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PROCEEDINGS OF THE 8TH ANNUAL CONFERENCE ON ENVIRONMENTAL TOXICOLOGY 4, 5 and 6 OCTOBER 1977

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TECHNICAL REVIEW AND APPROVAL

AMRL-TR-77-97

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

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

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

FOR THE COMMANDER

A. A. Thomas

ANTHONY A. THOMAS, MD
Director
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PREFACE

The Eighth Conference on Environmental Toxicology was held in Dayton, Ohio on 4, 5, and 6 October 1977. Sponsor was the University of California, Irvine under the terms of Contract F33615-76-C-5005 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. Arrangements were made by the Toxic Hazards Research Unit of the University of California, Irvine, and the papers presented at this Conference by personnel of the University of California, Irvine represent research conducted under the cited contract. Ralph C. Wands*, Director, Advisory Center on Toxicology, National Research Council, National Academy of Sciences, Washington, D.C. served as Conference Chairman, and Mrs. Lois Doncaster, University of California, served as Conference Coordinator. Special acknowledgement is made to Ms. Sharon Hinton, University of California, Irvine who typed the entire proceedings and to Ms. Mildred Pinkerton, Aerospace Medical Research Laboratory, who edited and prepared all copy for printing.

*Now affiliated with the Cosmetic Ingredient Review, Washington D,C.

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INTRODUCTORY REMARKS

ENVIRONMENTAL TOXICOLOGY FROM ANOTHER PERSPECTIVE

Colonel Roy L. DeHart, USAF, MC

Commander
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Ladies and Gentlemen, I am pleased to have this opportunity to welcome you to this Eighth Conference on Environmental Toxicology. The University of California, in collaboration with the Aerospace Medical Research Laboratory, has structured for you a program which should prove informing and intellectually stimulating. In reviewing the programs of previous conferences, I note that in the past this place in the program was occupied by an expert. We have all heard the classical definition of an expert--"an individual from out of town, carrying a briefcase, with a stack of 35 millimeter slides." This morning I meet none of these prerequisites. I am neither from out of town, nor have I a briefcase, and to your good fortune, I have no slides. However, having accepted your invitation, I do have some introductory remarks.

A number of years ago, the late Adlai Stevenson, while an Ambassador of the United Nations, was invited to speak at a luncheon at the Washington Press Club. He began much as I have, thanking those responsible for inviting him, but he continued "...Having accepted an invitation to appear before you, I have an obligation to speak. You, the audience, have an obligation to listen. My only hope is that we each complete our assigned tasks at approximately the same time."

I would like to bring to you this morning a perspective perhaps different from that which will permeate this conference. The perspective comes not from the workbench, the laboratory, or university or government research institutions but from the world of health care application and practice. Among the popular concerns of our culture today is environmental toxicology. Weekly we are informed by the printed word, visual scene, or verbal impact of new hazards to life, ours or our progeny. As a former hospital commander and practicing physician, I have been asked by patients and commanders for an opinion, a recommendation, or comment on some newly discovered toxic hazard to health. A brief review of a number of events will make my point. Cyclamates and

bladder tumors; exhaust products and aerosols and the ozone layer; hexachlorophyll in soaps used on babies; carbon dioxide and the greenhouse effect; saccharin and bladder tumors; Tris in children's sleepwear; artificial color additives in food products; chlorinated surface water and cancer, and PPB in cattle feed. I can appreciate the anxiety in the general public generated by such pronouncements. It appears that we are exposed to environmental toxins at home, at work, or at the beach; where we eat, we play, we drink, we breathe. Yes, even in our sleep.

> "<u>25% of U.S. Workers Exposed to Job Hazards, Survey Shows.</u>
> By David Burnham, New York Times Service.
>
> Washington - An extensive survey of occupational hazards has found that while at work, one out of four Americans is exposed to some substance thought to cause death or disease."
>
> "...fewer than 5 per cent of the places where people work have industrial hygiene services, active plans to prevent or reduce the exposure of employees to hazardous substances and such physical conditions as radiation and excessive noise."
>
> "John F. Finklea, Head of the National Institute of Occupational Safety and Health which conducted the survey, said in response to an inquiry that the study provided new evidence that exposure to chemical hazards was 'pervasive in a large number of occupations' and that the availability of such preventive services as industrial hygiene was 'not what it should be, especially in the smaller firms.'"
>
> "In discussing the costs involved, according to one government analysis...it could cost as much as $54 billion to provide warnings and health surveillance services to the 21 million Americans now believed to be exposed to harmful conditions while working."
>
> "A major problem in completing the survey, which was based on inspections between 1972 and 1974, was that about 70 per cent of the substances found were identified by trade names rather than chemical composition, so that frequently neither the employer nor the employee knew of the potential hazards."

These anxieties are frequently brought by the patient to the attention of the physician in the hope there will be some reassuring word.

Let's move for a moment from the individual to the industrial environment just mentioned. Charged with the responsibility of the total community health, the Directors of Base Medical Services on our Air Force bases must assure a safe working environment to protect both our military and civilian workforce. Rules and regulations must be complied with. Threshold limit values cannot be exceeded, yet the mission must be accomplished. All too frequently, it appears that exposure levels are based on the technology available to identify a substance with little concern for epidemiological evidence. There appears to be a growing zero-exposure-mentality without regard to established dose-response curves. Impact of arbitrary zero-exposure levels will be to reduce efficiency, raise costs, or halt production, affecting not only the Air Force, but all major industry. As investigators and scientists, you have two responsibilities: one to your science, and one to the public. In your deliberations, consider carefully both responsibilities. At some point, a physician such as myself, a plant manager, or a mother, may have to explain and interpret the results of your investigative efforts.

One final comment regarding maintaining a perspective. Given the regulatory power and the ability to motivate, I could rapidly and effectively significantly reduce morbidity and mortality in the United States. The epidemiological evidence is clear. The results of retrospective and perspective studies are undeniable. The recommended solution is simplistic and individualistic, the cost benefits definable. What actions are necessary to bring forth such miracles and improve the health of our Nation? Stop smoking; wear seatbelts; maintain physical fitness. Simple, inexpensive, noncontroversial actions, yet they may never be attained nationally.

From the diversity and timeliness of the program, it appears that your two-and-one-half days will be well spent. On behalf of General Unger, the Commander of the Aerospace Medical Division, and myself, I wish you another successful, outstanding, and stimulating conference.

SESSION I

OCCUPATIONAL HEALTH ASPECTS OF COAL CONVERSION

Chairman

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PAPER NO. 1

COAL CONVERSION TECHNOLOGY

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INTRODUCTION

At present there are large disproportionations between domestic fossil energy uses and domestic fossil energy resources. The extent of these disproportionations is shown in Table 1. Currently, oil and gas account for 74% (46 + 28) of the total energy consumed in quads/year in the U.S., but these two fuels comprise only 7% (4 + 3) of U.S. resources. On the other hand, while coal is providing 20% of the fuel required for today's energy consumption, coal is by far the dominant energy resource in the U.S. Shale presently does not contribute to U.S. energy usage, and its potential as a resource, while significant relative to oil and gas, is considerably less than coal.

TABLE 1. DOMESTIC FOSSIL ENERGY PICTURE

	CURRENT U.S. USAGE		U.S. RESOURCES	
	QUADS/YR*	% OF TOTAL ENERGY CONSUMED	QUADS	% OF FOSSIL RESOURCES
OIL	33	46	950	4
GAS	20	28	900	3
COAL	14	20	23000	86
SHALE	0	0	1800	7
ALL FOSSIL	67	94		

* 1 QUAD IS 10^{15} BTU's

BASED ON 1977 STATISTICAL ABSTRACT (U.S. 1975); ERDA PRESENTATION, FEB. 1976 ACS SYM.(U.S. 1975)

The disproportionations between domestic fossil energy usage and resources are also strikingly apparent in a comparison of domestic supplies. This comparison is shown in Table 2. Resources of oil and gas, which are providing 74% of current usage as noted previously, are sufficient for 35 years of domestic energy supply at the current usage rate (without imports). In sharp contrast, coal resources are equivalent to 1600 years of domestic energy supply at the current usage rate. These data almost certainly define the role of coal in our energy future. As our most abundant source of energy, the growth of coal appears to be desirable.

TABLE 2. DOMESTIC FOSSIL ENERGY PICTURE

	CONSUMPTION, % OF CURRENT USAGE	YEARS DOMESTIC SUPPLY AT CURRENT USAGE RATE
OIL & GAS	74	35
COAL	20	1600

BASED ON 1977 STATISTICAL ABSTRACT (U.S. 1975); ERDA PRESENTATION, FEB. 1976 ACS SYM.(U.S. 1975)

It is clear that the data presented thus far do not take into account the large volumes of foreign crude oil imported into the U.S. today. These volumes are quite large having reached a level of 40-50% of our current crude requirements. However, this circumstance does not negate the future of domestic coal in our fuel picture. Imported crude oil has and will continue to provide substantial energy to the U. S. domestic market, but overriding questions of security of supply and the stabilization of world-wide crude reserves point to the need for increased coal utilization in the U.S.

As abundant as our coal resources are, coal is not directly suitable for all of our domestic uses. Our economy and technology require the availability of various forms of fuels, not just BTU's. This is illustrated in Table 3. Only in the case of electricity generation is coal directly suitable as a fuel. For household/commercial, transportation, and some industrial uses clean energy in the form of gas and liquids is required. Hence, there is a need for the development of improved coal conversion techniques.

TABLE 3. FUEL CONSUMPTION BY MARKET VECTOR

	PERCENT	DIRECT COAL SUITABILITY
HOUSEHOLD/COMMERCIAL	19	NO
TRANSPORTATION	26	NO
INDUSTRIAL	27	SOME
ELECTRICITY GENERATION	28	YES

APPROACHES TO COAL CONVERSION

The conversion of coal to both liquids and gases is essentially a process for adding hydrogen to coal. This is apparent from the fact that the hydrogen/carbon atomic ratio for coal is below 1.0 while this ratio for aromatics and fuel oil is about 1.0; for paraffins in gasoline, about 2.2; and for methane (natural gas), 4.0. From a simplistic view, the process conditions for hydrogen addition must be severe enough to break carbon-carbon bonds in the large molecules in the coal, producing fragments with which the hydrogen then reacts. Coal conversion chemistry includes three types of processing approaches: liquefaction, pyrolysis, and gasification. The conditions for conducting these processes are shown in Table 4. In liquefaction, these conditions are moderately high temperatures and high pressures, 700-900 F, and 1000-3000 PSIG. In gasification, higher temperatures and moderately high pressures, 1300-1800 F, and $\sim$500 PSIG, are required. The conditions for pyrolysis are temperatures between those of liquefaction and gasification (900-1200 F) and low pressures, $\sim$50 PSIG. The very high temperatures (3000 F+) and atmospheric pressure used in direct coal utilization or combustion differ appreciably from the conditions employed in the coal conversion techniques.

TABLE 4. COAL CONVERSION CONDITIONS

	SEVERITY TEMPERATURE, °F	SEVERITY PRESSURE, psig
LIQUEFACTION	700 - 900	1500
PYROLYSIS	900 - 1200	50 +
GASIFICATION	1300 - 1800	500

DIRECT UTILIZATION	3000 +	0

Although the origins of the present petrochemical industry can be traced to coal pyrolysis, this conversion technique will probably not be used to produce the major quantities of liquids and gases needed to augment our oil and gas resources. For this purpose, direct coal liquefaction and gasification are more likely.

Liquefaction Processes

While coal liquefaction has a lengthy history dating from the coking of coal (pyrolysis) in the mid-1800's in Germany and England, direct coal liquefaction process development began in Germany in the mid-1920's with work on the Pott-Broche process. Germany also developed the Bergius process in the 1930's which used a catalyst to effect hydrogen addition. Although not a direct coal conversion process, the Fischer-Tropsch process developed in Germany in the early 1920's produced liquids from synthesis gas ($CO + H_2$) to which the coal was first converted. It is significant that all three of these processes provided gasoline and oil for Germany during World War II.

