforcement that follows responding [70]. For example, reinforcement can occur after a certain number of responses have been made or after a specified period of time, or a combination of both. A schedule of reinforcement specifies the relation between a specific response and the delivery of reinforcement. For example, Fixed Interval schedules require that a specific period of time elapse before a response is reinforced, whereas a Fixed Ratio schedule requires that a certain number of responses occur before reinforcement is delivered. Interval and ratio schedules can also be variable where the time or number of responses varies around some average.

Like motor activity, schedule-controlled behavior reflects the net integrated sensory, motor, and cognitive abilities of an organism. Schedule-controlled behavior has been used extensively in animal behavioral toxicology where it has been shown to be sensitive to a wide range of neurotoxicant agents [7,9]. Schedule-controlled behavior is useful in that the experimental animal frequently serves as its own control. Schedule-controlled behavior has been used to quantify effects of solvents [71], pesticides [72] and metals [69,73,74,75].

CONCLUSION

Behavioral procedures are being used with greater frequency to identify and characterize chemical-induced alteration in nervous system function. In the future, the extent to which behavioral studies will be used in risk assessment will depend on demonstrated validity and an understanding of the neural substrate affected by exposure to neurotoxic agents.

REFERENCES

1. **Weiss, B.** 1988. Quantitative perspectives on behavioral toxicology. *Toxicol. Lett.* 43:285-293.

2. **Tilson, H.A.** 1990. Neurotoxicology in the 1990s. *Neurotoxicol. Teratol.* 12:293-300.

3. **World Health Organization (WHO).** 1986. Principles and methods for the assessment of neurotoxicity associated with exposure to chemicals. In: *Environmental Health Criteria.* Document 60. World Health Organization, Geneva.

4. **Anger, W.K.** 1984. Neurobehavioral testing of chemicals: Impact of a recommended standards. Neurobehav. *Toxicol. Teratol.* 6:147-153.

5. **Weiss, B. and V. Laties.** 1975. *Behavioral Toxicology.* Plenum Press, New York.

6. **Annau, Z.** 1986. *Neurobehavioral Toxicology.* Johns-Hopkins Press.

7. **Tilson, H.A.** 1987. Behavioral indices of neurotoxicity: What can be measured. *Neurotoxicol. Teratol.* 9:427-443.

8. **Cory-Slechta, D.A.** 1989. Behavioral measures of neurotoxicity. *Neurotoxicology* 10:271-296.

9. **Rice, D.C.** 1988. Quantification of operant behavior. *Toxicol. Lett.* 43:361-379.

10. **National Academy of Sciences [NAS].** 1975. *Principles for Evaluating Chemicals in the Environment.* National Academy of Sciences, Washington, DC.

11. **Kimmel, C.A.** 1988. Current status of behavioral teratology: Science and regulation. *CRC Rev. Toxicol.* 19:1-10.

12. **U.S. EPA.** 1991. *Neurotoxicity Testing Guidelines.* National Technology Information Service, Springfield, VA.

13. **Gad, S.C.** 1982. A neuromuscular screen for use in industrial toxicology. *J. Toxicol. Environ. Health* 9:691-704.

14. **Moser, V.C., J.P. McCormick, J.P. Creason, and R.C. MacPhail.** 1988. Comparison of chlordimefon and carbaryl using a functional observational battery. *Fund. Appl. Toxicol.* 11:189-206.

15. **Haggerty, G.C.** 1989. Development of Tier I neurobehavioral testing capabilities for incorporation into pivotal rodent safety assessment studies. *J. Am. Coll. Toxicol.* 8:53-70.

16. **O'Donoghue, J.L.** 1989. Screening for neurotoxicity using a neurologically based examination and neuropathology. *J. Am. Coll. Toxicol.* 8:97-116.

17. **Moser, V.C.** 1989. Screening approaches to neurotoxicity: A functional observational battery. *J. Am. Coll. Toxicol.* 8:85-94.

18. **Schaeppi, U. and R.E. Fitzgerald.** 1989. Practical procedure of testing for neurotoxicity. *J. Am. Coll. Toxicol.* 8:29-34.

19. **O'Keeffe, R.T. and K. Lifshitz.** 1989. Nonhuman primates in neurotoxicity screening and neurobehavioral toxicity studies. *J. Am. Coll. Toxicol.* 8:127-140.

20. **Moser, V.C.** 1991. Applications of a neurobehavioral screening battery. *J. Am. Coll. Toxicol.* 10:661-669.

21. **Tilson, H.A. and V.C. Moser.** 1992. Comparison of screening approaches. *Neurotoxicology* 13:1-14.

22. **Reiter, L.W. and R.C. MacPhail.** 1982. Factors influencing motor activity measurements in neurotoxicology. In: C.L. Mitchell, ed. *Nervous System Toxicology*, pp. 45-65. New York, Raven Press.

23. **MacPhail, R.C., D.B. Peele, and K.M. Crofton.** 1989. Motor activity and screening for neurotoxicity. *J. Amer. Coll. Toxicol.* 8:117-125.

24. **Crofton, K.M., J.L. Howard, V.C. Moser, M.W. Gill, L.W. Reiter, H.A. Tilson, and R.C. MacPhail.** 1991. Interlaboratory comparison of motor activity experiments: Implications for neurotoxicological assessments. *Neurotoxicol. Teratol.* 13:599-609.

25. **Maurissen, J.P.J. and J.L. Mattsson.** 1989. Critical assessment of motor activity as a screen for neurotoxicity. *Toxicol. Ind. Health* 5:195-202.

26. **Newland, M.C.** 1988. Quantification of motor function in toxicology *Toxicol. Lett.* 43:295-319.

27. **Racine, R.** 1972. Modification of seizure activity by electrical stimulation II. Motor seizure. Electroencephalogr. *Clin. Neurophysiol.* 32:281-294.

28. **Bogo, V., T.A. Hill, and R.W. Young.** 1981. Comparison of accelerod and rotarod sensitivity in detecting ethanol- and acrylamide-induced. A performance decrement in rats: Review of experimental considerations of rotating rod systems. *Neurotoxicology* 2:765-787.

29. **Carlini, E.A., M.T.A. Silva, L.C Cesare, and R.M. Endo.** 1967. Effects of chronic administration of B-(3,4-dimethoxyphenyl)-ethylamine and (B-3,4,5-trimethoxyphenyl)-ethylamine on the climbing rope performance of rats. *Med. Pharmacol. Exp.* 17:534-542.

30. **Graham, R.C.B., F.C. Lu, and M.G. Allmark.** 1957. Combined effect of tranquilizing drugs and alcohol on rats. *Fed. Proc.* 16:302.

31 **Edwards, P.M. and V.H. Parker.** 1977. A simple, sensitive and objective method for early assessment of acrylamide neuropathy in rats. *Toxicol. Appl. Pharmacol.* 40:589-591.

32. **Tanger, H.J., R.A.P. Vanwersch, and O.L. Wolthius.** 1984. Automated quantitative analysis of coordinated locomotor behaviour in rats. *J. Neurosci. Meth.* 10:237-245.

33. **Lee, C.C. and P.J. Peters.** 1976. Neurotoxicity and behavioral effects of thiram in rats. *Environ. Health Perspect.* 17:35-43.

34. **Pryor, G.T., E.T. Uyeno, H.A. Tilson, and C.L. Mitchell.** 1983. Assessment of chemicals using a battery of neurobehavioral tests: A comparative study. *Neurotoxicol. Teratol.* 5:91-118.

35. **Hong, J.S., H.A. Tilson, L.L. Uphouse, J. Gerhart, and W.E. Wilson.** 1984. Effects of chlordecone exposure on brain neurotransmitters: Possible involvement of the serotonin system in chlordecone-induced tremor. *Toxicol. Appl. Pharmacol.* 73:336-344.

36. **Gerhart, J.M., J.S. Hong, L.L. Uphouse, and H.A. Tilson.** 1982. Chlordecone-induced tremor: quantification and pharmacological analysis. *Toxicol. Appl. Pharmacol.* 66:234-243.

37. **Mollinengo, L. and M. Orsetti.** 1976. Drug action on the "grasping reflex" and on swimming endurance: An attempt to characterize experimentally antidepressant drugs. *Neuropharmacology* 15:257-260.

38. **Bhagat, B. and M. Wheeler.** 1973. Effect of nicotine on the swimming endurance of rats. *Neuropharmacology* 12:1161-1165.

39. **Elsner, J., C. Fellmann, and G. Zbinden.** 1988. Response force titration for the assessment of the neuromuscular toxicity of 2,5-hexanedione in rats. *Neurotox. Teratol.* 10:3-13.

40. **Anger, W.K.** 1990. Worksite behavioral research: Results, sensitive methods, test batteries and the transition from laboratory data to human health. *Neurotoxicology* 11:629-720.

41. **Maurissen, J.P.J.** 1988. Quantitative sensory assessment in toxicology and occupational medicine: applications, theory and critical appraisal. *Toxicol. Lett.* 43:321-343.

42. **Pryor, G., J. Dickinson, R.A. Howd, and C.S. Rebert.** 1983. Transient cognitive deficits and high-frequency hearing loss in weanling rats exposed to toluene. *Neurobehav. Toxicol. Teratol.* 5:53-57.

43. **Fox, D.A., A.A. Wright, and L.G. Costa.** 1982. Visual acuity deficits following neonatal lead exposure: cholinergic interactions. *Neurobehav. Toxicol. Teratol.* 4:689-693.

44. **Maurissen, J.P.J., B. Weiss, and H.T. Davis.** 1983. Somatosensory thresholds in monkeys exposed to acrylamide. *Toxicol. Appl. Pharmacol.* 71:266-279.

45. **Merigan, W.H.** 1979. Effects of toxicants on visual systems. *Neurobehav. Toxicol.* 1 [Suppl.]:15-22.

46. **Stebbins, W. and D.B. Moody.** 1979. Comparative behavioral toxicology. *Neurobehav. Toxicol.* 1 [Suppl.]:33-44.

47. **Hastings, L.** 1990. Sensory neurotoxicology: use of the olfactory system in the assessment of toxicity. *Neurotoxicol. Teratol.* 12:455-459.

48. **Daniel, D.A. and H.L. Evans.** 1985. Effects of acrylamide on multiple behavioral end points in the pigeon. *Neurobehav. Toxicol. Teratol.* 7:267-273.

49. **Eastman, C.L., J.S. Young, and L.D. Fechter.** 1987. Trimethyl ototoxicity in albino rats. *Neurotoxicol. Teratol.* 9:329-332.

50. **Crofton, K.M., K.F. Dean, M.G. Menache, and R. Janssen.** 1990. Trimethyl effects on auditory function and cochlear morphology. *Toxicol. Appl. Pharmacol.* 105:123-132.

51. **Fechter, L.D. and L. Carlisle.** 1990. Auditory dysfunction and cochlear vascular injury following trimethyltin exposure in the guinea pig. *Toxicol. Appl. Pharmacol.* 105:133-143.

52. **Illingworth, R.S.** 1959. Congenital anomalies associated with cerebral palsy and mental retardation. *Arch. Dis. Child.* 34:228-238.

53. **Smith, D.W. and K.E. Bostian.** 1964. Congenital anomalies associated with idiopathic mental retardation. *J. Pediatr.* 65:189-199.

54. **Peele, D.B. and A. Vincent.** 1989. Strategies for assessing learning and memory, 1978-1987: A comparison of behavioral toxicology, psychopharmacology and neurobiology. *Neurosci. Biobehav. Rev.* 13:33-38.

55. **Heise, G.A.** 1984. Behavioral methods for measuring effects of drugs on learning and memory in animals. *Med. Res. Rev.* 4:535-558.

56. **Yokel, R.A.** 1983. Repeated systemic aluminum exposure effects on classical conditioning of the rabbit. *Neurobehav. Toxicol. Teratol.* 5:41-46.

57. **Riley, A.L. and D.L. Tuck.** 1964. Conditioned taste aversions: A behavioral index of toxicity. *Ann. NY. Acad. Sci.* 443:272-292.

58. **Peele, D.B., J.D. Farmer, and J.E. Coleman.** 1989. Time-dependent deficits in delay conditioning produced by trimethyltin. *Psychopharmacology* 97:521-528.

59. **Overstreet, D.H.** 1977. Pharmacological approaches to habituation of the acoustic startle response in rats. *Physiol. Psychol.* 5:230-238.

60. **Walsh, T., M. Gallagher, E. Bostock, and R.S. Dyer.** 1982. Trimethyltin impairs retention of a passive avoidance task. *Neurobehav. Toxicol. Teratol.* 4:163-167.

61. **Tilson, H.A., S. Shaw, and R.L. McLamb.** 1987. The effects of lindane, DDT, and chlordecone on avoidance responding and seizure activity. *Toxicol. App. Pharmacol.* 88:57-65.

62. **Walsh, T., D.B. Miller, and R.S. Dyer.** 1982. Trimethyltin, a selective limbic system neurotoxicant impairs radial-arm maze performance. *Neurobehav. Toxicol. Teratol.* 4:177-183.

63. **Vergieva, T. and H.R. Zaikov.** 1981. Behavioral change in rats with inhalation of styrene. *Hig. Zdraveopazvane.* 24:242-247.

64. **Tilson, H.A., B. Veronesi, R.L. McLamb, and H.B. Matthews.** 1990. Acute exposure to tris[2-chloroethyl]phosphate produces hippocampal neuronal loss and impairs learning in rats. *Toxicol. Appl. Pharmacol.* 106:254-269.

65. **Paule, M.G. and D.E. McMillan.** 1986. Effects of trimethyltin on incremental repeated acquisition [learning] in the rat. *Neurobehav. Toxicol. Teratol.* 8:245-253.

66. **Bushnell, P.J.** 1988. Effects of delay, intertrial interval, delay behavior and trimethyltin on spatial delayed response in rats. *Neurotoxicol. Teratol.* 10:237-244.

67. **Rice, D.B. and K.F. Karpinski.** 1988. Lifetime low-level lead exposure produces deficits in delayed alternation in adult monkeys. *Neurotoxicol. Teratol.* 10:207-214.

68. **Bushnell, P.J., S.S. Padilla, T. Ward, C.N. Pope, and V.B. Olszyk.** 1991. Behavioral and neurochemical changes in rats dosed repeatedly with diisopropyl fluorophosphate [DFP]. *J. Pharmacol. Exp. Therap.* 256:741-750.

69. **Rice, D.B.** 1990. Lead-induced behavioral impairment on a spatial discrimination reversal task in monkey exposed during different periods of development. *Toxicol. Appl. Pharmacol.* 106:327-333.

70. **Ferster, C.B. and B.F. Skinner.** 1957. *Schedules of Reinforcement.* Appleton-Century-Crofts, New York.

71. **Glowa, J.R.** 1985. Behavioral effects of volatile organic solvents. In: L.S. Seiden and R.L. Balster, eds. *Behavioral Pharmacology: The Current Status,* pp. 537-552. Alan Liss, New York.

72. **MacPhail, R.C.** 1985. Effects of pesticides on schedule-controlled behavior. In: L.S. Seiden and R.L. Balster, eds. *Behavioral Pharmacology: The Current Status,* pp.519-535. Alan R. Liss, New York.

73. **Cory-Slechta, D.A. and T. Thompson.** 1979. Behavioral toxicity of chronic postweaning lead exposure in the rat. *Toxicol. Appl. Pharmacol.* 47:151-159.

74. **Cory-Slechta, D.A., B Weiss, and C. Cox.** 1985. Performance and exposure indices of rats exposed to low concentrations of lead. *Toxicol. Appl. Pharmacol.* 78:291-299.

75. **Rice, D.C., S.G. Gilbert, and R.F. Willes.** 1979. Neonatal low-level exposure in monkeys: locomotor activity, schedule-controlled behavior, and the effects of amphetamine. *Toxicol. Appl. Pharmacol.* 51:503-513.

USE OF ALTERNATIVE END POINTS WITH THE UPITT II METHOD FOR ASSESSING THE TOXICITY OF SMOKE

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SUMMARY

A small-scale combustion module and animal exposure system for evaluating flaming combustion and toxicity of smoke from burning polymers has been developed at the University of Pittsburgh and designated the UPitt II method [1]. It is a radical departure from previous attempts to evaluate toxicity of smoke from burning polymers and represents a significant improvement over previous systems because well-defined burning conditions can be established over a wide range of imposed heat flux and ventilation levels, not possible to examine with previous methods. Thus, smoke toxicity can be evaluated under a range of burning conditions and differences in toxic potency and time to effect can be evaluated [2].

A Potential Smoke Hazard (PSH) index was developed which integrates the material performance characteristics which determine ease of ignition of a polymer with toxic potency, time to death, and rate of generation of toxicants [3]. This paper describes sublethal endpoints that can be used with the UPitt II method and modifications to the PSH index for evaluating the toxicity of smoke generated by burning polymers.

INTRODUCTION

Numerous hazards to humans result from exposure to the products of combustion of polymers. Predominant among these are effects from heat and flames, visual obscuration due to the density of smoke or to eye irritation, narcosis from inhalation of asphyxiants, and irritation of the upper and/or lower respiratory tracts. These effects, often occurring almost simultaneously in a fire, contribute to physical incapacitation, loss of motor coordination, faulty judgement, disorientation, restricted vision, and panic. The resulting delay or prevention of escape may lead to subsequent injury or death from further inhalation of toxic gases and/or the suffering of thermal burns. Survivors from a fire may also experience postexposure pulmonary complications and burn injuries which can lead to delayed death.

In most fires, obscuration of vision by smoke is the first threat to safe egress, although visual obscuration per se is not necessarily a dangerous physiological threat to life. The hazards of thermal injury are usually present only in the proximity of the fire itself. However, it is the physiological and

behavioral effects of the inhalation of smoke that can be manifest even at considerable distances from the fire.

Assessment of the overall physiological and behavioral effects of exposure of humans to fire and its smoke is an extremely difficult and complex task requiring quantification of what constitutes a hazardous "dose" of smoke. This is one of the most technically difficult aspects of assessing toxicity of smoke from fires because smoke is a mixture of airborne solid and liquid particles and gases which are evolved when a material undergoes pyrolysis or flaming combustion. Furthermore, the nature of the smoke changes continuously from the initial phase until the end of each fire [1,4]. The toxic and irritant properties of the smoke produced are therefore time-dependent. This dictates a dynamic (i.e., flow-through) animal exposure system be employed.

Three fundamental variables are needed to describe the conditions of real fires. These are the imposed heat flux, or irradiance, the ventilation and the mass loss rate [4]. The UPitt II apparatus is unique in that it permits control of both heat flux and ventilation, and measurement of the mass loss rate of the burning specimen [1]. Previous small-scale methods to evaluate toxicity of smoke from burning polymers all have the same limitations [5]. The most significant drawback is that the conditions under which the specimens are thermally decomposed are difficult to define in terms of energy supplied to the specimens and ventilation available for combustion. For those methods where the energy source or ventilation parameter has been adequately characterized there is very little change permitted in burning conditions due to the restrictions imposed by the apparatus used. For most of these methods there is no possibility of maintaining continuous flaming conditions for a given specimen for a long period of time such as 30 min [5]. Thus, evaluation of the toxicity of the smoke produced is restricted to one set of conditions in each apparatus used [5].

Concentrations of common fire gas toxicants, such as carbon monoxide (CO) and hydrogen cyanide (HCN), are usually measured during combustion toxicity experiments and are expressed as parts per million (ppm) by volume [1,5,6]. Therefore, the "dose" can be expressed as the product of the concentration and time of exposure (i.e., ppm-min). In the case of a changing concentration of a gaseous toxicant, the "dose" is actually the integrated area under a concentration versus time curve. However, this remains an approximation of the dose because it does not define the amount of toxicants actually entering the body. To do this requires measurement of respiratory variables such as breathing frequency and minute volume.

For other fire gases such as the irritants, which may be present in unknown concentrations, one can still deal with the concept of dose, or more correctly, exposure concentration, as it applies to smoke. Because the smoke concentration is difficult to quantify, an approximation is made that the smoke concentration is proportional to the mass loss of the material during a fire [5]. The integrated

area under a mass loss per unit volume versus time curve thus becomes a measure of the smoke exposure (e.g., mg-min/L). The yield of toxicant from a material, for example CO, may be very high or very low for the same mass loss depending upon different burning conditions [1,2,3]; therefore, using only the mass loss to characterize the toxicity of a material is not very informative.

There is a further complication with combined toxicants that is more difficult to deal with. Each individual toxicant may have physiological effects other than that of its principal specific toxicity. These effects, particularly those involving the respiratory system, may alter the rate of uptake of other toxicants. For example, HCN is known to cause hyperventilation early in an exposure, with a reported fourfold increase in the respiratory minute volume (RMV) of monkeys [7]. However, respiration eventually slows as narcosis results. It has been suggested that this initial hyperventilation in primates may result in faster incapacitation from HCN than would be expected, along with more rapid carboxyhemoglobin (COHb) saturation should CO also be present [7]. Carbon monoxide, by decreasing the oxygen-transport capacity of the blood, leads to a condition of metabolic acidosis and a slowing of respiration rate. Carbon dioxide, on the other hand, is a respiratory stimulant which increases the rate of uptake of other toxicants, producing a faster rate of formation of COHb from inhalation of CO. Irritants, such as hydrogen chloride (HCl), slow respiration in rodents due to sensory irritation [8,9], but increase RMV in primates from pulmonary irritation [10]. With primates, there is also evidence of the irritant HCl causing bronchoconstriction which may interfere with oxygen (O_2) reaching the alveoli for diffusion into the blood. The situation is extremely complex with all these effects possible in the inhalation of the mixture of toxicants produced in a real fire.

A contributing element to fire deaths, particularly in victims with sublethal COHb concentrations, is likely to be HCN [11,12,13]. Some 60 to 80% of fire victims had blood cyanide values above background levels. Despite the recognition that CO is the primary toxicant produced during fires, HCN must not be overlooked, especially since it is nearly 20 times more potent than CO. A cyanide value of 1 mg/L of blood has been proposed as the fatal threshold value [11,13]. Although there is no evidence for synergistic effects between CO and HCN, these two asphyxiants appear to be additive [11,12,14,15].

In the case of mixtures of HCl and CO, empirical analysis of toxicological data shows that exposure doses leading to lethality may also be additive [16]. A rapid respiratory acidosis was seen in the blood of rats exposed to HCl which, when coupled with the metabolic acidosis produced by CO, resulte n severely compromised animals. Observations with rodents also suggest complications occurring immediately following exposure. This implies that HCl may be much more dangerous than previously thought when in the presence of CO, or, conversely, CO intoxication may be much more serious in the presence of an irritant. There is also some suggestion that the incapacitating effects of CO may be enhanced in primates upon simultaneous exposure to HCl, the presence of which causes

the partial pressure of oxygen in arterial blood to be decreased [10]. This may have significance with human exposure, such as prolonged hypoxemic conditions following rescue or escape.

There may also be additivity between HCl and HCN as shown by the incidence of postexposure deaths from concentrations of the toxicants, each of which alone would not be expected to result in any postexposure lethality [9]. Deaths were often several days after the exposure.

The toxic potency of CO_2 is quite low and it is not normally considered to be a significant factor in fire deaths. Carbon dioxide stimulates respiration, leading to an increased rate of formation of blood COHb from inhalation of CO. However, the same equilibrium COHb saturation is reached as in the absence of CO_2. An increased incidence of lethality, particularly postexposure, has been observed with certain combinations of CO and CO_2 in rats [17]. The effect may be associated with the combined insult of respiratory acidosis, caused by CO_2, and metabolic acidosis, caused by CO, a condition from which the rodent has difficulty recovering postexposure. Whether or not these effects of CO_2 occur with primates has not been determined.

Escape is the key to survival from a fire, with tenability being the time available for escape. The time factor has two components, only one of which is the time for an exposed person to accumulate a hazardous dose. Of equal importance is the time required to generate and transport the quantity of smoke which would result in a hazardous dose. Thus, tenability is very much a function of the fire growth rate and resulting time-dependent yield of toxicants. These in turn depend on fundamental physical and chemical properties of materials which influence the way they burn.

In order to determine just what constitutes a dose that is hazardous to humans, and the time during a fire at which this untenable condition occurs, one must turn to laboratory experiments using animals exposed under conditions simulating those of real fire atmospheres. This requires that fire conditions must be well defined.

THE UPITT II METHOD

The UPitt II method has been described previously in great detail [1]. The toxic potency of smoke generated by Douglas fir under realistic burning conditions was determined using the UPitt II method [2]. Combining data from all burning conditions evaluated, the median lethal smoke concentration ($SCLC_{50}$) for a 30-min exposure to smoke from Douglas fir was 113 mg/L (95% confidence interval was 108 to 118 mg/L) [2]. No deaths were observed at a smoke exposure concentration below 76 mg/L for a 30-min exposure. Therefore, a "survivable exposure concentration" (SEC) for smoke from Douglas fir was set at 76 mg/L for an exposure period of 30 min following the beginning of heat flux to this material, regardless of the burning conditions which follow [2]. Lethality data were also analyzed for time to death to obtain median time to death, survival distribution function, and the statistically derived cumulative hazard function. These showed

that although the toxic potency of the smoke (i.e., the $SCLC_{50}$), was similar for the burning conditions investigated, the time to effect was different [2]. The cumulative hazard function was highest under the most efficient flaming conditions which produced low CO. Thus, the lethal effect of smoke from Douglas fir was not predicted from a simple analysis of CO as was previously suggested [1,5,6].

Additional polymers were selected for evaluation on the basis of expected toxicants that would be produced under burning conditions selected to enable comparison to Douglas fir data. Polyvinyl chloride (*PVC*) which produces HCl; a rigid polyurethane foam (*RPU*) which produces HCN; and polycarbonate (*PC*) and polymethylmethacrylate (PMMA), both of which produce CO; were evaluated with the UPitt II apparatus. As found using Douglas fir, the cummulative hazard function for exposure to smoke of a polymer varied with the burning conditions [3]. However, the $SCLC_{50}$ over the range of burning conditions evaluated was a good predictor of lethality [3].

DISCUSSION OF LETHALITY AS AN END POINT

The toxic potency of the polymers was directly compared by analysis of experimental data obtained with the UPitt II method because the same range of burning conditions was used to evaluate each polymer. The relative ranking, based solely on toxic potency (e.g., $SCLC_{50}$), was from most to least potent:

$$PVC \leq RPU > PC > > DF$$

The relationship between the median time to death (MTD) and the SC at all burning conditions for each of the polymers can also be investigated to obtain an estimate of the time necessary to produce an effect starting at the beginning of heat flux to the specimens. The MTD for *RPU* was clustered around 11 min regardless of SC. The MTD decreased as SC increased for the other polymers investigated. A similar relationship was obtained between the time of first death and the SC. This indicates that a relationship exists between the integrated SC and the time of death (i.e., the higher the SC, the earlier the animals die). This has been shown previously by other investigators [18].

Although the MTD and time to first death allow comparisons of time to effect to be made for different materials, these values do not present all the data and represent discrete time points. The cumulative hazard funciton, Σh, calculated as described previously [3] uses all available data and allows a comparison of the rate of change of effect for exposure to smoke of the same material at different burning conditions, or between different materials at the same burning conditions, regardless of the principal toxicants involved. For polymers evaluated under the same burning conditions (heat flux of 35 kW/m^2 and ventilation of 41.5 L/min), the Σh for PMMA and *PVC* were similar and reached a high level in 5 min. The Σh for *DF* and *PC* were zero, and the Σh for *RPU* was intermediate. This kind of analysis can be made for any burning conditions.