Subsequent to World War II, there has been widespread activity in coal liquefaction process development in the U.S. The level of this activity has generally varied with the estimate of domestic petroleum reserves; in recent years the level has been quite high.

While only one coal liquefaction process is operating commercially today - the indirect Fischer-Tropsch process - development work has been carried out on a number of direct coal liquefaction processes. Three of these on which development is continuing are described below.

• Solvent Refined Coal (SRC) Process - Figure 1

In SRC-I, dried and crushed coal is slurried with a heavy recycle solvent and preheated. In the presence of molecular hydrogen, the coal is dissolved to form a product consisting of light oil, heavy oil, and solids. The reaction conditions are 825 F and 1000-1500 PSI. Separation and cooling of the heavy oil yield the solid solvent refined coal, which is reduced in both ash and sulfur and is improved over the source coal as a solid fuel.

While SRC-I as developed yields a solid fuel as the primary product, SRC-II is being developed to produce liquids as a major product. The approach to achieve this objective is to recycle some of the product slurry (which contains part of the ash from the original coal feed) in lieu of recycle solvent as in SRC-I to the dissolver. Significant liquid yields have been reported from this approach.

A 6 T/D pilot plant has been in operation in Wilsonville, Alabama since January 1974, and a 50 T/D pilot plant has been in operation at Ft. Lewis, Washington since 1975. Recent operations in the latter unit have been concerned with SRC-II.

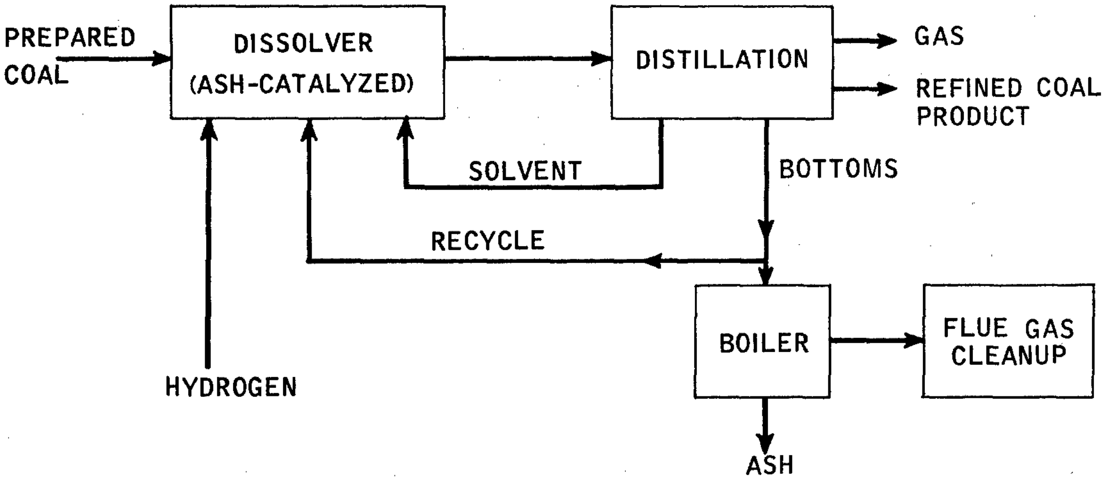

Figure 1. Solvent refined coal process.

• Coal Liquefaction Catalytic Process - Figure 2

In this process dried and crushed coal is slurried with a recycle solvent and preheated. The slurry and a hydrogen stream are reacted in an ebullating bed reactor containing a catalyst. The reaction conditions are 850 F and 3000 PSI. Synthetic crude, recycle solvent, light gases, and solids-containing bottoms are separated. These products, excluding the recycle solvent, are handled by processing the synthetic crude in a petroleum refinery, by using the light gases as a plant fuel, and by using the bottoms to manufacture hydrogen.

The process has been demonstrated in a 3 T/D pilot plant. A 200-600 T/D demonstration plant to be built at Catlettsburg, Kentucky is scheduled for operations in late 1978.

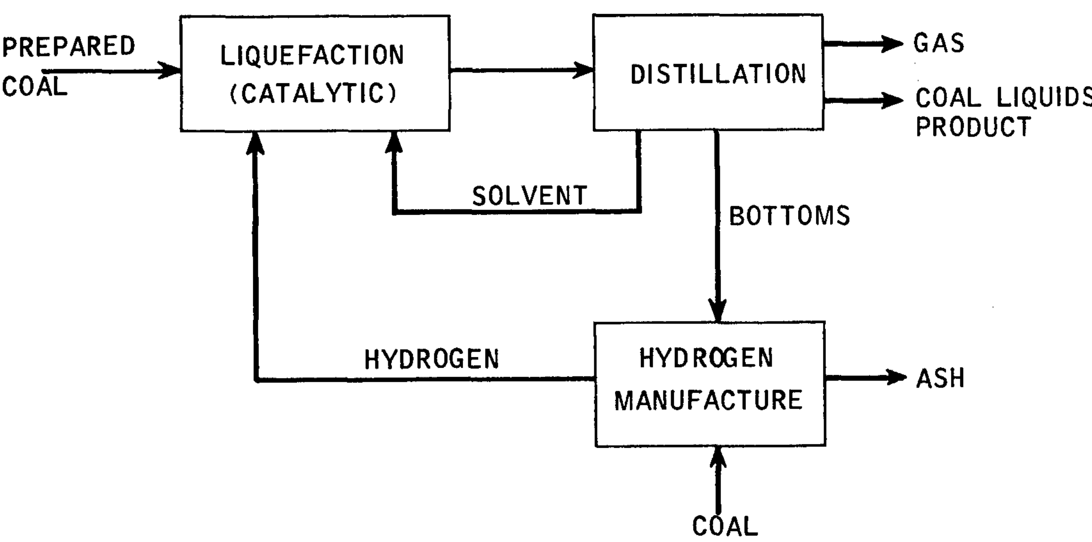

Figure 2. Coal liquefaction - catalytic process.

• Coal Liquefaction - Exxon Donor Solvent Process - Figure 3

In this process crushed coal is slurried with a hydrogenated donor recycle solvent and preheated. The slurry and a hydrogen stream are reacted at 840 F and 2000 PSI. Product liquids, recycle solvent, light gases, and solids-containing bottoms are separated. The recycle solvent is reacted with hydrogen in a fixed bed catalytic hydrogenation step prior to its return to the reactor. The light gases are used to produce hydrogen, and the bottoms are subjected to flexicoking to produce additional liquids and low BTU gas for plant fuel. The product liquids can be used either as fuel oil or upgraded by catalytic hydrogenation to a synthetic crude.

This Exxon process has been demonstrated in a 1 T/D pilot plant. The development program is continuing with laboratory and engineering studies and a 250 T/D pilot unit scheduled to be in operation in Baytown, Texas early in 1980.

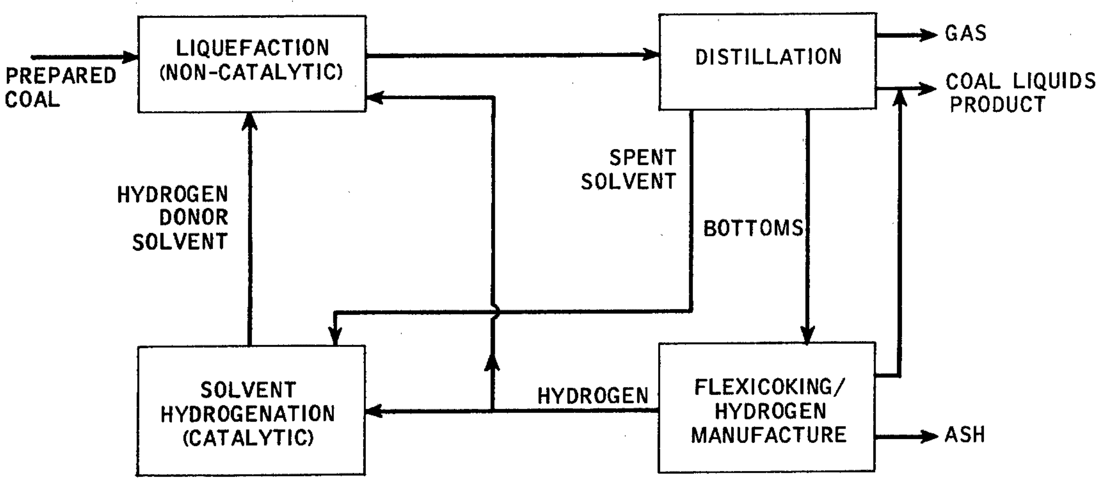

Figure 3. Coal liquefaction - donor solvent process.

Gasification Processes

The history of coal gasification is slightly longer than that of coal liquefaction, dating from the first gas producer built in 1832. Water gas, carburetted water gas, and coal gas were subsequent developments. Use of these products from coal for domestic and industrial heating ultimately gave way to natural gas as that fuel became economically attractive and plentiful. As in the case of coal liquefaction, it has only been in recent years that the projected decrease in natural gas reserves has focused attention on the application and/or development of coal gasification processes.

Currently there are two generic approaches to coal gasification with steam: thermal and catalytic. The Lurgi and Koppers-Totzek thermal processes are commercial, and there are numerous other thermal processes under development. The Exxon catalytic coal gasification process is entering the development stage. Both the thermal and catalytic techniques are described below.

- Coal Gasification - Thermal - Figure 4

In the Lurgi version of this process, heat is generated by oxygen injection in the bottom of the reactor; coal particles (1/8"-1/4") enter the reactor at the top and ash is withdrawn at the bottom. The process operates at a maximum temperature of 2000 F and 300-500 PSI. At the high reaction temperature, coal reacts with steam fed to the reactor to produce a mixture of hydrogen and carbon monoxide. This mixture is subjected to further processing (shift and methanation) to yield the final product. Low BTU gas instead of medium BTU gas can be produced if air is used in place of oxygen.

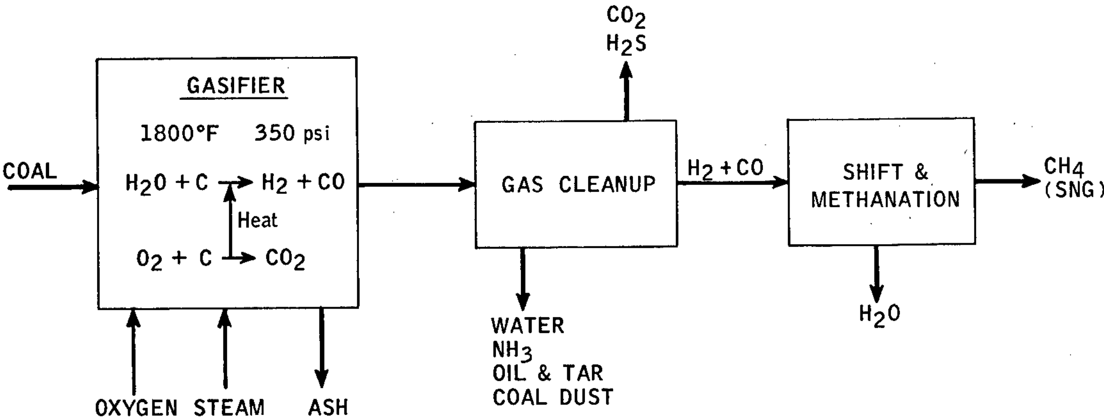

Figure 4. Coal gasification - thermal.

In the Koppers-Totzek process, pulverized coal (-200 mesh) and oxygen are injected at the bottom of the reactor; ash is also withdrawn from the bottom of the reactor. The process operates at a maximum temperature of 3300 F and at atmospheric pressure. As in the case of the Lurgi process, further processing of the gas from the reactor is required.