THE POTENTIAL SMOKE HAZARD INDEX

The above comparisons, although appropriate, are very restrictive in that they do not permit general conclusions, except for the toxic potency (e.g., $SCLC_{50}$) which included all burning conditions. The material performance data were not included, except mass loss rate. It is clear that material performance should be included because the stability of some materials precluded ignition at lower imposed heat flux. On the other hand, PVC evolved toxicants (HCl) even though ignition did not occur at the lowest heat flux evaluated. No deaths occurred at these heat flux levels, yet taking toxic potency alone and neglecting this fact is not appropriate when addressing the toxic hazard in fires. The hazard analysis must include material performance as well [19,20]. The UPitt II method and experimental design determines the time to ignition (T_{ign}) for a material at a variety of heat flux levels, and from these data the critical flux (q''_{cr}) and thermal inertia (TI) can be calculated [21]. These variables can then be taken into account to calculate an index of potential smoke hazard (PSH) [3].

The PSH incorporates the variables, q''_{cr} and TI, which determine "ease of ignition" of a material and rate of fire development (flame spread), the toxic potency and time to effect ($SCLC_{50}$ and MTD), and the rate of generation of toxicants from the mass loss rate (m). Thus, the formula for PSH is:

$$PSH = (S \times m)/(q''_{cr} \times SCLC_{50} \times MTD)$$

where S is the slope of the regression line of T_{ign} versus the irradiance level and is proportional to the thermal inertia [21].

In this formula, S and m are in the numerator because larger values of S (denoting lower TI) and m will increase the hazard of exposure to the smoke produced by a polymer. As the values of q''_{cr}, $SCLC_{50}$, and MTD increase, the hazard decreases and therefore these variables appear in the denominator. Thus, a low PSH value indicates that a material is relatively safer than materials with a higher PSH value.

The above formula was used to calculate the PSH for the experiments described above [3]. The average PSH at each heat flux are presented in Figure 66, which shows that PC had the lowest PSH; DF had the next lowest; the PSH for RPU and PVC were similar and higher than the value for DF; and PMMA had the largest PSH (approximately 40 times that of DF). General conclusions concerning the potential hazard of exposure to smoke from the different polymers indicate that PC is the least hazardous polymer when materials characteristics are considered in conjunction with toxicity data. Douglas fir has the next lowest PSH values at each heat flux, and the lowest relative increase in PSH from 21 to 50 kW/m^2 (e.g., 4.7 times) [3]. The PSH values for RPU increased 7.6 times over the same range of heat flux, and the higher PSH values compared to PC and DF were the result of the material properties (e.g., larger S, indicating lower thermal inertia) and the earlier times to death due to the

presence of HCN in the smoke. The PSH values for PVC increased 18.4 times over the same range of heat flux and was the largest increase found.

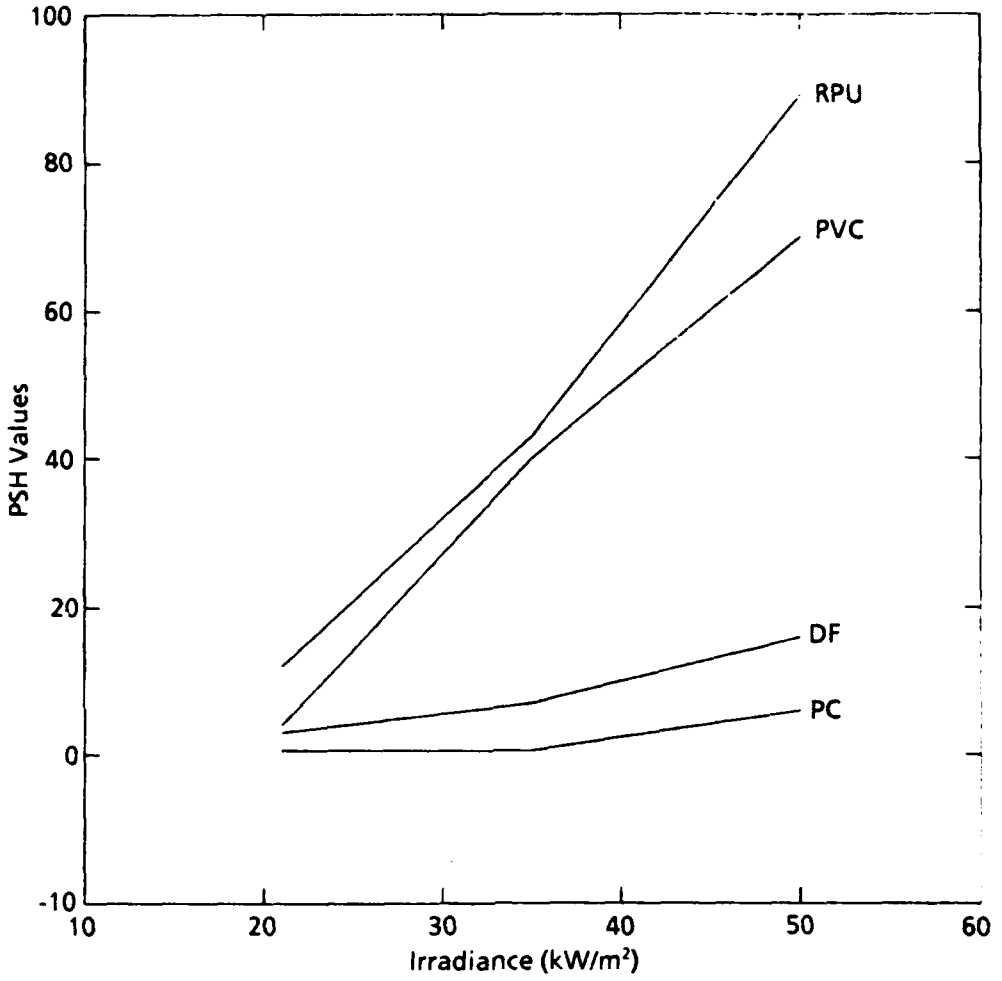

Figure 66. Plot of PSH Values vs. Irradiance Levels for the Polymers Investigated.

USE OF ALTERNATIVE END POINTS WITH THE UPITT II METHOD

An animal model was developed which could be used to evaluate incapacitating effect of smokes from burning materials rather than for evaluation of the lethal effect [22]. Another model capable of evaluating the incapacitating effect of asphyxiants as well as irritants permits evaluation of performance time prior to incapacitation [23]. The ergometer within a whole-body plethysmograph used in this model can easily be coupled to the UPitt II apparatus to evaluate smoke generated by any material under realistic burning conditions. More importantly, predictions for human exposures based on this animal model [23] are in line with the assessment of the effect of CO in humans calculated by previous researchers [24,25]. Using these existing models for time to

incapacitation can yield information of great utility for modeling hazards of human exposure to smoke.

Attention processing decrements in humans exposed to levels of CO representative of concentrations found in smoke from "survivable" fires [1,2,3] have been evaluated through electrophysiological measurement of auditory event-related potentials [26]. Attention-related components of the evoked potential appear to be a particularly sensitive measure of CO effects.

The effects to the lungs of smoke administered in nonlethal doses can be evaluated by a variety of techniques, such as determination of changes in pulmonary function [27] or chemical mediators [28]. Some of these methods are well suited for experiments involving repeated exposures of the same animals and can provide information on potential immune-mediated effects.

The PSH index can be modified and a survivability index can be calculated based on data from the above alternative end points, while retaining the material performance characteristics determined with the UPITT II method.

CONCLUSIONS

The UPitt II method has been succesfully used to determine the potential toxicity of smoke from burning polymers. Lethality data obtained with this apparatus under different burning conditions permits calculation of the toxic potency of a polymer in terms of the smoke concentration resulting in 50% mortality ($SCLC_{50}$) to exposed animals and estimation of a survivable smoke exposure concentration (SEC). Fundamental statistical analysis of time to death data and calculation of the cummulative hazard for exposure to the smoke permits for the first time a detailed comparison of the toxicity of smoke produced by a polymer under different burning conditions, or comparisons between the smoke of different polymers submitted to the same burning conditions.

The calculation of the PSH integrates the material performance characteristics which determine ease of ignition and rate of flame spread with toxic potency, time to effect, and the rate of generation of toxicants from a polymer.

This newly developed concept of PSH index can be expanded by using the alternative nonlethal end points, such as time to incapacitation, to calculate a survivability index.

DISCLAIMER

The views, opinions, and/or findings contained in this report are those of the author and should not be construed as official Department of the Army position policy, or decison, unless so designated by other official documentation.

REFERENCES

1. **Caldwell, D.J. and Y. Alarie.** 1990. A method to determine the potential toxicity of smoke from burning polymers. I. Experiments with Douglas fir. *J. Fire Sci.* 8:23-62.

2. **Caldwell, D.J. and Y. Alarie.** 1990. A method to determine the potential toxicity of smoke from burning polymers. II. The toxicity of smoke from Douglas fir. *J. Fire Sci.* 8:275-309.

3. **Caldwell, D.J. and Y. Alarie.** 1991. A method to determine the potential toxicity of smoke from burning polymers. III. Comparison of synthetic polymers to Douglas fir using the UPitt II Flaming Combustion/Toxicity of Smoke Apparatus. *J. Fire Sci.* 9:470-518.

4. **Drysdale, D.** 1985. An introduction to fire dynamics, pp. 164-183, Wiley, New York.

5. **Kaplan, H.L., A.F. Grand, and G.E. Hartzell.** 1983. Combustion Toxicology: Principles and Test Methods, p. 174. Lancaster, PA: Technomic Publishing Co., Inc..

6. **Babrauskas, V., R.H. Harris, E. Braun, B. Levin, M. Paabo, and R. Gann.** 1991. The role of bench-scale test data in assessing real-scale fire toxicity. NIST Technical Note, National Institute of Standards and Technology, Gaithersburg, MD.

7. **Purser, D.A., P. Grimshaw, and K.R. Berrill.** 1984. The role of hydrogen cyanide in the acute toxicity of the pyrolysis products of polyacrylonitrile. *Arch. Env. Health* 39:394-400.

8. **Barrow, C.S., Y. Alarie, J.C. Warrick, and M.F. Stock.** 1977. Comparison of the sensory irritation response in mice to chlorine and hydrogen chloride. *Arch. Env. Health* 32:68-76.

9. **Hartzell, G.E., S.C. Packham, A.F. Grand, and W.G. Switzer.** 1985. Modeling of toxicological effects of fire gases: III. Quantification of Post-exposure lethality of rats from exposure to HCl atmospheres. *J. Fire Sci.* 3:195-207.

10. **Kaplan, H.L., A.F. Grand, W.G. Switzer, D.S. Mitchell, W.R. Rogers, and G.E. Hartzell.** 1985. Effects of combustion gases on escape performance of the baboon and the rat. *J. Fire Sci.* 3:228-255.

11. **Esposito, F.M. and Y. Alarie.** 1988. Inhalation toxicity of carbon monoxide and hydrogen cyanide gases released during thermal decomposition of polymers. *J. Fire Sci.* 6:195-241.

12. **Levin, B.C., M. Paabo, J.L Gurman, and S.E. Harris.** 1987. Effects of exposure to single or multiple combinations of the predominant toxic gases and low oxygen atmospheres produced in fires. ***Fundam. Appl. Toxicol.*** 9:236-250.

13. **Anderson, R.A. and W.A. Harland.** 1982. Fire deaths in the Glasgow area: III. The role of hydrogen cyanide. *Med. Sci. Law* 22:35-72.

14. **Hartzell, G.E., W.G. Switzer, and D.N. Priest.** 1985. Modeling of toxicological effects of fire gases: V. Mathematical modeling of intoxication of rats by combined carbon monoxide and hydrogen cyanide atmospheres. *J. Fire Sci.* 3:330-342.

15. **Matijak-Schaper, M. and Y. Alarie.** 1982. Toxicity of carbon monoxide, hydrogen cyanide and low oxygen. *J. Combust. Toxicol.* 9:21-61.

16. **Hartzell, G.E., A.F. Grand, and W.G. Switzer.** 1985. Modeling of toxicological effects of fire gases: VI. Further studies on the toxicity of smoke containing hydrogen chloride. *J. Fire Sci.* 5:368-391.

17. **Levin, B.C., M. Paabo, J.L. Gurman, S.E. Harris, and E. Braun.** 1987. Toxicological interactions between carbon monoxide and carbon dioxide. *Toxicology* 47:135-164.

18. **Alarie, Y. and R.C. Anderson.** 1979. Toxicological and acute lethal hazard evaluation of thermal decomposition products of synthetic and natural polymers. *Toxicol. Appl. Pharmacol.* 51:341-362.

19. **Alarie, Y.** 1985. The toxicity of smoke from polymeric materials during thermal decomposition, *Ann. Rev. Pharmacol. Toxicol.* 25:325-47.

20. **Lieu, P.J., J.H. Magill, and Y. Alarie.** 1981. Ratings of some polyphosphagene and polyurethane foams. *J. Comb. Toxicol.* 8:242-259.

21. **Delichatsios, M.A., T.H. Panagiotou, and F. Kiley.** 1991. The use of time to ignition data for characterizing the thermal inertia and the minimum (critical) heat flux for ignition or pyrolysis. Combustion and Flame.

22. **Malek, D.E., M.F. Stock, and Y. Alarie.** 1987. Performance evaluation under intoxicating atmospheres *Fundam. Appl. Toxicol.* 8:335-345.

23. **Malek, D.E. and Y. Alarie.** 1989. Ergometer within a whole-body plethysmograph to evaluate performance of guinea pigs under toxic atmospheres. *Toxicol. Appl. Pharmacol.* 101:340-355.

24. **Tyuma, I., Y. Udea, K. Imaizumi, and H. Kosaka.** 1981. Prediction of the carboxyhemoglobin levels during and after carbon monoxide exposures in various animal species. *Jap. J. Physiol.* 31:131-143.

25. **Haldane, J.** 1895. The action of carbonic oxide on man. *J. Physiol.* 18:430-461.