- Exxon Catalytic Coal Gasification - Figure 5

In this process, coal is crushed, impregnated with a solution of alkali metal salts, dried, and charged to a fluid bed gasifier operating at 1300 F and 500 PSI. The coal is gasified with a mixture of steam and a recycle stream of hydrogen and carbon monoxide. Preheat is supplied by a conventional gas-fired furnace. The process does not require a source of oxygen, and downstream shift and methanation steps are not required. The process involves catalyst recovery and reuse.

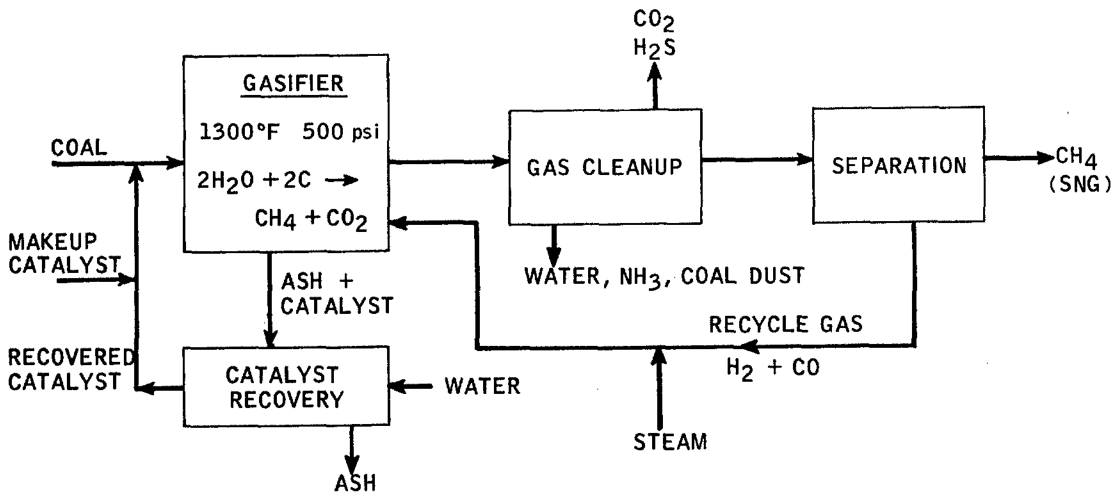

Figure 5. Coal gasification - catalytic.

Predevelopment work on the process in a 10-15 lb/hour unit is scheduled to be complete by the end of the year, and planning is in progress to continue the development in a program utilizing a 1 T/D process development unit.

As our domestic energy source shifts toward coal, the compositional differences between coal and natural gas and crude oil will require certain adaptations in the conversion processing of coal and in the use of coal products. The higher concentrations of nitrogen and inerts (ash) in coal versus natural gas and crude oil, as shown in Table 5, present difficult but not insoluble problems. In addition, since the liquid products from coal are high in aromatics, this characteristic will be beneficial in some applications (motor gasoline) and detrimental in others (jet fuel).

TABLE 5. COMPOSITION OF FOSSIL FUELS

	TYPICAL WEIGHT PERCENT IN		
	NATURAL GAS	CRUDE OIL	COAL
CARBON	75	85	70
HYDROGEN	25	13	5
SULFUR	0	2	4
NITROGEN	0	0.1	1
OXYGEN	0	0.1	10
INERT	0	ppm	10

At this time, differences between converted coal and crude oil as energy sources appear to be of degree rather than kind. Problems (such as the health and safety aspects of this emerging industry) resulting from these differences need to be defined and resolved in an evolutionary manner if the technology is to be in place when we need it. The solutions should be developed in a cost/effective and collaborative manner. Sensational and exaggerated approaches will not be constructive. There is, however, no doubt that liquid and gaseous products from coal will find their place in our energy picture.

SUMMARY

TABLE 6. OVERALL

- U.S. NEEDS COAL CONVERSION INDUSTRY
- TECHNOLOGY IS BEING DEVELOPED
- SHIFT TO COAL REQUIRES ADAPTATION
- NEED COST/EFFECTIVE APPROACHES

As shown in Table 6, the status of U.S. coal conversion technology may be summarized as follows:

- Domestic energy needs require a coal conversion industry.
- The technology for that industry is being developed now.
- The shift to a coal conversion technology will require adaptation from both processing and product usage standpoints.
- And finally, the successful development of a coal conversion industry will result only from cost/effective approaches.

PAPER NO. 2

DEFINING AN ADEQUATE INDUSTRIAL HYGIENE PROGRAM FOR COAL CONVERSION TECHNOLOGY IS A COMPLEX PROBLEM*

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TABLE 1. THE ESSENTIAL ELEMENTS OF AN ADEQUATE INDUSTRIAL HYGIENE PROGRAM FOR ANY GIVEN PROBLEM INCLUDE:

1. Informing employees of the hazards.
2. Installing effective engineering controls.
3. Establishing protective clothing requirements.
4. Monitoring exposures (both air and skin).
5. Maintaining adequate records.
6. Interfacing with medical.

TABLE 2. AN EFFECTIVE INDUSTRIAL HYGIENE PROGRAM FOR ANY NEW PROCESS DEMANDS A FAMILIARITY WITH THE TECHNICAL LITERATURE.

1. Identify <u>all</u> areas of concern.
2. What's <u>known</u> about similar processes?
3. Relate this knowledge to present problem.

TABLE 3. THE PROCESS CHEMISTRY MUST BE SHARPLY DEFINED <u>AND</u> UNDERSTOOD.

1. What chemicals are likely to be present?
2. At what points in the process?
3. Where are leaks most likely to occur?
4. Are control techniques available to prevent exposure?

NOTE: Armed with this information you can proceed in an orderly manner to identify specific tasks involved and develop a health protection program.

*This paper was prepared for oral presentation and only the visual aids were submitted for publication.

TABLE 4. WHAT ARE THE MAJOR PROCESSES PRESENTLY ENVISIONED?

1. Gasification
2. Liquefication
3. Pyrolysis

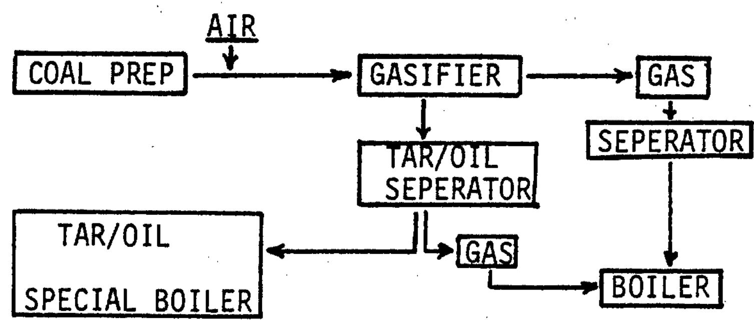
TABLE 5. GASIFICATION INVOLVES TWO BASIC PROCESSES:

1. Air blown for low BTU gas.
2. Oxygen blown for high BTU gas.

TABLE 6. PRIMARY STEPS IN LOW BTU GASIFICATION INCLUDES:

1. Coal prep.
2. Gasifier.
3. Process gas and tar/oil fraction.
4. Cleanup of both streams.
5. Recombine gas.
6. Fire in boiler.

TABLE 7. SCHEMATIC OF A SIMPLE GASIFIER INCLUDE PRIMARILY 5 ELEMENTS:



NOTE: So what industrial hygiene problems can be identified/ envisioned at each step ?

TABLE 8. TWO PROBLEMS ARE APPARENT IN THE COAL PREPARATION AREA:

1. Noise from crushers.
2. Coal dust.

NOTE: Both can be adequately managed with existing techniques. These problems can be reduced to defining the severity of the problem and using engineering controls to reduce exposures to an acceptable level.

TABLE 9. FUGITIVE ENVISIONS (LEAKS) IN THE GASIFIER AREA PRODUCE POSSIBLE EXPOSURES TO:

1. PNA's and condensable organics.
2. Sulfur compounds.
3. CO, NH_3, H_2S, etc.
4. Metal carbonyls (Fe & Ni).

TABLE 10. SIMILAR EXPOSURES ARE POSSIBLE IN THE SEPARATOR AREA AND THE GAS BOILERS.

1. PNA's and condensable organics.
2. Sulfur compounds.
3. CO, NH_3, H_2S, etc.
4. Metal carbonyls (Fe & Ni)

TABLE 11. THE CARCINOGENIC POTENTIAL IS THE MAJOR CONCERN.

1. By contact during maintenance.
2. By inhalation as a result of a leaking system.

TABLE 12. STANDARDS SPECIFIC TO THE CARCINOGEN PROBLEM SUGGESTED BY NIOSH OR PROMULGATED BY OSHA INCLUDE:

1. Total particulate - 0.2 mg/m^3
2. PPOM* - 0.2 mg/m^3
3. BaP - (tentative) - 0.0002 mg/m^3

*Benzene soluble fraction of total particulate. Sometimes called CTPV and/or PNA's. Coal tar pitch volatiles (CTPV) or polynuclear aromatics (PNA's).

Adequate standards exist for the other potential industrial hygiene problems and for the most part control technology has been shown effective.

TABLE 13. EXISTING SURVEILLANCE TECHNIQUES FOR COAL CONVERSION TECHNOLOGIES INCLUDE:

1. Surface contamination by longwave UV light.
2. Air analyses for PPOM, total particulate, BaP and other selected PNA's.

TABLE 14. MEDICAL SURVEILLANCE IS AN ESSENTIAL ELEMENT IN ANY HEALTH PROTECTION PROGRAM.

Specific to coal conversion technology and in addition to routine physical exams are:

1. Skin examinations.
2. Sputum cytology tests.

TABLE 15. THE ESSENTIAL ELEMENTS OF AN ADEQUATE INDUSTRIAL HYGIENE PROGRAM FOR COAL CONVERSION TECHNOLOGY INCLUDE:

1. Informing employees of the hazards.
2. Installing effective engineering controls.
3. Establishing protective clothing requirements.
4. Monitoring exposures (both air and skin).
5. Maintaining adequate records.
6. Interfacing with medical.

PAPER NO. 3

TOXICOLOGY -- RESULTS OF EXPOSURE OF MICE AND MEN TO COAL HYDROGENATION PRODUCTS

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The material presented here today was first published in some detail in four articles in the Archives of Environmental Health, 1, 181-233, 1960. However, it was decided that republication in these proceedings would be useful in view of the renewed interest in coal conversion.

The first commercial coal hydrogenation plant was built in Leuna, Germany, in 1926 and, with later improvements, products were evolved somewhat comparable in quality to those derived from petroleum oils. In that country, a total of 12 hydrogenation plants were built which supplied, eventually, approximately 85% of the aviation gasoline for Germany in World War II. These plants, however, did not produce chemicals. In the early 1930's, Union Carbide began such experimentation and in 1952, after 17 years of research, engineering and the operation of laboratory-sized plants, the first large-scale pilot plant production of chemicals from coal hydrogenation was begun at Institute, West Virginia. The design capacity was 300 tons of coal per day. The coal was bulldozed onto a belt conveyer, ground in the primary crushing equipment, and then pulverized to a smaller size in secondary equipment. After being dried to remove moisture, the coal dust was mixed with oil to form a paste. This coal-in-oil paste was pumped to its maximum pressure, followed by preheating to the desired temperature, for feed to the hydrogenation reactor or "liquefication unit". The preheated paste was reacted with hydrogen in a pressure converter at high temperature and pressures.

The hydrogenated coal paste was then treated to remove the unreacted solids. The product that remained was broken down into four main streams; the light oil stream, said to boil up to 260 C; the middle oil stream, described as boiling between 260 and 320 C; the heavy oil stream, boiling above 320 C, which was recycled either as pasting oil or distilled

to obtain polycyclic aromatic hydrocarbons; and the pitch stream, a hot molten material which solidified at 200 C; this was the residue after all volatile materials had been removed. The chemicals separated from these various streams were listed by Sexton (Table 1).