26. Personal communication with **A.B. Callahan**, Naval Submarine Medical Research Laboratory, Groton, CT (manuscript in preparation).

27. **Pfaff, J., K. Parton, R. Lantz, H. Chen, D. Carter, and M. Witten. Date.** Effects of JP-8 jet fuel exposure on pulmonary function. Report Abstract for U.S. Air Force Office of Scientific Research Grant No. 91-0199.

28. **Witten, M.L., R.C. Lantz, R. Grad, S. Seidner, A.K. Hubbard, S.F. Quan, and R.J. Lemen.** 1991. Effect of smoke inhalation on immediate changes in lung chemical mediators. *Res. Comm. Chem. Pathol. Pharmacol.* 74:259-272.

OZONE- AND ENDOTOXIN-INDUCED MUCOUS CELL METAPLASIAS IN RAT AIRWAY EPITHELIUM: NOVEL ANIMAL MODELS TO STUDY TOXICANT-INDUCED EPITHELIAL TRANSFORMATION IN AIRWAYS

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SUMMARY

Mucous (goblet) cell proliferation and hypersecretion of airway mucus are important characteristics of human respiratory disorders, especially chronic bronchitis and cystic fibrosis. These changes in secretory patterns also occur in animals experimentally exposed to chemical irritants such as ozone (O_3), sulfur dioxide (SO_2), and cigarette smoke. The cellular and molecular mechanisms involved in irritant-induced mucous cell metaplasia (MCM; transformation of airway epithelium, normally devoid of mucous cells, to a secretory epithelium containing numerous mucous cells) are still unclear. We used two experimental models of toxicant-induced MCM in rat airways to study the cellular and molecular changes that occur during the development of this respiratory tract lesion. MCM can be induced in the nasal transitional epithelium of rats by repeated exposure to ambient levels of ozone. In addition, MCM can be induced in the tracheobronchial airways of rats repeatedly exposed to endotoxin, a lipopolysaccharide-protein molecule found in the outer walls of gram-negative bacteria. The pathogenesis of ozone- or endotoxin-induced MCM has been partially characterized using a variety of morphometric and histochemical techniques. Toxicant-induced changes in the numbers and types of airway epithelial cells have been estimated using morphometric methods designed for estimating the abundance of cell populations. Nasal pulmonary airway tissues are also processed for light microscopy and stained with Alcian Blue (pH 2.4)/Periodic Acid Schiff (AB/PAS) for detection of acidic and neutral mucosubstances (the specific glycoprotein product of mucous cells), respectively, within the tissue. Computerized image analysis is used to quantitate the amount of the stained mucous product within the airway epithelium. To better characterize the molecular and cellular events in the pathogenesis of ozone- or endotoxin-induced MCM in the rat airway epithelium, we are conducting studies to determine when, and in which epithelial cells, the mucin gene is expressed after exposure to the toxicant. In these studies, rats undergo single or repeated exposures to ozone or endotoxin and are then sacrificed immediately or a few days after the end of the exposures. Airway tissues are microdissected from specific regions of the exposed respiratory tract, and changes in mucin core polypeptide mRNA are evaluated by Northern analysis using human and rat mucin cDNA. In future studies using in situ hybridization, we will establish when, and in which epithelial cells, the expression of high molecular weight airway mucin is initiated in response to ozone or endotoxin. These cellular and molecular analyses are being conducted to

better characterize the toxicant-induced changes involved in the metaplastic process. Results from these studies will help us to understand the pathogenesis of MCM and may provide insight into methods of treatment for these common airway lesions.

INTRODUCTION

Metaplasia is defined as "the abnormal transformation of an adult, fully differentiated tissue of one kind into a differentiated tissue of another kind [1]." The term is derived from the Greek word, metaplasis, which means transformation. Metaplasia is an acquired condition, in contrast to heteroplasia which is a developmental condition. Patients with chronic rhinitis, chronic bronchitis, chronic bronchopneumonias, cystic fibrosis, and chronic asthma have abnormally high numbers of mucous cells in their respiratory epithelium [2-7]. This phenomenon has been referred to as MCM. This phenomenon can also occur in laboratory animals experimentally exposed to chemical irritants such as O_3 [8-10], SO_2 [11,12], and cigarette smoke [13,14]. MCM has also been experimentally induced in hamsters and rats after intratracheal instillations of human neutrophil elastase [15,16] and in rats after injections of adrenergic and cholinergic drugs [17,18]. The cellular and molecular mechanisms involved in MCM, however, are still unclear. In our laboratory, we are using two new experimental models of MCM in rat airways to better understand the cellular mechanisms involved in toxicant-induced injury, repair, and adaptation. This article describes our current knowledge of ozone- and endotoxin-induced MCM in rats, the experimental advantages of the two rat models, and how these specific models of MCM, along with new techniques in molecular biology, can be used to better understand the cellular and molecular mechanisms involved in the pathogenesis of MCM.

OZONE-INDUCED MCM IN RAT NASAL EPITHELIUM

Ozone is the major oxidizing component in photochemical smog and is found in many urban areas during the summer. Currently, O_3 is the most pervasive problem of the major air pollutants for which National Ambient Air Quality Standards (NAAQS) have been designated under the Clean Air Act [19]. The U.S. Environmental Protection Agency (U.S. EPA) has estimated that 13 million moderately exercising healthy adults are exposed to O_3 in excess of the NAAQS concentration (0.12 ppm) for at least 1 h each week during the summer [20]. The episodic high concentrations of O_3 in large metropolitan areas like Los Angeles, California, and Mexico City, Mexico, pose significant threats to the health of their inhabitants. The current regulated concentration of 0.12 ppm O_3 is being questioned because documented studies indicate that pulmonary function in exercising children and adults exposed to concentrations below or slightly above the ambient concentration of the NAAQS is impaired [21,22]. In addition, laboratory animals have structural lesions in the upper and lower airways after repeated exposures to low ambient concentrations (0.12 to 0.30 ppm) of this oxidant [8-10, 23-26].

Bonnet monkeys exposed to 0.15 ppm ozone for 6 or 90 days (6 h/day) have marked epithelial changes in the proximal nasal passages [8,9] and intrapulmonary centriacinar regions [26]. The centriacinar lesions were similar to previously reported ozone-induced alterations in this region of the lung; this report was the first to describe the effects of ambient concentrations of O_3 on nasal epithelium. The nasal lesions were confined to the surface epithelium in the proximal nasal passages and were characterized by a marked increase of mucous cells in both transitional (NTE; nonciliated, stratified, cuboidal epithelium with few mucous goblet cells) and respiratory epithelium (ciliated, pseudostratified epithelium with numerous mucous goblet cells). The degree of this change was not dependent on the concentration of the O_3 (i.e., 0.15 versus 0.30 ppm), but on the duration of exposure (i.e., more mucous goblet cells after 90 days of exposure than after 6 days of exposure). Interestingly, a similar time dependancy, rather than concentration dependancy, in epithelial response was also evident in the epithelial alterations in the centriacinar regions of the lungs from these same monkeys. In the NTE of O^3-exposed monkeys, the numbers of nonciliated cells with secretory granules were approximately 15 to 20% greater than in air-exposed controls. In addition, the amount of intraepithelial mucosubstances in the NTE increased dramatically after O_3 exposure. For example, the amount of secretory material in the NTE was 300% greater in monkeys exposed to 0.15 ppm O_3 for 6 days (8 h/day), compared to filtered air (0 ppm O_3)-exposed controls [8,9]. Accompanying the increase of mucous cells in the nasal passages of O_3-exposed monkeys were ciliated cell necrosis (6 and 90 days of exposure), attenuation of ciliary length (6 and 90 days of exposure), and inflammatory cell influx (only after 6 days of exposure). These nasal epithelial alterations induced by O_3 may indicate alterations in normal physiologic functions (e.g., mucociliary clearance) which are important upper respiratory tract mechanisms that defend the lungs from the excessive burdens of harmful agents. This type of nasal damage could also be a factor in increasing susceptibility to acute infections of the upper respiratory tract. The findings of this study had added significance because of their presence in a species of nonhuman primate whose nasal cavity resembles, at a gross and microscopic level, that of humans [27].

If a similar lesion could be induced in a laboratory rodent, many studies to better understand the mechanisms of O_3-induced increase in mucous cells could be conducted that otherwise would be cost-prohibitive using nonhuman primates. In 1989, we reported that F-344/N rats exposed for 7 days to 0.8 ppm O_3, 6 h/day, developed MCM in the NTE lining the maxilloturbinates, lateral wall, and lateral aspects of the nasal turbinates in the proximal nasal passages [10]. (At our Institute, which is located at an elevation of 1,728 m, with an average barometric pressure of 625 mm Hg [0.82 sea level atmosphere], an O_3 concentration of 0.8 ppm gives a partial pressure equivalent to a sea-level O_3 concentration of 0.66 ppm.) The O_3-induced lesion in the F-344/N rat resembled those that were experimentally induced in the nasal cavity of bonnet monkeys. There were, however, some

noticeable differences. The increase in mucous cells in the rat nasal airway was restricted to the NTE that is normally devoid of mucous cells. The NTE in the rat is only 1 to 2 cells thick and usually contains no secretory cells and only a few widely scattered ciliated cells [28]. The NTE in the monkey, however, is a stratified epithelium (4 to 6 cells thick) with few ciliated cells and widely scattered mucous cells and other secretory cells [28]. Therefore, the ozone-induced, nasal epithelial alteration in the rat was a true metaplastic response (transformation of an epithelium with no mucous cells into an epithelium with numerous mucous cells) that differed from the hyperplastic response (increase in the number of mucous cells in an epithelium which normally contains some mucous cells) that was induced in monkeys after O_3 exposure. In addition, no epithelial alterations were evident in the nasal respiratory epithelium in the rat, unlike the monkey. Finally, no metaplastic alterations were evident in the NTE of rats exposed to 0.12 ppm, 6 h/day for 7 days. This was in contrast to the conspicuous mucous cell hyperplasia that was induced in monkeys after a 6-day (8h/day) exposure to 0.15 ppm O_3. These results suggest that the rat NTE may not be as sensitive to ozone-induced injury as monkey NTE.

The amount of histochemically stained mucosubstances within the the NTE (i.e., stored secretory product within mucous cells) has been determined to be a sensitive and reproducible quantitative indicator of the severity of the ozone-induced metaplasia [8,10,29]. To estimate the amount of intraepithelial mucosubstances in the nasal epithelium, transverse tissue sections from specifically designated locations throughout the nasal airway are processed for light microscopy and stained with AB/PAS for demonstration of acidic (stains blue) and neutral (stains red) mucosubstances [30]. The volume density of acidic (AB-stained) and neutral (PAS-stained) mucosubstances within the NTE is quantitated by using a computerized image analysis system. This system consists of a light microscope which is connected to a CCD camera (TM-840, Pulnix America, Sunnyvale, CA), an FG-100 digital image processing board (Imagine Technology, Inc. Woburn, MA), a color monitor, and a 386-based personal computer running commercially available image analysis software (Phoenix Technology, Inc., Seattle, WA). The areas of AB- and PAS-positive intraepithelial mucosubstances are calculated by the image analysis software program from the manually or automatically circumscribed perimeter of the stained material. The method we use to estimate the volume of stored mucosubstance per unit of surface area of epithelial basal lamina has been described in detail [27]. The data are expressed as the mean volume density (Vs; nL/mm^2 basal lamina) of AB-, PAS-, or AB/PAS-positive mucosubstances within the epithelium ± the standard error of the mean.

The differences in amounts of AB/PAS-stained mucosubstances in the NTE of the maxilloturbinate in F-344/N rats that were exposed to 0 or 0.8 ppm O_3 for 7 days, 6 h/day and sacrificed immediately after the end of the exposure are shown in Figure 67. The mean amount of intraepithelial acidic mucosubstances in the NTE of the maxilloturbinates immediately after the 7 days of O_3 exposure was 0.6 ± 0.2 nL/mm^2 (mean ± standard error of the mean), compared to

0 nL/mm^2 in controls exposed to filtered air (0 ppm O_3). Interestingly, the amounts of intraepithelial mucosubstances in the NTE do not decrease 7 days after the end of the exposure, but markedly increase. The volume densities of the mucosubstances in the NTE of the nasal turbinates of rats sacrificed 7 days after the end of exposure are approximately 3 times that in 0.8 ppm O_3-exposed rats sacrificed immediately after exposure (3.2 ± 0.5 nL/mm^2 compared to 1 ± 0.3 nL/mm^2) [10]. The persistence of this O_3-induced change in intraepithelial mucosubstances has not been adequately examined for time periods greater than 7 days after the end of exposure.