TABLE 1. AROMATIC AND ALIPHATIC CHEMICALS

Heavy Products Separation Unit	Light Products Separation Unit
Heavy Oil Stream	Stabilizer Overhead Fraction
Anthracene	Pentane
Dihydroanthracene	Pentene
Phenanthrene	Cyclopentene
Pyrene	Hexane
Chrysene	Methylcyclopentane
Perylene	Heptane
Picent	Heptene
	Hexene
Middle Oil Stream	Benzene
Dimethylnaphthalenes	
Biphenyl	
Acenaphthene	
Fluorene	

Investigation of potential carcinogenicity of the streams was undertaken by the Chemical Hygiene Fellowship of Carnegie-Mellon University to determine whether a problem existed. Subsequently, a plant medical director, with the cooperation of the plant safety engineers and product supervisor, initiated preventive medical measures to combat the problems as delineated by the information supplied from the animal experiments. The industrial hygienists attempted to measure the extent of the exposure which was sometimes noticeable in the plant as fume formation (Ketcham and Norton, 1960).

The following is a summary of the carcinogenic studies performed. The literature in the field of experimental carcinogenesis of chemicals derived from coal and of synthetic products similar in structure is voluminous. The relationship between experimental cancer in or on laboratory animals, on the one hand, and cancer produced on man by the same material, on the other, is scanty. A historical background of industrial carcinogens has been presented in a book by Eckardt. Such materials as soot, shale oil, certain aromatic amines, chromates, asbestos, coal tar, and isopropyl oil, as well as physical

agents such as X-ray and certain other radiations have been implicated as producing occupational cancer. Eckardt has presented evidence of the reduction in carcinogenic activity by the removal of a carcinogenic oil from the skin of a mouse with soap and water. He also reported the failure of six so-called protective creams to prevent mouse skin cancer.

Male mice, primarily C3H, were used in our studies. The fur was removed, starting at 90 days of age, from the back of the mice by the use of electric clippers. The mice received three applications per week - one brushful of each chemical being applied to the midline of the back on Monday, Wednesday, and Friday of each week. Observations were made for papillomas and carcinomas during each painting period and the readings were summarized every month. Groups of 30 mice were used for each material.

A positive and at least one negative control group were painted and examined with each study. The former consisted of applications of 0.2% methyl cholanthrene in benzene, while the latter were applications of the solvent used, such as benzene, or of water alone. All materials were applied undiluted if liquids or in solution in a proper solvent if a solid. The mice were observed until death or at least until the end of their 450th day of age.

Samples from several streams and residues of the coal hydrogenation process were applied to the skin as described. Additional special studies were performed. One was the application of either of two barrier creams to the back of the mice one hour before the painting with pasting oil - the coal-in-oil paste to be discussed at length later. Similarly, four hours after painting with pasting oil, the efficiency with which this material was removed with soap or with one of two soapless cleansers was studied. A sponge was used to apply the cleanser to the skin. The mouse was then rinsed with water and dried with cleansing tissues. Another study was performed to see the effect of dilution of pasting oil with benzene.

In each case, when a tumor was seen on the skin, a plot of its position was made and a record of its progress was kept. The median tumor or cancer latent periods were calculated, using a method suggested by Horton. These latent periods are the lengths of time necessary to reach a 50% tumor or cancer index. These indices are 100 times the number of mice with skin tumors or with cancers divided by the "effective" group, where "effective" group is the number of mice given adequate exposure.

It would have been desirable to test all of the streams and products of the coal hydrogenation process at one time, using one stock of mice. This was not possible for two reasons: (1) large enough groups of mice were not available, and (2) changes were made in the process. Therefore, some comparisons of two highly carcinogenic materials that were painted in several of the studies should indicate if the factors of stock and of time would radically affect comparison of results.

A positive control carcinogen, methyl cholanthrene, was used in six of the studies in which coal hydrogenation chemicals were painted. As is apparent in Table 2, the final tumor and cancer indices were not affected by the stock of mice used. If tumors start at a sufficiently early stage in the life of the mice, essentially all of those alive will develop papillomas and carcinomas when painted with a carcinogen of this potency. More important than these indices is the median latent period, the time in days for the average mouse (50% of the effective group) to develop tumors.

TABLE 2. EFFECT OF DIFFERENT STOCKS OF MICE ON CARCINOGENIC POTENCY OF METHYL CHOLANTHRENE (M.C.)*

Study No.	Mice	Tumor Index	Cancer Index	Median Latent Period, Days	
				Tumor	Cancer
4	C3H-JAX	100	100	145	190
5	R.A.P.	96	82	105	165
6	R.A.P.	89	89	118	165
7	C3H-R	92	84	144	168
11	C3H-JAX	100	97	114	138
12	C3H-JAX	100	97	111	150

*All were painted 0.2% in benzol.

While there was some variation in these periods, it does not seem to be related to the stock of mice used. As these studies were all performed at different times, as well as with different stocks of mice, it is remarkable that the agreement of these latent periods is so close. The data in Table 3 are indications of the relative potency of pasting oil, the "oil" used to carry the ground coal. With the exception of sample 3, the tumor and cancer indices of the pasting oils are similar. Sample number 3 was a purchased coal tar (creosote) oil which was blended with the plant-produced pasting oil as required to maintain a proper vehicle balance. All of the mice

that received this sample had died by the end of the eighth month of painting. Its potency, as measured by the median latent period of methyl cholanthrene divided by that for the coal tar oil, was similar to those of the various pasting oil samples. Among the pasting oils, the potency of the later produced and tested sample, number 4, appeared to be somewhat higher than the two previously tested. All, however, were high. As pasting oil was recycled and, therefore, subjected to repeated contact with coal at high temperatures and pressures, this probable increase in potency is logical.

TABLE 3. POTENCY OF DIFFERENT SAMPLES OF UNDILUTED PASTING OIL (P.O)

Sample Number	Study Number	Mice	After One Year Tumor Index	After One Year Cancer Index	Median Latent Period (M.L.P.), Days Tumor	Median Latent Period (M.L.P.), Days Cancer	(M.L.P. of M.C. / M.L.P. of P.O.) X 100 Tumor	(M.L.P. of M.C. / M.L.P. of P.O.) X 100 Cancer
1	4	C3H-JAX	100	100	225	245	64	78
2	7	C3H-R	94	67	225	318	64	53
3*	7	C3H-R	67*	42*	165	246	87	68
4	11	C3H-JAX	100	90	114	150	100	92
4	12	C3H-JAX	78	56	123	180	90	83
4	15	C3H-R	100	64	144	240	---	--

*Purchased cresote oil, used to mix with coal tar oil. All mice dead within eight months.

As the potency of methyl cholanthrene and of pasting oil did not vary much with different stocks or in the different studies, it is assumed that differences between the results of painting samples from the various streams and products of coal hydrogenation units were measures of the potency of these samples. Figure 1 is a diagrammatic presentation of the tumor and cancer indices of samples of 15 of these coal process materials after one year of painting. The two products, heavy and light oil, produced only papillomas. As the former was too viscous to test undiluted, it was painted as a 10% suspension. Tumors were found until 8 months but none from the 8th to 12th month with this product. The light oil product took one and one-half years to give the tumor indices listed; at one year those of 13 and o for tumor and cancer, respectively, were noted.

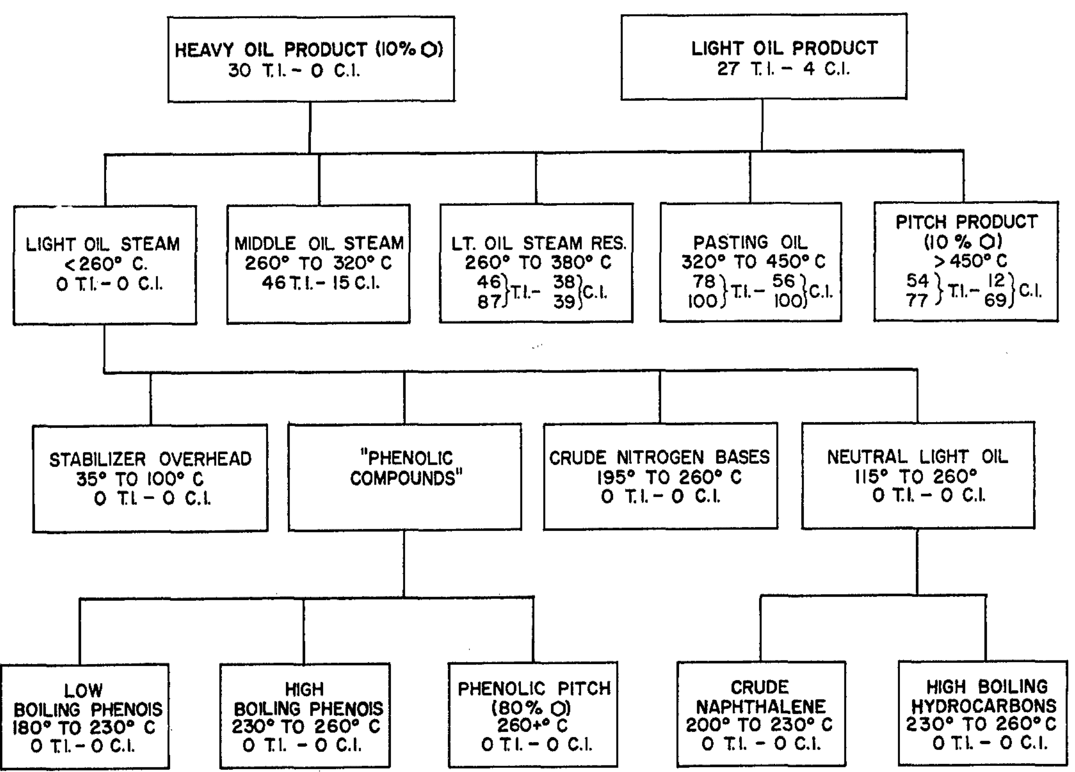

Figure 1. Products, stream and fractions of coal hydrogenation process tested (with boiling points and tumor indices indicated).

The light oil stream and eight fractions of this stream were all without tumorigenic action. No tumors whatsoever were found on the skin of these mice. All, with the exception of phenolic pitch, were tested undiluted. This pitch was also the only fraction of the light oil stream which had a boiling point range above 260 C.

Experimenters as early as 1921 showed that the higher boiling fractions of tar (over 300 C) produced the highest proportion of skin carcinomata experimentally. This was more recently discussed by Combes, Hueper and others. The findings of the carcinogenic activity of certain coal hydrogenation materials verify this temperature relationship. The middle oil stream, light oil stream residue, pasting oil, and pitch product were all carcinogenic for mice. Pitch product, the residue boiling higher than 450 C, was tested 10% in benzol. Two samples resulted in tumor indices of 54 or 77 and cancer indices of 12 or 69. The numerical results are semiquantitative as are those of any biological assay. The potency of the 10% residue, when expressed as percentage of the 0.2% methyl cholanthrene control, was 42% for tumors and 43% for cancers.

Table 4 is a presentation of the fact that for the other three carcinogenic materials, the tumor indices and potencies increased and induction periods decreased as their boiling points rose.

TABLE 4. RELATIONSHIP OF CARCINOGENICITY AND BOILING POINT RANGE OF ALL STREAMS EXCEPT LIGHT OIL

		Mean Values of			
				M.L.P. of M.C. / M.L.P. of Material X 100	
Material	Boiling Point Range, C	Tumor Index	Cancer Index	Tumor	Cancer
Middle Oil Stream	260 to 320	46	15	35	31
Light Oil Stream Residue	260 to 380	66	38	46	45
Pasting Oil	320 to 450	94	75	80	76

Pasting oil, it has been seen, was as highly carcinogenic as any other fraction. As this recycled material was as likely to be contacted by the workers as any of the other streams or products, detailed studies were performed to find the effect on carcinogenicity of diluting the oil, of preventing its contact with the skin, or of removing it from the skin after contact.

Table 5 indicates the effect of diluting pasting oil with benzene. The tumor and cancer indices were similar with all but the 0.4% concentration of the oil. Even at this dilution 40% of the mice had papillomas and 15% had carcinomas. The columns to note, however, are the median latent periods. These increased as the concentration of pasting oil decreased. It took 114 days for the average tumor-bearing mouse to develop a papilloma with the undiluted pasting oil as compared to a year, or more than two years, for those painted with the 2% or the 0.4% dilutions of the oil.

TABLE 5. EFFECT OF DILUTION OF PASTING OIL ON ITS TUMOR PRODUCTION

Concentration of Pasting Oil in Benzol	Maximum No. of Months Painted	Tumor Index	Cancer Index	Mean Latent Period, Days Tumor	Mean Latent Period, Days Cancer
100.0%	6	100.0	89.5	114	150
50.0%	11	85.4	50.0	147	231
10.0%	12	100.0	95.2	180	234
2.0%	22	88.9	51.8	384	474
0.4%	21	40.0	15.0	>630	>630
0.2% Methyl Cholanthrene	6	100.0	96.6	114	138

The question arose of the relative importance of the concentration or of the total dosage the mice received. To attempt an answer to this, the concentration was used that was slow in tumor production but still resulted in a high percentage of mice with tumors, namely, 2% pasting oil in benzene. Table 6 lists the relationship between painting the mice with one or five brush strokes per day with 2% pasting oil versus one brush stroke of 10% pasting oil. Theformer produced fewer tumors and the median latent periods were delayed with this one brush stroke, 2% concentration. Five brush strokes of 2% pasting oil, however, increased tumor production and resulted in a shorter latent period. This was still not as short as that for one brush stroke of 10% pasting oil.

TABLE 6. EFFECT OF DILUTION AND TOTAL DOSE OF PASTING OIL ON ITS TUMOR PRODUCTION

Concentration of Pasting Oil in Benzol	No of Brush-strokes per Painting	Maximum No. of Months Painted	Appearance of First Tumor: Months of Painting	Appearance of First Tumor: No. of Mice Alive	Tumor Index	Cancer Index	Median Latent Periods, Days: Tumor	Median Latent Periods, Days: Cancer
2.0	1	22	7	28	41.7	25.0	>660	>660
2.0	5	19	6	23	100.0	81.2	259	376
10.0	1	17	4	27	91.7	79.2	198	252
100.0	1	11	3	26	100.0	63.6	144	240

As diluting the pasting oil was ineffective in preventing tumors from it, even though this did lengthen the induction periods, attempts were made to prevent skin contact by barrier creams or to remove the oil by the use of waterless soaps or soap and water. These are summarized in Table 7. An extraneous infection invaded these mice concurrent with their treatments. Thus the results on waterless soap 1 were inconclusive. For most of the other groups, however, the infection came late enough in the study to allow comparisons. In each case the final tumor indices were practically 100% and in the majority the cancer indices were high. The barrier creams, applied one hour before the pasting oil and in two groups followed in four hours by a soap and water wash, did not appreciably delay the time of tumor production as measured by the median latent period. In each case when soap and water were used a slight additional delay appeared.

TABLE 7. EFFECTS OF CERTAIN BARRIER CREAMS AND SOAPS ON TUMORS PRODUCED BY PASTING OIL

Hour of Application of				Maximum			Median Latent	
Barrier Cream	Pasting Oil	Waterless Soap	Soap and Water (S.W.)	No. of Months Painted	Tumor Index	Cancer Index	Period, Days Tumor	Cancer
1-0 hr.	1 hr. later			10.0	88.5	46.2	132	234
1-0 hr.	1 hr. later		4 hr. later	11.0	100.0	61.5	186	258
2-0 hr.	1 hr. later			5.5	85.7	35.7	99	(180)
2-0 hr.	1 hr. later		4 hr. later	11.0	100.0	77.7	132	210
	0 hr.			9.0	77.8	55.6	123	180
	0 hr.	1-4 hr. later		6.0	34.6	7.7	(246)	---
	0 hr.	1 and S.W. both	4 hr. later	6.0	9.1	0.0	---	---
	0 hr.	2-4 hr. later		11.0	94.4	88.9	174	210
	0 hr.	2 and S.W. both	4 hr. later	12.0	93.8	87.5	270	279
	0 hr.		4 hr. later	11.0	100.0	100.0	222	240
Methyl cholanthrene 0.2% in benzol				6.0	100.0	96.7	111	159

The mice that received waterless soap 2 had a slightly longer median latent period than those without this treatment. When this was followed by a soap and water wash the induction period was further lengthened. These latter times were similar to those for soap and water alone, which appeared to be as effective a treatment as any other. It must be re-emphasized, however, that none of these barrier creams or wash treatments did more than slightly delay the appearance of tumors.

Men were unavoidably exposed during the operation and maintenance of the unit. In 1955, 3 years after the startup, the first suspected skin cancer was observed on the buttock of a 36-year-old man. In Henry's (1947) statistics on 1,335 pitch and tar workers, the shortest time for a papilloma to develop was eight months, and for an epithelioma, 10 months. The longest interval of time from the onset of work to the manifestation of an epithelioma was 73 years in his report. The maximum number of cases in his series appeared after 20 to 24 years of exposure.

The shortest exposure in the coal hydrogenation series of cases in which cancer of the skin developed (pathologically confirmed) was after nine months. The clinical effects are summarized by Sexton (1960b). During the period of operation of the coal hydrogenation units, from May 1952 to July of 1956, a total of 359 men were assigned regularly to work in this plant area. Their median age was 36 years. Fifty-one men, in quarterly or semi-annual inspections, presented 63 recorded skin abnormalities. Forty-three of these cases were diagnosed as precursors of skin cancer; 10 men were diagnosed as having cutaneous cancer. Their ages varied from 29 to 46 years with a median of 38 years of age. The incidence was compared on the crude basis, as well as the age-adjusted bases for the West Virginia population and for the population of the United States.

It was reported that the normal skin cancer incidence is 30 to 40 per 100,000 per year. The incidence in the coal hydrogenation unit was at least 20 times higher than expected.

The unit was shut down in July 1956. The incidence we have discussed was until the end of December 1958. The men have been followed since then - at first there were 1 to 2 new cases of skin cancer (small, malignant) every year. Now the incidence is about 1 every 5 years. There has been no evidence of increases in systemic (including lung) cancers. UCC was going to do an epidemiological study when NIOSH announced they planned this. They visited the area over a year ago, discussed their plan but haven't followed up with an epidemiological study as yet (10-3-77).

Therefore, this study was considered important, not in that it reported a new industrial carcinogen, but rather in that it is one of the few reported instances where cancer was suspected before a unit started; where the products as soon as available were tested experimentally for their carcinogenic action and this suspicion confirmed; where the workers in the unit were thoroughly warned of these results, urged to avoid contact, provided with information about the procedure to use to remove contamination from the skin and provided with changes of clothing; and where, even with all of these precautions, careful medical examination of these men at repeated intervals did uncover an unusually high number of cases of skin cancer. Thus, this study helped confirm the value of animal experimentation in predicting areas of caution for industrial and other human exposure. It also indicates that extreme care must be practiced in the handling of a similar process and that exposure must be held to a minimum.

Cancer is dosage-related. Time to tumor is also dosage-related. Therefore, if contact is minimized, risk will be minimized.

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PAPER NO. 4

OCCUPATIONAL HEALTH COSTS VS. BENEFITS IN COAL CONVERSION

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This is not the usual sort of cost vs. benefits analysis. Macroeconomic and microeconomic concerns will not be generated. I will not delve into the variables that make up complicated algorithms of total cost "guesstimates" such as medical treatment and supply costs, insurance premiums paid by both employers and employees, lost time-lost productivity-lost profits projections, and litigation costs where settlements are sought. You are not about to hear of discounted dollar factors and year 2000 costs in terms of 1977 dollars. Furthermore, as a health scientist, I cannot accept the economists' penchant for assigning dollar values to human life and suffering for the purpose of creating benefit tiers derived from various cost alternatives.

I am pleased, however, to discuss the relationship between profitable operations and adequate health protection. I will also address relative cost factors for occupational safety and health investments made before plants are constructed compared to future costs after the industry is built. I hope to impress upon you certain guidelines that can be used in industrial and research decision making now, while the somewhat embryonic coal conversion (coal gasification and coal liquefaction) industry is developing with a prognosis for hearty growth.

First, let us explore some recent examples of the costs that have resulted from the need to retrofit industrial facilities as a result of evidence of occupational diseases. In the case of vinyl chloride, we learned that the most cost-effective means of production is not necessarily the most hazardous. In 1974, industry contractors projected costs as high as $65 billion for compliance with the proposed Federal

standard of 1 part vinyl chloride per million parts of air. However, capital costs of one large company for compliance with the standard approximated $34 million, while this company recovered costs by leasing its clean-up technology (Epstein, 1976). A second company documented economic advantages of compliance, due to the recycling of recovered vinyl chloride (Epstein, 1976). Thus, the industry first saw crippling investments in the $billions, and spent $thousands fighting increased regulation. In the end, the industry may reap $millions through more efficient production and sales of control technology. This is the definitive "best-case" situation.

A second example is the styrene-butadiene rubber industry. In the 1940's with 90 percent of the natural rubber supply cutoff, the Federal government financed the building of 15 styrene-butadiene rubber plants (Morton, 1973). Three decades later, we are finding that styrene-butadiene rubber employees have a six-fold risk, as compared with other rubber workers, of dying of cancer of the lymphatic and hemopoietic systems (McMichael et al., 1976). New occupational safety and health regulations probably will be developed accordingly. Research and development of control technology, and retrofit of existing styrene-butadiene rubber plants will be expensive, but they are essential for worker health protection.

To bring the matter of retrofit costs per occupational health needs directly into coal conversion technology development, consider the experience of the Synthetic Fuels Pilot Plant at Cresap, West Virginia. This Office of Coal Research coal liquefaction facility was operated in the 1960's. It was plagued by fires and leaks, and as a result surfaces were contaminated with carcinogenic coal tars (Evans, 1977). Since 1974, the plant has been completely renovated at a cost of $13.5 million (U.S. ERDA, 1976). Much of this cost reflects the installation of new pumps and piping to reduce leakage, and the installation of equipment to meet environmental protection standards. In this case, better process engineering was also a major factor in promoting better occupational safety and health.

What are the safety and health hazards in coal conversion plants, and what measures can be taken to protect workers in these facilities? Injury-producing hazards are seen primarily as the potential for explosions or fires, and leaking hot gases or liquids. Workers may be exposed to toxic agents

by inhalation of gases or airborne particulates, by absorption through the skin following deposition of airborne material or by contact with contaminated surfaces, and by accidental ingestion. In maintenance operations, liquid and solid residues may be encountered which would not constitute normal operational hazards (NIOSH, 1977).

The range of toxicants and the resulting possible health effects is extremely wide, from simple chemicals like carbon monoxide to complex mixtures of organic carcinogens. This situation is further complicated by the special problems associated with carcinogens: long latent periods before effects are observed, doubt about "safe" levels, and uncertain multiagent interactions (NIOSH, 1977).

Full protection for workers cannot be assured by protective measures, monitoring procedures, and/or medical tests that are simply the sum total of controls for each individual toxicant. The complexity of the potential hazards calls for innovative control strategies (NIOSH, 1977).

A comprehensive occupational safety and health program consists of safe work practices, engineering controls, protective equipment and clothing, workplace monitoring, medical examinations and record keeping, a health education program, personal hygiene, and enforcement of regulated areas.

Figure 1 is a sample job safety analysis form, which represents a safe work practice that should be required for all routine operations. Such a form is a tool that allows the safety supervisor to assess the hazards and necessary precautions associated with each specific job in the coal conversion facility. It is also a simple method to inform workers and the responsible shift supervisor of the safety equipment and rules that apply to specific job steps. Maintenance tasks should also include safe work permits, signed in advance by both the shift supervisor and safety officer (NIOSH, 1977).