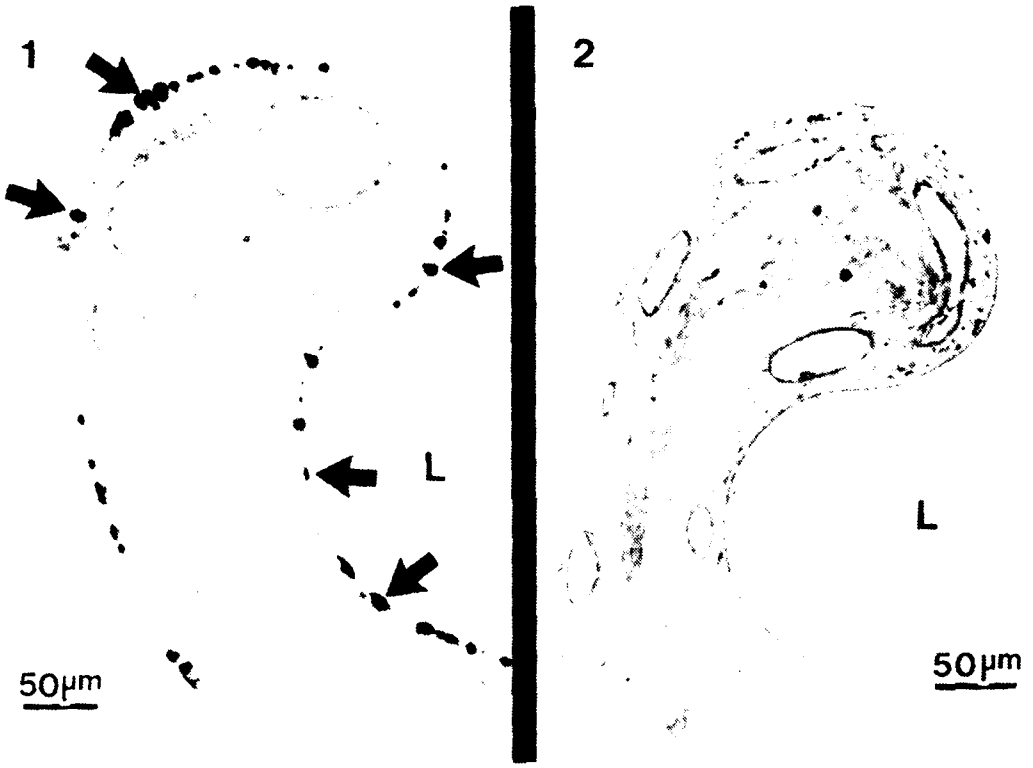

Figure 67. Light Photomicrographs of Maxilloturbinates from Rats Exposed to 0.8 ppm (1) or 0 ppm (air-control); 2). Ozone-induced MCM is present in the surface epithelium of 1. E = surface epithelium, B = -bone of turbinate; L = nasal airway lumen; arrows = AB/PAS-stained, intraepithelial mucosubstances.

In other studies, we demonstrated that the cellular population in the NTE is dramatically altered in rats exposed for 7 days to 0.8 ppm O_3, 6 h/day. After this repeated exposure to O_3, the NTE is markedly hyperplastic (i.e., an increase in total epithelial cells) and metaplastic with approximately 15% of the epithelial cell population consisting of mucous cells compared to a normal mucous cell density in the NTE of 0% [29]. This metaplastic NTE was also less responsive, compared to controls, to a 6-h challenge of 0.8 ppm O_3 1 week after the end of the MCM-inducing, 7-day exposures (0.8 ppm, 6 h/day) [29]. Epithelial response was estimated by the O_3-induced increase in DNA synthesis within the NTE, using immunohistochemistry to detect nuclear incorporation of bromodeoxyuridine (BrdU;

an analog of thymidine). Normal NTE exposed for 6 h to 0.8 ppm O_3 had approximately ten times more BrdU- labelled epithelial cells, whereas the metaplastic NTE had no increase BrdU-labelled epithelial cells, compared to air (0 ppm O_3)-exposed controls. This data suggest that MCM is an adaptive tissue response to injury in order to prevent further toxicant-induced damage to NTE cells.

It is also known that O_3-induced MCM and epithelial hyperplasia in the NTE of rats can be induced with only a 3-day exposure of 0.8 ppm, 6 h/day and the amount of intraepithelial mucosubstances at 7 days after the start of the exposures is similar to the amount found in the NTE after a 7-day exposure to the same concentration of O_3 [30]. The fact that the type and magnitude of these alterations induced by 3 and 7 days of O_3 exposure were similar suggests that, once initiated, the development of O_3-induced phenotypic changes within the epithelium was not dependent on further O_3 exposure. Four additional days of exposure to 0.8 ppm O_3 had no significant effect on the location or magnitude of the O_3-induced MCM over that observed in rats exposed to 0.8 ppm O_3 for 3 days followed by 4 days of exposure to air.

An influx of neutrophils [31] and an increase in epithelial DNA synthesis [32,33] always precedes the MCM induced by ozone, and these inflammatory and proliferative responses decrease as the MCM develops in the NTE. The relationship(s) between neutrophils, or epithelial cell proliferation, and MCM is unclear. Research must be designed to determine if MCM is dependent on neutrophil influx and/or increased DNA synthesis and epithelial proliferation. The basic question is whether O_3 can directly stimulate the NTE cells to undergo transformation to mucous cells, or is MCM the secondary result of O_3-induced inflammatory or proliferative events? Research especially designed to answer this question must still be conducted.

Recently, the effects of O_3 on the structure and function of the nasal epithelium of F-344/N rats exposed to 0, 0.12, 0.5, or 1 ppm O_3 for 20 months, 6 h/days, 5 days/week have been examined [34]. Rats were sacrified 1 week after the end of the exposure. Immediately after death, mucous flow rates throughout the nasal passages were determined using *in vitro* video motion analysis. Following assessment of mucociliary function, the nasal tissues were processed for light microscopy and stained with AB/PAS for detection of intraepithelial mucosubstances. Rats exposed to 0.5 and 1 ppm O_3 had markedly slower mucous flow rates over the lateral walls and turbinates of the anterior third of the nasal passages compared to rats exposed to 0 and 0.12 ppm O_3. Interestingly, these internasal regions in the rats exposed to 0.5 and 1 ppm O_3 had marked mucous goblet cell metaplasias and 200 and 400 times greater amounts of intraepithelial mucosubstances than controls (0 ppm O_3), respectively. There were no significant differences in the mucous flow rates between 0 ppm- and 0.12 ppm-exposed rats, and no O_3-induced morphologic alterations were present in the nasal epithelium of rats exposed to 0.12 ppm O_3. This is the first study to demonstrate that the O_3-induced MCM in the rat NTE is associated with a functional alteration (i.e., decreased mucous flow rates). This alteration

could significantly alter an important respiratory defense mechanism of the upper airways (i.e., mucociliary clearance) and leave the more distal pulmonary airways vulnerable to potentially injurious concentrations of inhaled xenobiotics or infectious agents.

ENDOTOXIN-INDUCED MUCOUS CELL METAPLASIA IN PULMONARY AIRWAYS

Endotoxins are lipopolysaccharide-protein molecules released from the walls of gram-negative bacteria and are believed to be the principal etiologic agents responsible for the acute inflammatory responses in gram-negative, bacteria-induced pneumonias and sepsis. Inhalation exposure to endotoxin from gram-negative bacteria may also be an important factor in several airborne-related occupational diseases or syndromes, including byssinosis [35], mill fever [36], bagassosis [37] and sick building syndrome [38,39].

Airway infections induced by endotoxin-producing bacteria are often characterized by an influx of inflammatory cells (i.e., neutrophils) and hypersecretion of mucus from airway mucosa. Endotoxin is a potent chemotaxinogen for neutrophils [40,41], and an inducer of mucous hypersecretion [42] and mucous cell metaplasia/hyperplasia in the airways of rats [43,44]. We examined the upper and lower airways of rats that had been intranasally instilled with endotoxin or saline, once a day for 3 days. We have found that endotoxin instillations dramatically increase the amount of intraepithelial mucosubstances in both the nasal and intrapulmonary airways, but especially in the large main axial airways of the lung lobes. Compared to saline-instilled controls, there were approximately 1200 and 1000% more AB/PAS-stained mucosubstances in surface epithelia lining airway generations 8 and 11, respectively, along the main axial airways of the right caudal lobe in endotoxin-instilled rats sacrificed 1 day after the last instillation. This increase was predominantly due to an increase in the numbers of secretory cells (63 ± 5 cells per mm/basal lamina in the endotoxin-instilled group compared with 37 ± 3 cells/mm basal lamina in the control group).

In addition, there were conspicuous changes in the histochemical character of the mucosubstances within the airway epithelial cells. Instead of the predominantly PAS-positively stained secretory material in the airway epithelium of saline-instilled controls, the endotoxin-exposed airway epithelium contained secretory cells with both AB- and PAS-positively stained secretory material. This striking increase in the AB- staining of acidic mucosubstances after endotoxin instillations is similar to the increases in acidic mucosubstances reported in tracheobronchial and pulmonary axial airways of rats exposed to SO_2 [11,12] or tobacco smoke [13,14], and the nasal epithelium of rats and monkeys repeatedly exposed to O_3 [8,9,10]. Figure 68 photomicrographically illustrates the increase in intraepithelial mucosubstances in the conducting axial airway of endotoxin-instilled lungs. The secretory cells in the endotoxin-exposed airways were mucous cells with large,

electron-lucent secretory granules, whereas the secretory cells in the saline-exposed (control) airways were serous cells with small, electron-dense secretory granules.

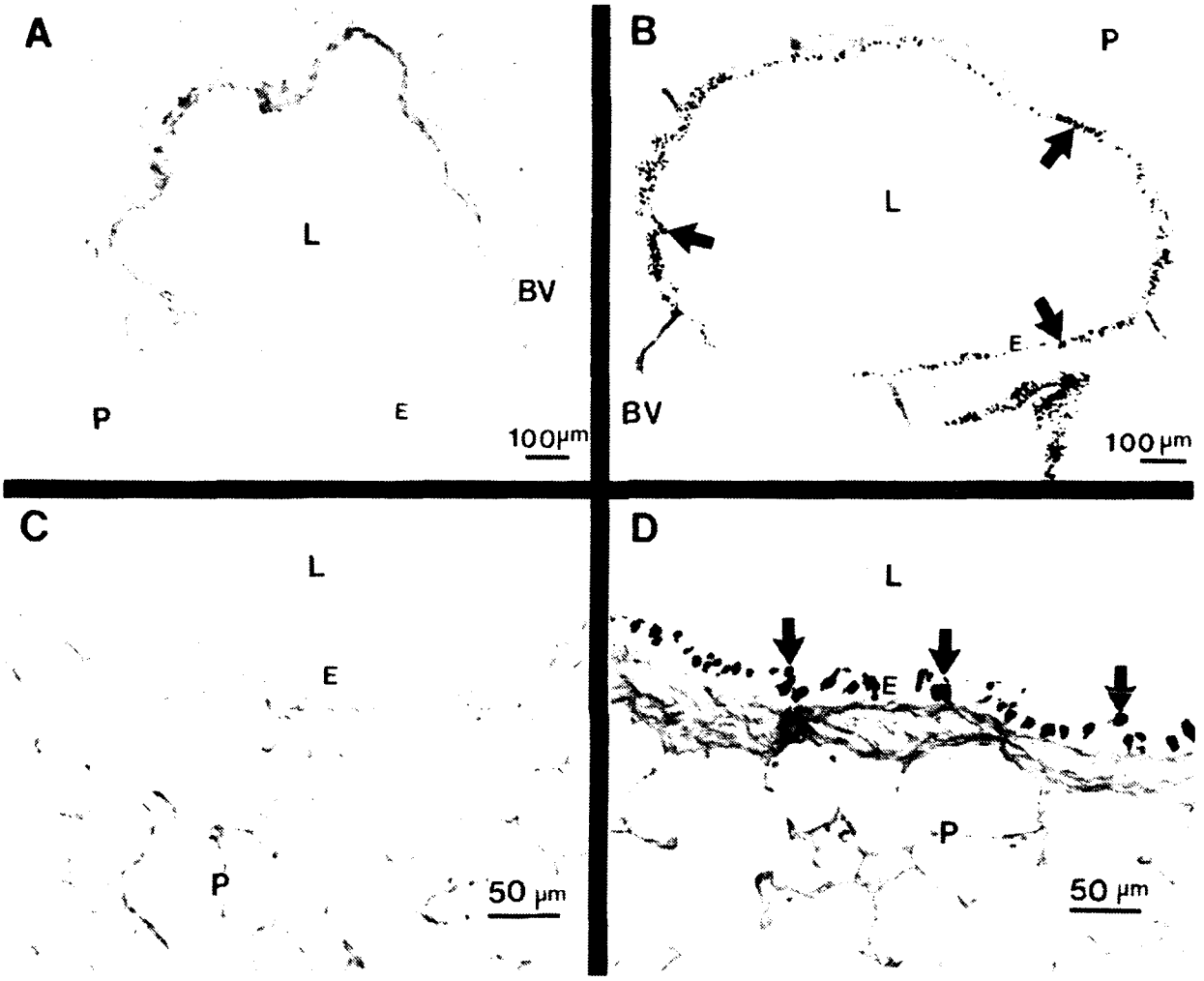

Figure 68A and 68B. Light Photomicrographs of Conducting Axial Airways in the Left Lung Lobes of a Rat Intratracheally Instilled with Saline (A) or Endotoxin (B) and Sacrificed 4 Days After the Instillation. C and D, Higher Magnifications of Axial Airway Epithelium in the Boxes of Photomicrographs A (C) and B (D). L = conducting axial airway lumen; E = axial airway epithelium; P = alveolar parenchyma; BV = blood vessel; arrows = intraepithelial mucusubstances stained with AB/PAS.

The fact that no serous cells were found in endotoxin-exposed airways in that study indicates that serous cells were either killed by endotoxin and replaced by mucous cells, or that exposure to endotoxin stimulated serous cells to transform into mucous cells. Because there was no histologic or ultrastructural evidence of necrosis or exfoliation in any of the endotoxin-exposed tissue, most of the endotoxin-induced increase in mucous cells was probably due to transformation of serous cells already present in the epithelium before exposure. Future studies must be must be conducted to test this hypothesis.

A similar increase in mucous cells in large intrapulmonary airways of hamsters intratracheally instilled with endotoxin has also been reported by Stolk et al. [45]. In contrast to the few intranasal instillations of endotoxin (i.e., 500 μg of endotoxin in 100 μL of saline, once a day for 3 days) that we gave to our rats, Stolk et al. intratracheally instilled 500 μg of lipopolysaccharide in 200 μL of saline twice a week over a period of 3.5 to 5 weeks. In addition to the MCM, these authors found that the endotoxin-instilled hamsters developed pulmonary emphysema. They also demonstrated that simultaneous instillations of a highly selective inhibitor of neutrophil elastase resulted in a 50% reduction of the endotoxin-induced emphysema and a 35% inhibition of endotoxin-induced MCM. They concluded that intratracheal instillation of endotoxin in hamsters causes recruitment of neutrophils into the lung and that neutrophil-derived products, including elastase, can induce pulmonary emphysema and MCM.

Recently, we collaborated with Dr. Terry Gordon and co-workers at the Institute of Environmental Medicine, New York University Medical Center, Tuxedo, NY, to examine the effects of inhaled, occupational levels of endotoxin on airway epithelium in the lungs of rats. Preliminary results suggest that MCM with increased amounts of intraepithelial mucosubstances can be induced by exposures of 0.3 $\mu g/m^3$ endotoxin, 3 h/day for 3 days. If we use estimates of minute ventilation (0.1 L/min) and total deposition of inhaled particles in the respiratory tract (50%), we predict that approximately 9 ng endotoxin/rat were retained in the lungs of rats during a 3-h exposure to 0.3 $\mu g/m^3$ endotoxin. These results demonstrate that the surface epithelium lining the intrapulmonary airways of rats is extremely sensitive to minute amounts of inhaled endotoxin.

ADVANTAGES AND APPLICATIONS OF THE EXPERIMENTAL MODELS OF MUCOUS CELL METAPLASIA INDUCED BY OZONE AND ENDOTOXIN

Both O_3- and endotoxin-induced models of MCM have several characteristics that make them attractive for use in inhalation studies examining the toxicity of these inhaled xenobiotics or in studies designed to better understand the pathogenesis of MCM. Both models can be induced by concentrations of the toxicant that can be found in some urban or occupational atmospheres. This suggests that these toxicant-induced MCMs could be used as morphologic end points to help determine the ambient air quality standards for these air pollutants. These models can also be induced in a few days, rather than in a few weeks or months as with other toxicants. This feature of rapid induction is cost effective for the researcher and allows the investigator to examine and characterize cellular or biochemical changes in the tissue after hours or days of exposure that may be important for the onset of the phenotypic change.

The O_3-induced change in the NTE of the rat is unique because it is truly a secretory metaplasia. That is, the NTE with no secretory cells is transformed into a secretory NTE with numerous mucous cells. This rapidly induced morphologic change allows for the study of cellular and molecular

mechanisms involved in toxicant-induced injury, epithelial adaptation (or metaplastic induction and maintenance), and repair of the epithelium.

The endotoxin-induced epithelial alteration of the intrapulmonary airways of the rat is technically a hyperplastic response (i.e., increase in number of secretory cells) in an epithelium that normally contains some secretory cells. The change in secretory cell types (serous cells to mucous cells) after endotoxin exposure also makes the response by definition a mucous cell metaplasia. Therefore, this rapidly inducible model is especially suited to help determine the progenitor cells for mucous cells in hyperplastic and metaplastic epithelium. Because endotoxin exposure also induces a marked and persistent inflammatory cell influx, the interactions of epithelial cells with neutrophils (or their secreted products) could be further investigated.

Recent advances in biotechnology add to the attractiveness of these models to better understand the molecular mechanisms of MCM. Molecular biologic approaches have only recently been applied to investigate the mechanisms involved in the airway secretory apparatus. The possibilities for application of this methodology to investigate physiologic and pathologic processes are now becoming apparent. Human airway mucin cDNA has been isolated from a cDNA library constructed in bacteriophage λ from human bronchial polyA + RNA by using a 950 bp human cDNA for intestinal mucin [46]. Using this or similar probes, investigators have begun to probe total and poly A + airway RNA from rats with normal and metaplastic airways. Basbaum et al. [46] reported that rats exposed to 400 ppm SO_2 gas in air for 3 h/day, 5 days/week for 0, 1, 2, or 3 week(s) had 8- to 9-fold increases in mucin mRNA as a function of SO_2 exposure. In collaboration with Dr. Carol Basbaum and co-workers at the University of California, San Francisco, CA, we also found in preliminary studies using similar mucin cDNA probes that rats with endotoxin-induced MCM have conspicuously more mucin mRNA compared to saline-exposed controls [47]. These studies suggest that mucin gene transcription can be induced by toxicant exposure and may represent a primary event in the development of MCM and mucous hypersecretion. Whether the observed increase in mucin mRNA is the result of de novo mucin gene transcription or an increase in mucin message stability remains to be determined. By using mucin cDNA probes in future experiments with the endotoxin- or O_3-induced models of MCM, it will be possible to determine when and in which airway epithelial cells mucin mRNA accumulates. Analysis of 5' regulatory regions will provide insight into molecular mechanisms regulating toxicant-induced gene expression. In addition, the time between toxicant-induced mucin mRNA accumulation and the appearance of histochemically detectable stored product can be determined for these rapidly inducible MCMs. More sensitive and specific probes of mucin gene transcription after endotoxin or O_3 exposure may be used in the future as homologous mucin cDNAs are isolated from F-344/N rat airway cDNA libraries.

In situ hybridization is a method by which the expression of a specific gene can be studied at the cell level. For airway epithelial tissue, which consists of a heterogenous population of cells, this method offers obvious advantages over studies of lung or nasal tissue lysates, which assess the overall content of a particular mRNA by Northern or RNA dot blot analysis but cannot relate this information to a specific cell type. *In situ* hybridization is similar to immunohistochemistry, except that the probe used for detection is not an antibody, but rather a specific fragment of complementary RNA or DNA. By using isolated airway mucin cDNA probes on nasal tissues excised from O_3-exposed rats or intrapulmonary airways exposed to endotoxin, it will be possible to identify particular epithelial cells that have increased expression of the mucin message. With the combination of *in situ* hybridization and histochemistry for mucosubstances in thoughtfully designed experiments, it will also be possible to identify the progenitor cells for the mucous cells in the O_3- and endotoxin-induced metaplasias.

CONCLUSION

In summary, we have reviewed two novel animal models of MCM in airway epithelium. We have tried to point out the principal advantages of these models to better understand the cellular and molecular mechanisms involved in the pathogenesis of MCM. Both the nasal MCM induced by O_3 exposure and the pulmonary airway MCM induced by endotoxin exposure are produced more rapidly after exposure (within a week after the start of exposure) compared to other MCM-inducing agents. These MCMs are similar in morphology to those described in the airways of humans with chronic rhinitis, chronic bronchitis, cystic fibrosis, and chronic asthma, and other toxicant-induced MCMs in laboratory animals. The acute histologic changes that occur during the onset of both O_3- and endotoxin-induced MCM have been well characterized. Only a few studies examining the cellular and molecular events prior to the expression of the intracellular mucous material have been conducted. Results of future studies should provide answers to unknown mechanisms involved in the transformation of an airway epithelium with few or no mucous cells to an airway epithelium with abnormally high numbers of these secretory cells. Like other experimental models of MCM, we must be cautious in extrapolating our findings from studies using these animal models to human conditions with MCM that are not associated with the toxicants used for the experimental induction of this metaplastic response.

ACKNOWLEDGEMENTS

The authors thank Ms. Lois Herrera and Ms. Pat Lawson for their excellent technical assistance in many of our studies described in this review. We also thank Ms. Paula Bradley for her editorial assistance in the preparation of this manuscript. In addition, we thank our colleagues, Drs. Rogene Henderson, Charles Hobbs, Fletcher Hahn, and David Bice, for reviewing this article and supplying helpful comments and suggestions.

This research was supported by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract DE-AC04-76EV01013 in facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

REFERENCES

1. **Hensyl, W.R.** 1990. Stedman's Medical Dictionary, pp. 954. Baltimore: Williams and Wilkins.

2. **Robbins, S.L., R.S. Cotran, and S. Kumar.** 1984. The respiratory system: Lung. In: Pathologic Basis of Disease, pp. 725-726.

3. **DeHaller, R. and L. Reid.** 1965. Adult chronic bronchitis: Morphology, histochemistry, and vascularization of the bronchial mucous glands. *Med. Thorac.* 22:549-567.

4. **Reynolds, H.Y. and W.W. Merrill.** 1981. Airway changes in young smokers that may antedate chronic obstructive lung disease. *Med. Clin. North Am.* 65:667-690.

5. **Lamb, D. and L. Reid.** 1972. The tracheobronchial submucosal glands in cystic fibrosis. *Br. J. Dis. Chest* 66:230-247.

6. **Lev, R. and S.S. Spicer.** 1965. A histochemical comparison of human epithelial mucins in normal and in hypersecretory states, including pancreatic cystic fibrosis. *Am. J. Pathol.* 46:23-47.

7. **Robins, S.L., R.S. Cotran, and S. Kumar.** 1984. The respiratory system: Nasal cavities and accessory air sinuses. In: W.B. Saunders, ed., *Pathologic Basis of Disease*, pp. 763-764.

8. **Harkema, J.R., C.G. Plopper, D.M. Hyde, J.A. St. George, and D.L. Dungworth.** 1987. Effects of an ambient level of ozone on primate nasal epithelial mucosubstances: Quantitative histochemistry. *Am. J. Pathol.* 127:90-96.

9. **Harkema, J.R., C.G. Plopper, D.M. Hyde, J.A. St. George, D.W. Wilson, and D.L. Dungworth.** 1987. Response of the macaque nasal epithelium to ambient levels of ozone: A morphologic and morphometric study of the transitional and respiratory epithelium. *Am. J. Pathol.* 128:29-44.

10. **Harkema, J.R., J.A. Hotchkiss, and R.F. Henderson.** 1989. Effects of 0.12 and 0.80 ppm ozone on rat nasal and nasopharyngeal epithelial mucosubstances: Quantitative histochemistry. *Toxicol. Pathol.* 17:525-535.

11. **Lamb, D. and L. Reid.** 1968. Mitotic rates, goblet cell increase and histochemical changes in mucous in rat bronchial epithelium during exposure to sulfur dioxide. *J. Pathol. Bacteriol* 96:97-111.

12. **Spicer S.S., L.W. Charkrin, and J.R. Wardell, Jr.** 1974. Effect of chronic sulfur dioxide inhalation on the carbohydrate histochemistry and histology of the canine respiratory tract. *Am. Rev. Respir. Dis.* 110:13-24.

13. **Jones, R., M. Phil, and L. Reid.** 1978. Secretory hyperplasia and modification of intracelluar glycoprotein in rat airways induced by short periods of exposure to tobacco smoke and the effect of anti- inflammatory agent pheylmethyloxidiazole. *Lab. Invest.* 39:41-49.

14. **Rogers, D.F. and P.K. Jeffery.** 1978. Inhibition by oral N-Acetylcysteine of cigarrette smoke-induced "bronchitis" in the rat. *Exp. Lung Res.* 10:267-283.

15. **Snider, G.L., E.C. Lucey, T.G. Christensen, P.J. Stone, J.D. Calore, and A. Catanese.** 1984. Emphysema and bronchial secretory cell metaplasia induced in hamsters by human neutrophil products. *Am. Rev. Respir. Dis.* 129:155-60.

16. **Nields, H.M., G.L. Snider, R. Breur, and T.G. Christensen.** 1991. Reversible pancreatic elastase-induced bronchial secretory cell metaplasia in the rat. *Exp. Pathol.* 41:185-193.

17. **Sturgess, J. and L. Reid.** 1973. The effect of isoprenaline and pilocarpine on (a) bronchial mucus-secreting tissue and (b) pancreas, salivary glands, heart, thymus, liver, and spleen. *Br. J. Exp. Pathol.* 54:388-403.

18. **Jones, R. and L. Reid.** 1979. Beta-agonists and secretory cell number and intracellular glycoprotein in airway epithelium: The effect of isoproterenol and salbutamol. *Am. J. Pathol.* 95:407-422.

19. **Steinfeld, J.H.** 1991. Rethinking the ozone problem in urban and regional air pollution, pp. 1. Washington, DC: National Academy Press.

20. **Paul, R.A., W.F. Biller, and T. McCurdy.** 1987. National estimates of population exposure to ozone. Paper 87-42.7 in *Proceedings of the 80th Annual Meeting and Exhibition of the Air Pollution Control Association* Pittsburgh, PA: American Pollution Control Association.

21. **Spektor, D.M., M. Lippmann, and P.J. Lioy.** 1988. Effects of ambient ozone on respiratory function in active normal children. *Am. Rev. Respir. Dis.* 137:313-320.

22. **Spektor, D.M., M. Lippmann, G.D. Thurston, P.J. Lioy, J. Stecko,G., O'Connor, E. Garshick, F.E. Spelser, and C. Hayes.** 1988. Effects of ambient ozone on respiratory function in healthy adults exercising outdoors. *Am. Rev. Respir. Dis.* 138:821-828.

23. **Plopper, C.G., D.L. Dungworth, W.S. Tyler, and C.K. Chow.** 1979. Pulmonary alterations in rats exposed to 0.2 and 0. ppm ozone: A correlated morphological and biochemical study. *Arch. Environ. Health* 34:390-395.

24. **Boorman G.A., L.W. Schwartz, and D.L. Dungworth.** 1980. Pulmonary effects of prolonged ozone insult in rats: Morphometric evaluation of the central acinus. *Lab. Invest.* 43:108-115.

25. **Barry, B.E., F.J. Miller, and J.D. Crapo.** 1985. Effects of inhalation of 0.12 and 0.25 parts per million ozone on the proximal alveolar region of juvenile and adult rats. *Lab. Invest.* 53:692-704.

26. **Harkema, J.R., C.G. Plopper, J.A. St. George, N.K. Tyler, and D.L. Dungworth.** 1985. Comparison of nasal and centriacinar epithelial changes induced by low ambient levels of ozone. *Am. Rev. Respir. Dis.* 131:A169.

27. **Harkema, J.R., C.G. Plopper, D.M. Hyde, and J.A. St. George.** 1987. Regional differences in quantities of histochemically detectable mucosubstances in nasal, paranasal, and nasopharyngeal epithelium of the bonnet monkey. *J. Histochem. Cytochem.* 35:279-286.

28. **Harkema, J.R.** 1991. Comparative aspects of nasal airway anatomy: Relevance to inhalation toxicology. *Toxicol. Pathol.* 19:321-336.

29. **Harkema, J.R., E.C. Averill, and J.A. Hotchkiss.** 1991. Adaptation of rat nasal epithelium to ozone exposure. *The Toxicologist* 11:183.