<table>
<tr><td colspan="2">JOB HAZARD
BREAKDOWN</td><td colspan="2">JOB DESCRIPTION</td><td>JHB NUMBER</td></tr>
<tr><td colspan="2"></td><td colspan="2">COMPONENT</td><td>BUILDING</td></tr>
<tr><td colspan="2">REVIEWED BY
INDUSTRIAL SAFETY</td><td colspan="2">PREPARED BY</td><td>DATE</td></tr>
<tr><td>DATE</td><td>INITIALS</td><td colspan="3">REVIEW DATES</td></tr>
<tr><td colspan="2">SAFETY EQUIPMENT REQUIRED</td><td>TOOLS & EQUIPMENT REQUIRED</td><td colspan="2">JOB PREPARATION</td></tr>
<tr><td colspan="2"></td><td>HAZARDOUS MATERIALS</td><td colspan="2">RELATED REQUIREMENTS
RADIATION WORK PROCEDURE YES ☐ NO ☐
NUCLEAR SAFETY SPEC. YES ☐ NO ☐</td></tr>
<tr><td colspan="2">JOB STEP</td><td>HAZARD</td><td colspan="2">SAFETY RULES AND SAFE PRACTICES</td></tr>
<tr><td colspan="2"></td><td></td><td colspan="2"></td></tr>
</table>

Figure 1. Job safety analysis sample form.

Figure 2 shows a sample pump and shut off valve arrangement that constitutes a simple but highly effective engineering control. Shutoff valves that are strategically located in the piping design of a plant are a guard against accidental exposures downstream of maintenance operations or "nonroutine operational events" such as purge gas pressurization during startup or shutdown (NIOSH, 1977). It is no more difficult or costly to design a plant initially in this fashion than it is to design a plant without these built-in hazard controls.

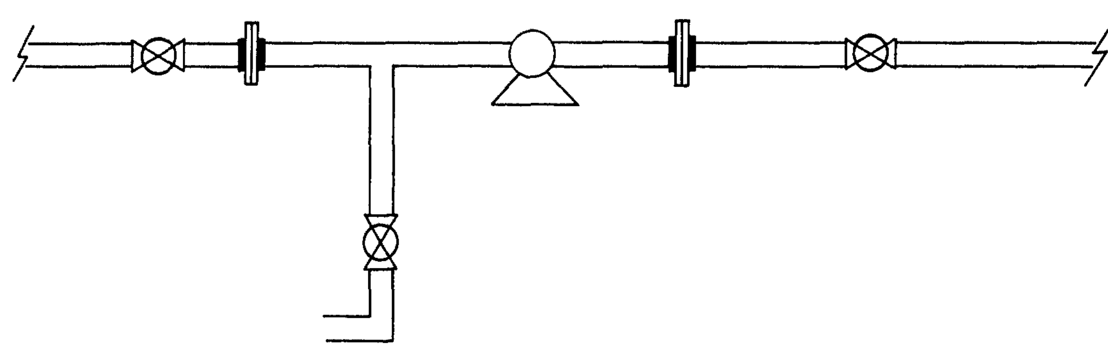

Figure 2. Pump and shutoff valve. Source: NIOSH Recommended Health and Safety Guidelines for Coal Gasification Pilot Plants.

Figure 3 depicts a sample layout for clean and dirty locker rooms that can assure good personal hygiene among workers in a coal gasification facility. The objectives are to prevent plant soiled work clothing or gear from contaminating clean street or work clothing, and to assure that these items are not taken from the plant facility. The latter is especially important for keeping tar-contaminated work clothing out of the family laundry. Again, it is neither more difficult nor more costly to design available locker room space with health protection in mind than it is to design these facilities without concern for reducing hazardous exposures.

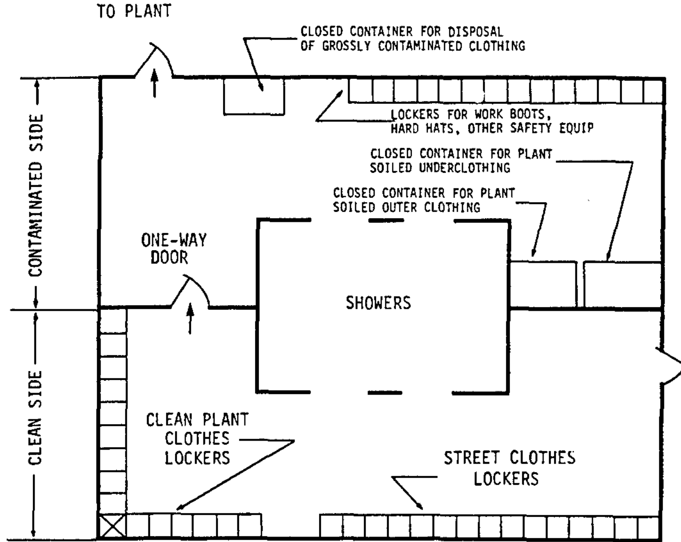

Figure 3. Sample layout for clean and dirty locker rooms. Source: NIOSH Recommended Health and Safety Guidelines for Coal Gasification Pilot Plants.

These three examples emphasize careful forethought in developing standard operating procedures and designed-in engineering controls and personal hygiene facilities. They demonstrate the need to accomplish occupational safety and health objectives prior to constructing coal conversion facilities.

Research and development for control technology must be accomplished now, simultaneously with process development for the conversion of coal to liquid and gaseous fuels. Dollars spent to build margins of safety into these facilities will always be few relative to the dollars required to retrofit later. The building of safe plants today will also help to assure the future integrity of coal conversion plants, and thereby help to protect the investments made in this industry. Additionally, the investment now for built-in worker protection will likely pay dividends in increased production efficiency. Thus, for the coal conversion industry, occupational health costs on the "front end" actually are benefits in terms of dollars saved from process efficiency, minimized retrofit, and reduced human illness and injury.

To conclude, let me emphasize that we have a tremendous opportunity from a public health perspective with respect to the development of the coal conversion industry in the United States. For the most part, there should be no surprises. We have a good idea of what the hazards associated with this technology are likely to be, and we can readily determine the research and development needs for further hazard identification and control. Investments can be made now to design hazard control technology into these industrial facilities. Shareholders should not reap annual dividends, and consumers should not receive lower cost products at the expense of the workers whose toil produces the profits and products. If occupational health and safety considerations are properly addressed in the initial development of coal conversion technology, then these plants will not, hopefully, contribute to serious health problems 20 to 30 years from now for today's workers.

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OPEN FORUM

DR. STERNER (University of California, Irvine): I would like to ask Mr. Levine if we are likely to end up with a number of different successful operations or different methods of coal conversion or do you think it will funnel down so that one or two methods will survive? Are the various processes competitive enough so that perhaps a number of systems will be evolved?

MR. LEVINE (Exxon Research and Engineering Company): I think there will be several commercial processes for liquefaction and for gasification. I don't think there will be a proliferation of processes. I think the chemistries of the various processes will be very similar. If you look at the fundamental physics and chemistry of the processes, they just can't be very different. There will be differences in the way that you handle the materials but basically the chemistries will be the same. As of right now, there is just the thermal commercial process for gasification. I think that it will not be done at extremely high temperatures. They will have to be done somewhere under 2000 degrees. I think that from the toxicologist's viewpoint you will have a workable situation to deal with. There probably won't be a wide range of chemical processes with a large number of operations for either liquefaction or gasification. There is one caveat I would like to make about the area of pyrolysis. I didn't speak in great detail about pyrolysis because if you want to make liquid or gaseous fuels, you are going to use a liquefaction or gasification process. Pyrolysis can be an attractive process in combination with other approaches and specifically with respect to gasification. It's very attractive to get some of the liquids off of the feed before you gasify. I think there could be much more variability in the chemical species formed with pyrolysis.

DR. CULVER (University of California, Irvine): My question is for Mr. Bolton. Has any positive result come out of your efforts to use sputum cytology as a method for monitoring?

MR. BOLTON (Oak Ridge National Laboratory): Let me answer that indirectly. When we went into this program, we established some very rigid specifications for the control group. We matched them, almost perfectly, from the standpoint of hourly payrolls, from the standpoint of education, and other factors with the coal processing workers. The one positive finding that came out of that program was a diagnosis of one in situ carcinoma in the control group which has been treated

and the prognosis for this employee looks pretty good. It was a case of early detection of a tumor that was not apparent on x-rays. It was found in the sputum cytology tests. We have not, at this point, found a sufficient number of metaplasias to make any kind of statement about it. If you look at the sputum cytology studies conducted at the uranium mines in the west, the statistics are extremely impressive. There's a correlation between smoking and radon exposure, for example, compared with nonsmoking. It's just undeniable that this test is extremely important. It's not a test that anyone submits to eagerly. It's like most medical examinations. It has some discomfort and we get a certain amount of resistance to being tested. We have, therefore, made it a mandatory job requirement.

DR. STERNER: Let me just suggest on that particular point, once you get a positive test or worse yet, a questionable test, then you have to have the individual undergo bronchoscopy. You have to get down into the lung and find out what is involved. Then you really begin to get into difficulty. Bronchoscopy is an invasive type of clinical procedure which can cause not only the individual a great deal of difficulty but the management of the medical surveillance program a considerable amount of difficulty.

MR. BOLTON: The other problem is that the program has to be designed in a fail safe mode. If we find a person with marked atypia, that person is going to be removed from further exposure. We will follow his condition but we are not going to further expose him to the chemical process.

MR. WANDS (National Academy of Sciences): I'd like to address a couple of questions to Mr. Levine. Will there be changes of processes depending upon the kind of coal that is used? For example, are the vast beds of brown coal susceptible to these kinds of conversions? Do they basically differ in either their products or their risks? And the second question I would like to ask concerns the waste products of the various processes. What kind of problem do they present occupationally and environmentally?

MR. LEVINE: On the question related to the coal feed, coal is high variable. It is an extremely important consideration in the process development. There are cases, which we read about recently in the newspaper, of processes being developed on one coal which had subsequent serious problems when applied to another type of coal. I can't answer the question in detail because I don't think there is one simple answer. I'll try to select some examples. There are pronounced differences in eastern coals and western coals in the U.S. but I believe that

you will be able to handle all these coals eventually with the kinds of processes that I outlined in my presentation for liquefaction. Among gasification procedures, the thermal processes seem to be very sensitive to coal feed. These are other companies' processes so I won't make a comment on more of the specific differences. The work that we've done on a catalytic process suggests that it tends to make coals look more similar. We feel that one of the contributions we could make to this industry would be to find a more pervasive type of process technology for gasification. The catalyst approach does directionally do that. You mentioned the brown coals, and although I can't address myself to brown coal specifically, I don't know of any coal that can't be converted. I think the judgement on using various coals is basically economics. There will be some coals that won't be attractive economically. The second question asked was about the waste products. A very simple way of looking at that problem was shown in the next to the last slide I used. When you think of our energy, the system is a big box and you are pumping coal, gas and liquids into it. At some point down the road, you might see past some of those gases and crude oil. Part of them could be replaced by additional coal input. And then inside of that box, you might see another box which is the coal. Some of it goes to direct energy utilization which is what we're doing now, and some of it goes to conversion which is not being done on a very large scale right now. As a result of that second box, there would be some waste products introduced into the system. One would be ash disposal which is the same ash disposal resulting in direct energy utilization from coal. The question raised was what is the difference between that ash and the ash you would get from a conversion process? Some of the gasification processes convert all the carbon. There are no major differences in the ash that we know about. The inorganics are still there. There are possible subtle differences which I don't think we are aware of yet. What the impact of this will be, I'm not really prepared to answer. I certainly am not going to tell you there are no problems, but I think we should be persuaded by the fact that it is not a new operation and these are not new materials. There is validity in the fact that they should be safe for a land fill type of operation. The other changes are you have higher sulfur content in the ash with the conversion processes, and with the sulfurization process, there is a net gain during conversion. What happens to nitrogen is a question mark. In some product applications, you want to remove nitrogen because it is harmful to either the hardware that you are going to use or it would not meet an emission standard. There is a technology to remove the nitrogen oxides from effluent wastes from the processes used. Again, the solution is a question of dollars.