30. **Hotchkiss, J.A., J.R. Harkema, and R.F. Henderson.** 1991. Effect of cumulative ozone exposure on ozone-induced nasal epithelial hyperplasia and secretory metaplasia in rats. *Exp. Lung Res.* 15:589-600.

31. **Hotchkiss, J.A., J.R. Harkema, J.D. Sun, and R.F. Henderson.** 1989. Comparison of acute ozone-induced nasal and pulmonary inflammatory responses in rats. *Toxicol. Appl. Pharmacol.* 98:289-302.

32. **Johnson, N.F., J.A. Hotchkiss, J.R. Harkema, and R.F. Henderson.** 1990. Proliferative responses of rat nasal epithelia to ozone. *Toxicol. Appl. Pharmacol.* 103:143-155.

33. **Hotchkiss, J.A. and J.R Harkema.** 1992. Endotoxin or cytokines attenuate ozone-induced DNA synthesis in rat nasal transitional epithelium. *Toxicol. Appl. Pharmacol.* 114:182-187.

34. **Harkema, J.R., E.G. Bermudez, K.T. Morgan, and P.W. Mellick.** 1992. Effects of chronic ozone exposure on the nasal mucociliary apparatus in the rat. *Am Rev. Respir. Dis.* 145:A98.

35. **Rylander, R. and A. Nordstrand.** 1974. Pulmonary cell reactions after exposure to cotton dust extract. *Br. J. Ind. Med.* 31:220-223.

36. **Pernis, B., E.C. Vigliani, C. Cavanga, and M. Finuli.** 1961. The role of bacterial endotoxins in occupational diseases caused by inhaling vegetable dusts. *Br. J. Ind. Med.* 18:120-129.

37. **Salvaggio, J., H.A. Bucchner, and J.A. Seabury.** 1966. Precipitins against extracts of crude bagasse in the serum of patients. *Ann. Intern. Med.* 61:748-758.

38. **Burge P.S., A. Hedge, S. Wilson, J.H. Bass, and A. Robertson.** 1987. Sick building syndrome: A study of 4373 office workers. *Ann. Occup. Hyg.* 31:493-504.

39. **Harrison J., C.A.C. Pickering, E.B. Faragher, P.K.C. Austwick, S.A. Little, and L. Lowton.** 1992. An investigation of the relationship between microbial and particle indoor air pollution and the sick building syndrome. *Resp. Med.* 86:225-235.

40. **Issekutz, A.C. and S. Bhimji.** 1982. Role of endotoxin in the leukocyte infiltration accompanying Escherichia coli inflammation. *Infect. Immun.* 36:558-566.

41. **Synderman, R., H. Gewurz, and S.E. Mergenhagen.** 1969. Interactions of the complement system with endotoxin lipopolysaccharide: Generation of factor chemotactic for polymorphonuclear leukocytes. *J. Exp. Med.* 128:269-278.

42. **Harkema, J.R., J.A. Hotchkiss, A.G. Harmsen, and R.F. Henderson.** 1988. *In vivo* effects of transient neutrophil influx on nasal respiratory epithelial mucosubstances. *Am. J. Pathol.* 130:605-615.

43. **Harkema, J.R. and J.A. Hotchkiss.** 1992. *In vivo* effects of endotoxin on intraepithelial mucosubstances in rat pulmonary airways: Quantitative histochemistry. *Am. J. Pathol.* 141:307-317.

44. **Harkema, J.R. and J.A. Hotchkiss.** 1991. *In vivo* effects of endotoxin on nasal epithelial mucosubstances: Quantitative histochemistry. *Exp. Lung Res.* 17:743-761.

45. **Stolk, J., A. Rudolphus, P. Davies, D. Osinga, J.H. Dijkman, L. Agarwal, K.P. Keenan, D. Fletcher, and J.A. Kramps.** 1992. Induction of emphysema and bronchial mucus cell hyperplasia by intratracheal instillation of lipopolysaccharide in the hamster. *J. Pathol.* 167:349-356.

46. **Basbaum, C., M. Gallup, J. Gum, Y. Kim, and B. Jany.** 1990. Modification of mucin gene expression in the airways of rats exposed to sulfur dioxide. *Biorheology* 27:485-489.

47. **Royce, F.H., T. Tsuda, J.A. Hotchkiss, J.R. Harkema, and C.B. Basbaum.** 1992. Mucin gene expression in rat airway exposed to endotoxin. *Am. Rev. Respir. Dis.* 145:A352.

POSTER ABSTRACTS

1. THE RISK ASSESSMENT OF CHEMICAL MIXTURES: FINE TUNING THE HAZARD INDEX

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Most toxicity and risk assessments of chemical mixtures are based on the assumption of additivity that yields a hazard index (HI). The HI is the sum of the hazard quotients (i.e., the ratio between the exposure level and an allowable level of toxicant).

The Environmental Protection Agency (EPA) guidelines for health risk assessment of chemical mixtures (U.S. EPA, 1986) recommend the calculation of a separate HI for each effect of concern. An HI >1 raises concern for potential health effects. The Risk Assessment Guidance for Superfund (RAGS), (US EPA, 1989) recommends the calculation of separate hazard indices for all major effects, including those that occur at higher doses than the critical effect. The RAGS states that the Reference Dose (RfD) can be used to calculate a chemical's hazard quotient for each effect, but acknowledges that use of the RfD may overestimate the hazard for effects that occur at higher exposure levels than the critical effect. The EPA's Science Advisory Board (SAB) has recommended the development of additional procedures to promote consistency in risk assessment for mixtures with more than one end point or effect.

This research project investigated the feasibility of developing target-organ toxicity doses (TTDs) for chemicals of interest to the Superfund program and their utility in Superfund baseline risk assessment. The approach used to develop the TTDs was analogous to the RfD derivation, using No Observed Adverse Effect Levels (NOAEL) and Lowest Observed Adverse Effect Levels (LOAEL) for each effect of concern and applying appropriate uncertainty factors. A simulated chemical mixtures risk assessment was conducted using the current method (RfDs for all effects) and the proposed method (using RfDs for the critical effect and TTDs for other effects). The results indicate that currently available data allow for the derivation of TTDs for several chemicals as well as provide specific guidance towards the conduct of more realistic chemical mixtures risk assessments. Further studies are needed to evaluate this approach with a larger group of chemicals.

2. A GENERALIZED MODEL FOR DEFINING ADDITIVITY FOR TOXICITY OF CHEMICAL MIXTURES

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Most of the current representations of additivity for toxicity of chemical mixtures are based on modeling the response as a function of the individual doses of the mixture component chemicals. One problem with this approach is that it is difficult to separate the influence of the total mixture dose on the response from the influence of the relative proportions of the components. A simple mathematical transformation facilitates the examination of dose–response relationships by allowing the blend (relative proportions of the components) of the mixture to be held fixed while changing the total dose of the mixture, and conversely. By segregating these critical factors, a clearer picture of the dose–response relationship emerges. This leads to a single empirical approach to defining additivity that includes both dose addition and response addition as special cases. The new twist of "proportional response addition" may have implications for molecular interpretation that do not require the assumption of independence.

3. HAZARDOUS WASTE SITE RISK ASSESSMENT: AN ILLUSTRATIVE CASE STUDY

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The Risk Assessment Guidance for Superfund was developed based on the National Academy of Sciences paradigm by the United States Environmental Protection Agency (EPA), Office of Emergency and Remedial Response specifically for assessment of risk at hazardous waste sites. After a site is listed on the National Priorities List, the first step of the cleanup process is to conduct a remedial investigation (RI) to determine the nature and extent of contamination, and the magnitude and likelihood of actual or potential harm to public health, welfare, and the environment posed by the actual or threatened release of hazardous substances. As an integral part of the RI, the baseline risk assessment includes chemical characterization of the site followed by evaluation of factors, such as the extent of contamination in various environmental media, the potential of contaminants to migrate, their persistence and toxicity, site-specific factors that influence the possible routes of human and environmental exposure to contaminants, and human populations that could be exposed and the potential risks resulting from exposure. The assessment utilizes chemical-specific levels obtained from the IRIS (Integrated Risk Information System) data base and other EPA documents. The nature and extent of risks to human health and the environment posed by the site being investigated guide decisions about whether remedial action is warranted, and the selection and evaluation of remedial alternatives. An example of an integrated hazardous waste site risk assessment will be presented that will follow four specific steps: data collection and evaluation, toxicity assessment, exposure assessment, and risk characterization including the criteria and assumptions used.

4. THE RISK ASSESSMENT PROCESS AT THE U.S. EPA: CURRENT GUIDELINES AND REFERENCE NUMBERS

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Risk assessment has become an integral component of decision making for protection of human health that comprises a four-step process as described in the National Academy of Sciences paradigm (NAS, 1983). It summarizes all of the available scientific evidence on the toxicity of a chemical and/or a mixture of chemicals and serves as a vehicle to organize and present our knowledge and experience of evaluating appropriate data. To promote consistency in risk assessment, the United States Environmental Protection Agency (EPA) has established various tools. Among these are the Risk Assessment Guidelines (Fed. Reg. Vol. 51, 1986) that provide generic guidance to conduct health risk assessment of single chemicals or chemical mixtures including hazard identification and dose-response assessment for end points such as carcinogenicity, mutagenicity, and developmental effects. Guidance is also provided for consistent evaluation of human exposure to potentially hazardous materials. The EPA also decides chemical-specific reference levels called Reference Concentration (inhalation), Reference Dose (oral), and Unit Risk (carcinogenicity) by various routes of exposure. These are EPA consensus risk values reached by workgroups for gauging the potential effects of exposure to certain chemicals. These risk values are accessible to the risk assessors and managers through Integrated Risk Information System (IRIS, IRIS-2/TOXNET). This process of risk assessment is dynamic, is becoming more holistic, and has been adopted by various other federal agencies; it is bound to take a more definite shape and promote consistency in the risk assessment community for health risks to the human population and ecological effects on the environment.

5. TESTING A NOVEL APPROACH TO ESTIMATE HEALTH RISK ABOVE THE REFERENCE DOSE (RfD)/REFERENCE CONCENTRATION (RfC)

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Current methods to quantify noncancer health risks are limited to estimates of subthreshold dose. Unfortunately, situations exist where such subthreshold doses are exceeded, and little is known about the possible health risk. Recent publications have indicated that the regression of toxicity data viewed as categories of pathologic staging has the potential for exploring the likely health risk at doses above the Environmental Protection Agency (EPA's) estimates of Reference Dose (RfD) or Reference Concentration (RfC). Such regression can use all of the available toxicity data. Moreover, the analysis depends on the judgments of toxicologists and other health scientists as to the overall pathology exhibited by the dose group or individual animal. This regression model was used on two well known human toxicity data sets where the EPA has determined an RfD for arsenic oral exposure of 0.3 μg/kg/day, and inhalation exposure to manganese where EPA has determined an inhalation RfC of 0.4 $\mu g/m^3$. In general, the model was able to synthesize diverse data on these two chemicals, yielding what appear to be reasonable estimates of health risk at various doses above the arsenic RfD or the manganese RfC. Verification of these estimates and future research are discussed.

6. A COMPUTER PROGRAM FOR TESTING THE STATISTICAL COMPATIBILITY OF DATA SETS WITH A COMMON MULTISTAGE MODEL

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Frequently there is more than one data set available upon which a risk assessment might be based. Combining data sets that are biologically and statistically compatible would lead to improved estimates of risk because of the resulting increases in sample sizes and/or number of dose groups. A computer program implementing a likelihood ratio test to statistically compare data sets with a common linearized multistage model is demonstrated. In addition to performing the statistical test, the program provides graphical comparisons of the data sets and the resulting multistage models which can be output in a variety of different formats.

7. COMPARISON OF TWO METHODOLOGIES FOR ASSESSING HEALTH RISKS ASSOCIATED WITH INDIRECT EXPOSURES TO MSW COMBUSTOR EMISSIONS

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Humans can be exposed to pollutants emitted from municipal solid waste combustors via indirect pathways, such as through the food chain or groundwater contamination. "Health Risk Assessment Guidelines for Nonhazardous Waste Incinerators" by the State of California Air Resources Board (1990), and "Methodology for Assessing Health Risks Associated with Indirect Exposure to Combustor Emissions" by the United States Environmental Protection Agency (1990) are available to aid in the determination of health risks. These two methodologies were quantitatively compared using three hypothetical exposure scenarios representing typical, moderate, and worst-case exposure to cadmium and benzo[*a*]pyrene. The indirect pathways considered in the methodologies are qualitatively similar, but the algorithms for some routes of exposure and the parameters differ. The exposure levels calculated using these two methodologies differ by orders of magnitude. Sources of these differences are discussed.

8. DETERMINING HAZARDS ASSOCIATED WITH MUNICIPAL SOLID WASTE COMPOSTING

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As communities attempt to lessen their dependency on landfills, several waste diversion alternatives, such as incineration or composting, are emerging. The relative health and environmental hazards associated with municipal solid waste composting, including both the production and the use of compost, are evaluated. During the process, workers may be exposed to noise, pathogens, chemicals, dust, and pests. During the composting process and after application of the compost onto the soil, metals and organics may leach through the soil. Users of the compost, as well as the communities surrounding a municipal compost facility may be exposed to many of the same hazards either by direct contact with the compost or via indirect pathways such as through the food chain or by groundwater contamination. The impacts of compost use on soil, plants, and groundwater, and the potential hazards to the community, compost users, and workers are qualitatively presented.

9. SEVERITY MODELING INSTEAD OF A NO-OBSERVED-ADVERSE-EFFECT LEVEL (NOAEL) FOR NONCANCER RISK ASSESSMENT

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Traditional noncancer risk assessment by the United States Environmental Protection Agency has used a selected data point (e.g., a no-observed-adverse-effect level), divided by uncertainty factors to estimate a subthreshold exposure level for humans, called the Reference Dose (RfD). The RfD, however, lacks statistical treatment and is not designed for estimating risk of adverse health effects at existing exposure levels. Our alternative approach requires a toxicologist to assign the array of observed effects for each exposed animal to one of several ordered categories, based on overall toxic impact. The approach is superior to the standard dose–response model because it accounts for differences in toxic intensity for different effects as well as the combined impact of multiple effects.

A cumulative log odds model regresses severity on log (exposure level) and duration using all relevant toxicity data, including multiple types of effect. The results are expressed as "risk of any adverse effect" for a range of exposure levels. By graphing the data along with the exposure levels predicting 10% and 1% risk of adverse effect, one can demonstrate model performance. Dosimetrically adjusting the exposure levels improves biological interpretation but complicates the modeling. The data are now highly correlated: multiple tissue levels for the different effects in the same animal from the same external exposure.

10. DEVELOPMENT OF A PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) LEVELS IN THE BRAIN FOLLOWING SINGLE DOSE ADMINISTRATION

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2,4-Dichiorophenoxyacetic acid (2,4-D) has been reported to cause neurotoxicity, including learning deficits, memory loss, loss of coordination, lethargy, coma, and even death in experimental animals and humans. It has been widely used in forestry management and agriculture as a weed killer. Our present work was intended to develop a generic physiologically based pharmacokinetic (PBPK) model for the dosimetry of organic acids in the central nervous system (CNS) using 2,4-D as a model compound. A physiological model was constructed for rats and rabbits using physiological parameters from the literature; chemical-specific parameters were obtained both from the literature and from our experimental data. 2,4-D is a very strong acid and partially water soluble. Its penetration into the brain via the blood-brain barrier (BBB) is very slow, but exposure to 2,4-D does cause accumulation in the brain. Our model consists of brain, body, venous, and arterial compartments. In the model, brain uptake is membrane-limited via the BBB with saturable clearance from the cerebrospinal fluid into the venous blood by the choroid plexus. The body has both a central and a deep compartment with saturable clearance from the central compartment. The model was used to examine plasma time course curves with experimental data obtained at Dow Chemical Co. from rats given 2,4-D by intravenous (5 or 90 mg/kg) or oral ingestion (10, 50, or 150 mg/kg). The model was also applied to studies in which rabbit plasma, brain, and CSF concentrations were measured at 2 h after intraperitoneal injection (40 mg/kg). Analysis of these studies provided estimates of permeation constants and elimination parameters for future model development. A validated PBPK dosimetry model would be an invaluable tool in regulatory evaluation of safety data for the whole class of organic anions where it can be used to predict the pharmacokinetics of these chemicals in the CNS in animals and people.

11. RISK ASSESSMENT BASIS FOR A DRINKING WATER HEALTH ADVISORY FOR 2,4- AND 2,6-DINITROTOLUENE (DNT)

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The United States Army and the United States Environmental Protection Agency (EPA) have cooperated to produce Drinking Water Health Advisories (HAs) for munitions and other Army environmental contaminants. The EPA risk assessment methodology produces HAs and guidance levels at which adverse health effects are not expected to occur over specific exposure durations ranging from one day to a lifetime. Dinitrotoluene (DNT) isomers, primarily 2,4- DNT and 2,6-DNT, are present in the environment due to production and manufacturing wastestreams. The EPA recently has established advisory levels for 2,4-/2,6-DNT that are protective of human health. The reference dose (RfD) for 2,4-DNT is 2E-3 mg/kg/day and that for 2,6-DNT is 1E-3 mg/kg/day. One-day, 10-day, and longer-term HAs for each isomer range from 300 to 1000 µg/L. The RfDs and HAs are based primarily on neurotoxicity, methemoglobinemia, Heinz body formation, and biliary tract hyperplasia that developed in beagle dogs dosed orally with DNT in chronic and subchronic studies. Dinitrotoluene is classified by EPA as Group B2: probable human carcinogen; therefore, a lifetime HA is not appropriate. The estimated 10^{-4}, 10^{-5}, and 10^{-6} cancer risks for DNT in drinking water are 5 µg/L, 0.5 µg/L, and 0.05 µg/L, respectively.

12. PHYSIOCHEMICAL MODELING OF DERMAL ABSORPTION USING STELLA®

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Occupational dermatitis represents a major occupational health problem. However, an issue of potentially greater importance is the issue of systemic injury attending dermal absorption. Obviously, these phenomena are interrelated, because damaged skin allows more rapid penetration. Several data bases have either been developed or are currently being developed that may prove potentially useful.

These data bases are dependent upon the quality of data available for inclusion. Data bases suffer from the features of rigidity associated with the physiochemical constants used or other factors. Ultimately, we would like to address this problem in a satisfactory manner, being able to at least categorize substances as being of major, intermediate, or minor concern. Investigators are still developing methods for the systematic classification of major use industrial substances into basic categories of rapid, intermediate, or slow penetrants.

Comparisons at present among methodologies tend to be tedious. One possible solution to this problem is the use of models. The computer modeling program Stella® facilitates modeled flows through heterogeneous tissues by allowing different regions or anatomical structures to be connected in a pattern matching the system's morphology. Time-dependent and steady-state models are both possible; nonlinearity does not appear to create special problems.

13. BINDING OF 1,3,5-TRINITROBENZENE TO RAT BLOOD PROTEINS AND TISSUE DNA

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Nitroaromatic compounds, such as 1,3-dinitrobenzene (DNB), 1,3,5-trinitrobenzene (TNB) and tetryl are environmental contaminants of groundwater and soil. Wastewaters discharged from trinitrotoluene manufacturing processes contain a large number of aromatic compounds, including DNB and TNB. These can form covalent adducts by nucleophilic interactions with macromolecules such as DNA, RNA, and cellular proteins or stable adducts with blood proteins (e.g. hemoglobin). We have investigated the ability of TNB to form such adducts in rats to develop biological markers of exposure.

14C-TNB, when given as a single gavage dose (225 mg/kg; 9.47 mCi/mmol) to male Fischer 344 rats, formed stable adducts with the blood proteins and tissue DNA in a dose-dependent manner (225, 45, and 9 mg/kg). These adducts persisted throughout the life span of the rat red blood cell. In contrast to globin, the loss of radioactivity in albumin and globulin compartments was much faster in the first 4 days. On the other hand, DNA adducts in all tissues (kidney, lung, spleen, liver, and stomach) persisted throughout the study period (70 days). Our data clearly show that TNB forms stable adducts with DNA and blood proteins. The identification of TNB-protein and TNB-DNA adducts and the quantitation of these adducts may be used as markers of exposure to TNB and other related aromatic compounds in risk assessment, site characterization, and postexposure monitoring.

14. REVERSAL OF *ras*-INDUCED INHIBITION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION, TRANSFORMATION, AND TUMORIGENESIS BY LOVASTATIN

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The plasma membrane association and transforming activity of *ras* oncoproteins, $p21^{ras}$, are dependent upon posttranslational farnesylation. Farnesyl synthesis and $p21^{ras}$ farnesylation are inhibited by HMG-CoA reductase inhibitors such as lovastatin. In the present study, we examined whether lovastatin could reverse the transformed phenotype of a viral H-*ras*-transformed rat liver epithelial cell line (WB-*ras* cells) and if changes were associated with the enhancement of gap junctional intercellular communication (GJIC). WB-*ras* cells grow in soft agar, have reduced GJIC, and are highly tumorigenic. Membrane association of $p21^{ras}$ in these cells was inhibited by *in vitro* treatment with lovastatin (0.05-0.5 uM) for 48 h. Concomitantly, the cells displayed a more normal morphology, decreased growth in soft agar, and enhanced GJIC. These changes were prevented by cotreatment with mevalonic acid. The morphology and GJIC of rat liver epithelial cells transformed with other oncogenes (*src*, *neu*, and *raf/myc*) were not affected by lovastatin. Intrahepatic WB-*ras* tumors were induced in male rats by intraportal vein injection of WB-*ras* cells. The size and DNA labeling index of these tumors were decreased approximately 75% after administration of lovastatin (5 mg/kg orally, twice per day) for 2 weeks. These results suggest that lovastatin reverses the transformed phenotype of WB-*ras* cells by inhibiting $p21^{ras}$ plasma membrane association. The concomitant enhancement of GJIC in lovastatin-treated cell also suggests a role for reduced GJIC in the expression of the transformed phenotype.

15. PERFLUORODECANOIC ACID (PFDA) EFFECTS ON HEPATOCELLULAR ENZYME INDUCTION

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Perfluorodecanoic acid (PFDA) is a straight chain 10-carbon perfluorocarboxylic acid with a molecuiar weight of 514 that causes anorexia, weight loss, and hepatomegaly in rats. The hepatomegaly is primarily due to massive hepatocellular peroxisome proliferation. Other chemically unrelated compounds that induce peroxisome proliferation have been associated with hepatocellular carcinogenesis, and PFDA is currently being evaluated for its carcinogenic potential. Perfluorodecanoic acid has been chosen as a model compound to examine peroxisomal proliferation, β oxidation, ω oxidation, and potential carcinogenicity of other perhalogenated hydrocarbon compounds.

The objectives of this project are to define the mechanism(s) and kinetics of PFDA hepatotoxicity with emphasis on ω oxidation of unsaturated free fatty acids. Omega oxidation is a minor oxidative pathway which substitutes a $-CH_3$ group with a -COOH group. This procedure is followed by β oxidation in the peroxisomes and mitochondrion. Preliminary studies have indicated that PFDA treatment causes alterations in hepatocyte cell cycle kinetics and cellular protein content.

16. DEVELOPMENT OF AN *IN VITRO* METHOD TO ASSESS DAMAGE TO THE BARRIER PROPERTIES OF PIG SKIN

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The effect of liquid gun propellant (LP), a highly irritating mixture of hydroxylammonium nitrate, triethanolammonium nitrate, and water, on the barrier function of skin was assessed *in vitro* by measuring the penetration of ^{14}C-benzoic acid following exposure to LP. Weanling pigs were topically exposed to single doses (25 $\mu L/cm^2$) of test compound. After 1 to 5 days, pigs were sacrificed and skin sections, excised from the sites of application, were mounted on *in vitro* penetration chambers to measure cumulative 24-h penetration of ^{14}C-benzoic acid. Topical treatment with undiluted LP resulted in 8.2, 4.5, 2.8, 1.2, and 1.7-fold increases in permeability for ^{14}C-benzoic acid at 1, 2, 3, 4, and 5 days postexposure, respectively. In parallel studies, untreated skin was excised, placed in penetration chambers, and then exposed to saline or LP for 1 day. *In vitro* skin exposure to LP resulted in a 3.9-fold increase in the permeability of ^{14}C-benzoic acid. In contrast, *in vivo* topical exposure of pigs to an aqueous solution of hydroxylamine HCl for 1 day resulted in only a 2.1-fold increase in skin permeability to ^{14}C-benzoic acid. These studies demonstrated that (1) the effect of LP on skin barrier properties is greatest at 1-day postexposure and steadily diminishes thereafter and (2) both *in vivo* and *in vitro* dermal exposure to LP alter the barrier properties of skin. This technique, quantitation of transdermal transport of benzoic acid or other model compounds, can be used to assess the effect of chemical or physical agents on barrier function and repair processes of skin.

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"The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as official Department of the Army position, policy, or decision, unless so designated by other official documentation."

"Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NIH publication 85-23, 185 edition."

17. MUTAGENICITY OF EMISSIONS FROM THE M16 RIFLE

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Mutagenic activity was found to be associated with the particulate, but not the gas phase, of combustion products generated by firing the M16 rifle with the propellant WC844. Dimethyl sulfoxide (DMSO) extracts of emissions from the breech and muzzle were mutagenic in three strains of *Salmonella* (TA1537, TA1538, and TA98) both in the presence and absence of metabolic activation systems. The extracts were negative in strains TA100 and TA102. Aerosols generated by firing the M16 rifle were fractionated according to particle size with the model 100 micro-orifice uniform deposit impactor (MOUDI). As expected, the mass of the particulate fractions increased with particle size. The mutagenic activity associated with the smaller, respirable particles was far greater than that associated with larger particles. Large particles that settled rapidly out of the airstream were not mutagenic. When extracts of emission particulates were subjected to preparative high performance liquid chromatography (HPLC), over 20 fractions were recovered. A number of these fractions were found to be mutagenic after being concentrated by air drying under nitrogen, redissolved in DMSO, and subjected to the AMES test. Although HPLC fractions from larger particles were generally less mutagenic than those from smaller particles, the difference in activity between the smaller and larger particles could not be associated with loss of any one or more HPLC peaks.

18. CARBON FIBER: FRAGMENT OR FIGMENT? (Acute Inhalation Toxicity of Carbon Fiber Fragments Generated From the XM81 Grenade)

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Carbon fibers are lightweight, high-tensile strength synthetic strands used commercially and in military applications for aircraft and electromagnetic obscuration. Fibers with respirable diameters (<3.5 μm) and with lengths beyond 10 μm will deposit by interception in the bronchi. Recent field studies with the 81 grenade indicated that explosive dissemination produced fibers with diameters less than 3.0 μm) and lengths less than 100 μm, making some of the fibrous smoke respirable.

The purpose of this study was to explosively generate carbon fibers to determine if the resultant aerosol is respirable and if it adversely affects rodents. Groups of male Fisher 344 rats were exposed to one of three concentrations of carbon fibers for 30 min. Air-exposed and fuse/fuel-exposed rats served as the controls. Exposed rats and respective groups of controls were submitted for bronchoalveolar lavage; biochemical, physiological, and pathological evaluation at 24-h, 14-days, and 90-days postexposure.

In addition, Scanning Electron Microscopy analysis was used to characterize the size range of the fibers, and to determine fiber deposition in trachea and lung tissues. Aerosol samples were collected for mutagenicity testing.

Initial results indicate the presence of fiber fragments and combustion products in the respirable range. However, there were no adverse changes in the biological responses of the rats from short-term exposure to aerosols from the XM81 grenade.

U.S.G.P.O.: 1993–750-061/81009