MR. WANDS: I guess one of the things that I was concerned about in the waste products is that many of our coals, particularly those from the midwest region, are relatively high in trace metals which are somewhat hazardous. These may be concentrated in the ash and thus might be a source of environmental pollution of a different magnitude entirely. Is this a potential problem we should be concerned with? And secondly, are these then going to be utilizable resources of raw materials for further processing for recovery of special components such as the trace metals?

MR. LEVINE: The concentration of the heavy metals in the ash has been defined in terms of the loading. I don't have that data with me.

DR. NEWILL (Exxon Corporation): I think that whether there is a problem depends on the kind of disposal system that you're thinking of using. At the present, with the relatively small amounts of waste produced, it's probably going to be a land fill disposal system in places where the waste is protected from leeching into ground water supply. During the pilot plant phase, that's the way we will handle waste disposal. In the later phases, I think this problem will be given a great deal of consideration and we will solve how we are going to dispose of the waste before going into commercial production. We're concerned about concentrating inorganics also, and concerned enough that we're going to be sure that they don't get into the general environment.

MR. LEVINE: In response to the second part of the question, I don't believe that the concentrations of the heavy metals are great enough right now for serious consideration for recovery.

MR. BOLTON: The recovery of metals from ash has been looked at. While on the surface it looks like it might be economical, it isn't. The problem is in the form that the materials are present. It is virtually impossible to refine them out in any economical fashion.

MR. WANDS: This is not a new or unique problem perhaps, but at least there is a question for further study in the near future.

MR. BOLTON: I don't think that the ash from coal conversion processes is as available as the fly ash from coal power plants.

MR. HASS (Argonne National Laboratory): I'd like to direct several questions to Mr. Weil. One, were your mice exposed to light in any way? Two, since benzene exposure has been linked with leukemia, did you look for that particular endpoint? And thirdly, have there been any other studies that corroborated any of your results?

MR. WEIL (Carnegie-Mellon Institute): The natural light was northern light into the laboratory. This was back in the 1950's so we did not have fluorescent lighting. I covered benzene during the presentation. We only used that for the three very, very viscous samples. Benzene was really not involved in the response observed. It is a vehicle that was used in the mouse skin painting studies. We try to avoid its use and select something else if we can. Thirteen of these sixteen materials were tested undiluted with no benzene involved. These skin cancers found were caused by the polycyclic hydrocarbon in the coal products from coal hydrogenation. If there have been any repeat studies, I am not aware of them. Our experience has been that any organic material heated high enough will produce carcinogenic moieties.

DR. DREW (Brookhaven National Laboratories): Mr. Weil, were the three viscous mixtures that proved to be carcinogenic analyzed to identify common components?

MR. WEIL: No, we didn't conduct a complete analysis of the mixtures. The coal hydrogenation process was undergoing continual change. In fact, the reason they didn't succeed probably is that they may have moved from the laboratory scale to a large pilot plant scale too rapidly. The process kept breaking down and many of the workmen were exposed to the products of the process.

DR. MAC FARLAND (Gulf Science and Technology Company): One of the major problems that confront the toxicologist is that when he's asked to study the carcinogenicity of the coal conversion process, the operation may not be operational. He must wait for a stabilized commercial process to be developed to collect a meaningful sample of the mixture or mixtures to which workers or neighbors will be exposed before beginning the animal experiments. The second major problem lies in the reactivity of the products collected from the coal conversion process streams. Some of the components of the mixtures are phenolic compounds, and these are highly susceptible to oxidative reactions. It's very disconcerting for a toxicologist to undertake a skin painting study and notice that the solution being tested has slowly changed from a pale yellow color to a deep red. The question becomes one of how much impact will that chemical change, shown by the color change, affect the toxicological significance, particularly when a dose response is being determined.

DR. STEMMER (University of Cincinnati): We are very concerned about the carcinogenicity of these products. We are also interested in occupational exposures and air pollution hazards to the general population. Are there any studies being conducted that are addressed to these problems?

MR. BOLTON: I don't know if there are any ongoing studies addressing the problem. There have been a number of proposals submitted to attempt to assess the hazards involved in the entire process. The proposals were to study the complete process from removing the coal from the ground to delivery of the finished product to the consumer. I don't believe these proposals have been funded to date.

DR. STEMMER: If these products come to the market in the timetable suggested by Mr. Levine, in the late 1980's or early 1990's, will we begin then to look at their impact on the populus?

MR. BOLTON: I sure hope we don't wait that long. We should be doing that right now. There are, however, several different problems to be examined in respect to air pollution effects. Coal conversion processes for chemical manufacture and coal for low BTU gasifiers are entirely different problems. The state of the art for low BTU gasification is only in the early stages. There are perhaps 80 manufacturers for low BTU gasifiers (70-100 ton per day maximum capacity). Since these are state of the art and since changes in fuel sources in one type of gasifier can result in variations in waste products, it is impractical to conduct comprehensive toxicology studies. The costs would be astronomical to test all the variations possible.

MR. WANDS: Mr. Weil, you raised a point in your presentation about the dose relationship and the time factor involved. How long ago was it that the workers were exposed at the pilot plant you studied?

MR. WEIL: The last exposure was in 1956.

MR. WANDS: That's over 21 years ago. Has there been any increase in lung cancer among these workers since the end of their exposure?

MR. WEIL: There has been no increased incidence of lung tumors in this group. Perhaps I should say something about the epidemiology studies proposed to be conducted on these workers, about 350 in number. Although surveillance on the workers has continued, the data have not been evaluated statistically because NIOSH informed the plant medical director that they wanted to perform the study approximately 1 year ago. As of yesterday, there has been no further word from NIOSH so the data are untreated and the epidemiology study hasn't been conducted.

SESSION II

ASSESSMENT OF TOXIC EFFECTS OF COMBUSTION AND PYROLYSIS PRODUCTS

Chairman

CDR. Lawrence J. Jenkins, Jr., MSC, USN
Officer-in-Charge
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PAPER NO. 5

GUIDELINES FOR EVALUATION OF TOXICITY OF PYROLYSIS AND COMBUSTION PRODUCTS*

J. A. Winstead, Ph.D.

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INTRODUCTION

It is estimated that fire causes 12,000 deaths, 300,000 injuries, and $11.4 billion in property damage each year in the United States (National Commission on Fire Prevention and Control, 1973). These figures show the United States to be the industrialized world's leader in fire deaths per capita. The rate for second-ranking Canada is only half that high. Most fire victims die by inhaling smoke or toxic gases well before the flames have reached them.

One approach to decreasing fire deaths and property damage has been to reduce the flammability of materials used in building construction and transportation systems as well as in their furnishings. In fact, flammability standards have been established for numerous items such as carpets and mattresses. Flame retardants are often used to meet these standards. The number of flame retardants on the market has increased dramatically during the past decade.

This approach to reducing flammability does not address the problem of smoke and toxic gases. Flame retardants can undergo pyrolysis when exposed to heat and may produce more toxic products than under flaming conditions (Petajan et al., 1975). Untreated wool produces primarily carbon dioxide, water, carbon monoxide, and oxides of nitrogen under flaming conditions; however, under pyrolysis conditions, hydrogen cyanide and organic cyanides are produced. The deaths of many aircraft crash victims have been attributed to poisoning by combinations of carbon monoxide and hydrogen cyanide (Mohler, 1975).

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*This paper was adapted from a report (National Academy of Sciences, 1977) prepared by the Committee on Fire Toxicology, which was supported by the Consumer Product Safety Commission and the National Aeronautics and Space Administration under Office of Naval Research Contract N00014-75-C-0718.

The state-of-the-art in fire toxicology is considerably less developed when compared to other areas of toxicology. Relatively standard approaches are used to evaluate the toxicity of food additives, drugs, cosmetics, and pesticides, but none have been developed to evaluate the toxicity of combustion and pyrolysis products. The rapid growth of the synthetic polymer industry has significantly increased both the quantity and variety of materials affected by fire. Investigations of the toxicities of combustion and pyrolysis products, begun in the fifties, have resulted in many publications dealing with the types of gases produced from the different polymers. No standard approaches have yet been developed to assist engineers in the selection of materials by using the toxicity of combustion/pyrolysis products as a criterion.

Recognizing the deficiencies in the area of fire toxicology, the Advisory Center on Toxicology has assumed a leadership role by establishing the Committee on Fire Toxicology. With its support from the National Aeronautics and Space Administration and the Consumer Product Safety Commission, the committee is studying this multidisciplinary problem.

Based on its literature review, presentations by selected researchers to the committee, and the experience of its members, the committee concluded that there are no acceptable screening tests to evaluate relative toxicities of pyrolysis and combustion products of polymeric materials. All present methods have one or more shortcomings. It further concluded that the state-of-knowledge in fire toxicology precludes the establishment of a standard protocol for screening materials. However, the committee has developed some guidelines that would limit the variables so that the data obtained could serve as a basis for devising screening systems and results could be compared among laboratories.

GUIDELINES

To be practical, a screening method must be economical and relatively easy to conduct. This probably eliminates tests simulating a large-scale "real fire" situation; however, it is important that the toxic pyrolysis/combustion products produced in a screening system be representative of those occurring in a "real fire". In addition, the tests must be reproducible, sensitive and valid. Since the screening system is designed to identify highly toxic materials and to rank materials, the primary toxic agent might not be identified nor would the mechanism of toxicity be determined. The results from screening systems for determining the toxicity of pyrolysis/combustion products are

comparable to "range-finding" studies used to determine the toxicity of chemicals. In many cases, a screening system will not provide all the information required about the material; however, the results should certainly provide a sound basis for planning more definitive experiments.

PYROLYSIS/COMBUSTION CONDITIONS

Since pyrolysis and combustion occur under an almost infinite number of actual conditions, the laboratory situation should bracket likely conditions and should attempt to simulate the worst case that could occur in "real" fires. Fire reaction processes generally involve oxidation and/or decomposition, so that the product molecules are breakdown or oxidation products of the original material. The two major variables are heat transfer to the sample--particularly by radiation, which controls surface temperature--and the availability of oxygen. The two extremes are free burning of the sample with ready access of air and pyrolysis under reducing conditions; therefore, specimens should be tested under both pyrolysis modes and flaming combustion.

For the limited goal of a screening test, heat fluxes (temperatures) should be chosen at three levels - one just above that necessary to sustain pyrolysis, one just below that required for flaming combustion with normal oxygen concentration, and one that supports flaming combustion when supplied with a source of ignition. If a furnace is used, the static temperatures prior to introducing the specimen should be recorded. If a radiant heat source is used, the flux should be calibrated.

The heating mode is not specified in the committee's recommendations since the chemical decomposition is not governed explicitly by the mode of heat transfer. Therefore, the apparatus for burning or pyrolyzing the sample need not operate in a purely radiant or conductive mode; rather, it should be reproducible and relatively simple in construction, characterization and operation. For this reason, the committee prefers a shallow furnace with a weighable sample cup over elaborate radiant heating devices. The furnace should provide a uniform temperature in the sample region and be designed so that undue condensation and repyrolysis of the sample within the test chamber are avoided. Temperature, radiation flux, or some other characteristic parameter should be monitored to ensure constancy of conditions. The cup should be of resistant material such as quartz or aluminum oxide.

The sample should be prepared in a standard manner and its physical properties reported. Weight, volume, subdivision, porosity and geometry should be recorded. Sample size should be scaled so that it reaches the specified test end point within 30 minutes. The sample should be introduced rapidly into a preheated furnace or a preset radiant flux. The time of pyrolysis or combustion should be short compared with the animal exposure time. Pyrolysis or combustion time and the weight and character of any residue should be reported. The dose should be expressed as the amount decomposed in the chamber volume in units of mg/m^3.

ANIMAL EXPOSURE CONDITIONS

A single chamber for both pyrolysis/combustion and animal exposure is highly desirable. Both static and dynamic chambers are feasible; however, the committee's opinion is that the single static chamber with integral pyrolysis furnace is a simple approach that provides more realistic information. This not only approximates the real fire situation but prevents large losses of combustion particles and gases on the walls of any transfer apparatus. The sample holder and pyrolysis elements should be enclosed in the exposure chamber as close to the animal area as practical, consistent with providing thermal shielding from heat and hot gases. Adequate mixing of toxic gases and particulates, either by convection or mechanical agitation, is required. Other means of temperature control at the location of the exposed animals may include size of the chamber and the placement of the pyrolysis/combustion unit.

The chamber should be airtight to prevent toxic gases from leaking into the laboratory; however, there should be a safety pressure relief diaphragm vented to a laboratory hood. Construction materials should be inert and easy to clean between runs. Glass is an excellent choice except for fluoropolymers, which generate molecules that attack the glass surface. Care should be taken to avoid reaching explosive limits during pyrolysis. Safety precautions should be outlined. Compliance by operating and cleaning personnel should be enforced.

A small rodent species, such as the rat or mouse or a combination of both, should be used as the animal models. Enough animals must be used at each exposure condition to give statistically valid results.

The exposure time should range from 15 to 30 minutes, preferably 30 minutes. Measurement of exposure time should begin at the time pyrolysis or combustion is initiated. The temperature in the animal exposure chamber should not exceed 35 C; however, the best means for controlling temperature has not been determined.

In addition to temperature, carbon dioxide, carbon monoxide, and oxygen levels should be monitored in the chamber during exposure. The oxygen should be maintained about 16%. This is usually accomplished by limiting sample size. Other expected toxic degradation products such as hydrochloric acid or hydrogen cyanide should be monitored. The optical density should be recorded as a measure of smoke obscuration. Additional analyses for volatile gases may be desirable as an indication of reproducibility of pyrolysis/combustion and for deducing the mechanism of intoxication.

END POINTS

The end points for toxicity tests in animals should be applicable to the interpretation of effects in humans. Incapacitation is considered to be the most important end point as this is related to the ability to escape from the fire. Next in importance would be the latent impairment of organ function or damage to organ structure. Rats and mice are generally used because of practical considerations.

In the opinion of the committee, the simplest and least costly measurements should be those made frequently; the more difficult or costly ones should be reserved for understanding of mechanisms and resolution of special problems that arise. The committee specifically recommends that the chosen end points provide a measure of both incapacitation and delayed effects. As a minimum, the end points of a screening test should be as follows:

- Observation--Observations of animal behavior and physical condition during and after exposure are extremely important; thus, these should be done by a qualified investigator. Some type of quantitative test may be required to evaluate observations made by the investigator.

- Incapacitation--Incapacitation should be measured using a rotating wheel or an equivalent test. Measurements should be reproducible, sensitive, and valid. Packham et al. (1976) have reported that the activity wheel and a conditioned leg-flexion avoidance response are reproducible paradigms for carbon monoxide toxicity. Measurement of sensory irritation may provide a basis for assessing incapacitation. The rotating wheel or an equivalent test is a tentative recommendation until a more valid measure of incapacitation is determined.

- Mortality--Deaths during exposure and those occurring within 14 days postexposure should be recorded. Postexposure observations and mortality are a simple approach to investigating delayed effects.

- Carboxyhemoglobin determination--Carboxyhemoglobin levels should be measured at the end of the exposure to determine if the toxic response is due to carbon monoxide concentration or to other toxic components in the combustion products.

The second echelon should be used for more definitive and mechanistic studies, and includes: blood pH, partial pressures of oxygen and carbon dioxide, and blood cyanide; electrocardiogram; respiratory record by plethysmograph; organ functions; and gross and microscopic pathology. The animal data obtained by some or all of these methods should be interpreted as well as possible in terms of human ability to see or to hear the direction for escape, to judge a course of action, to move from or remain in the area, and to avoid serious sequelae that affect the heart, lungs, liver and kidney. This would pertain to normal healthy people as well as to those especially 'at risk' from preexisting conditions such as old age, coronary heart disease, or other disabilities.

Toxicity data should be relatable to human capability for escape and effective survival. This requires the comparison of different test materials with reference materials. The latter may be either a defined material in common use or a reference material, currently used for a particular purpose, that is being considered for replacement by the new material to be tested against it. In addition, the absolute toxicity can sometimes be judged in relation to the fire

conditions that might occur, the amount of material that would be used, and the amount of this material evolved or combusted under these circumstances.

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PAPER NO. 6

TOXICOLOGICAL ASPECTS OF A FIRE FATALITY STUDY

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SUMMARY

The work reported in this paper has been done under grants from the National Science Foundation and presently the Center for Fire Research/The National Bureau of Standards. The major collaborators involved in the toxicological aspects of the program were:

Yale H. Caplan, Ph.D.
Roy Altman, Ph.D.
Bernard C. Thompson, Ph.D.
Russel S. Fisher, M.D.
Byron Halpin, B.S.
David Shapiro.

Results of examining human fire fatalities in respect to their exposure to the toxic atmosphere are presented in this paper. The discussion includes the gaseous and particulate fractions of the smoke present in a fire.

Carbon monoxide is shown to be the primary lethal agent as a toxic gas, but hydrogen cyanide is also shown to be suspect as a toxic hazard. The interactions of the two gases are unknown and need to be explained.

Soot generated during the fire is the transport mechanism for such things as heavy metals. The results shown in this paper indicate that significant levels of several heavy metals are inspired during a fire. However, the role of the heavy metals is not understood at this time.

INTRODUCTION

Death and injuries due to fires have been accepted as a matter of fact in the United States for a long time. Statistics have been quoted annually as to the number of fire casualties inflicted each year. However, the numbers are

gross estimates, and although the estimate of the annual figure of 12,000 deaths per year (America Burning: The Report of the National Commission on Fire Prevention and Control) is about to be revised sharply downward to approximately 8000 deaths (Fristrom, 1977; Derry, 1977) the number will still be the best estimate available.

A major source of data upon which the above estimates have been based is the death certificate count supplied by the National Center for Health Statistics of HEW. However, very few of the official death causes found on these death certificates for fire victims result from a thorough autopsy. Thus, there are several vital questions that must be answered such as do these people die as a result of the fire and if so, what mechanisms are involved in such a fire death?

JHU/APL FIRE CASUALTY PROGRAM

The Fire Casualty Program of the Applied Physics Laboratory of The Johns Hopkins University (Berl et al., 1975) was designed to attempt to address some of the specific problems attendant with fire casualties. This program was started in September of 1971. The major thrust of this program was to attempt to gain an understanding of the problems associated with fire victims who have died in fires in the State of Maryland and have been exposed to the toxic atmosphere of the fire. With this concept one recognizes that the toxic atmosphere will be constituted in the gas and particulate fractions of the smoke. One must further realize that there will be physiological, biological, and toxicological reactions involved.

The JHU/APL program requires close cooperation and coordination of many organizations. Some of the significant organizations involved are the State Fire Marshal's Office, the State Medical Examiner's Office, and Baltimore City and other local fire departments. There are six major areas of participation in which the organizations function. They are:

1. Program Coordination - APL/JHU coordinates the study.

2. Postmortem Analyses - The Maryland State Medical Examiner provides detailed pathological and toxicological analyses of fire fatalities for Maryland. The autopsies are performed at the Baltimore facility of the State Medical Examiner. Toxicological studies include tests for carboxyhemoglobin and alcohol blood levels as well as possible drug involvement. As a result of a special blood cyanide study, a technique for obtaining accurate and meaningful blood cyanide levels is anticipated.

3. Field Investigations - The Maryland State Fire Marshal and his staff, as well as local county and Baltimore City fire investigators, provide standard in-depth data about the physical aspects of fires. They also notify an APL fire investigator who visits the fire scene to attempt to obtain samples of materials that were involved in the fire, soot samples, and the data available from other investigators.

4. Biochemical and Chemical Analyses - Special analyses are being provided to analyze the biological and other samples obtained at the fire scene and during autopsies. Special lung tissue and fluid outgassing, tracheobronchial trees, blood cyanide, heart and carbon monoxide interactions, fire material, and soot studies have been initiated to provide data that can be used in defining the fire fatality problem. These analyses are provided by the Medical Examiner's Office and APL/JHU.

5. Medical Analyses - The medical experts analyze the postmortem and biochemical data, when available, and provide meaningful interpretations of the results. At this time, a judgment is made about the cause of death. The Medical Examiner's Office is the prime organization here.

6. Data Analyses - The total set of data for each case is analyzed to try to tie together the physical, medical, and other data in a comprehensive form. It is now that one hopes meaningful conclusions about fire fatalities can be drawn that will aid one in understanding the total problem and suggesting practical solutions. This area is primarily the function of the JHU/APL staff.

GENERAL RESULTS

There were some general results obtained during autopsy that are pertinent to the toxicological aspects of the program. The pathology indicated that the victims exposed to the fire atmosphere had moderate to very heavy soot depositions in the respiratory system. There was severe edema and pulmonary congestion in the respiratory system as well.

In approximately half of the cases, the blood carboxyhemoglobin level of the victim was over 50% which is the level taken to be lethal. In another 30%, carbon monoxide plus some other agent such as preexisting heart disease and severe alcohol intoxication was considered to be the cause of death. However, several of these findings are still being examined such as the heart interactions in an attempt to verify the statements.

STUDY PROTOCOL

A protocol was developed for the Fire Fatality Program. A complete autopsy was performed at the Maryland State Medical Examiner's facility in Baltimore, Maryland. Samples of blood, urine, bile, liver, kidney, and spleen were collected for toxicologic analyses including alcohol, carbon monoxide, drugs and cyanide. These analyses were conducted at the Medical Examiner's Office. Samples of lung and trachea soot scrapings were collected and analyzed for gases and metals at the Applied Physics Laboratory.

CARBON MONOXIDE

Carbon monoxide is generated in all fires and must be considered as the primary toxicant. Table 1 indicates the distribution of the blood carboxyhemoglobin saturation levels in victims reported on in this paper. These cases are a subset of the overall case load. One can readily see that about 59 percent had a level above the 50 percent saturation point. These we believe were primarily victims of carbon monoxide poisoning. The remaining 41 percent must be considered as having died for some other reason such as carbon monoxide poisoning plus preexisting heart diseases or a possible combination of toxicants.

TABLE 1. DISTRIBUTION OF FIRE VICTIMS ACCORDING TO BLOOD CO.

COHb (%)	Number of Victims	%
0-9	38	13.1
10-19	20	6.9
20-29	25	8.6
30-39	16	5.5
40-49	21	7.2
50-59	28	9.7
60-69	35	12.1
70-79	65	22.4
$\geq$80	42	14.5
Total	290	100.0

ALCOHOL INVOLVEMENT

Alcohol has been the only drug-like material found to be a problem. In fact it is a major problem in fatal fires both from the standpoint of the fatal outcome as well as the ignition of the fire. Table 2 indicates that a positive level of blood alcohol was found in 130 of the victims. The data

also show that 88 percent of these victims had a blood alcohol level that would be considered to be an intoxicating level (> .1%).

TABLE 2. DISTRIBUTION OF FIRE VICTIMS ACCORDING TO BLOOD ALCOHOL

Alc (%)	Number of Victims	%
.01-.04	10	7.7
.05-.09	6	4.6
.1-.14	20	15.4
.15-.19	33	25.4
.2-.24	21	16.1
.25-.29	19	14.6
.3-.34	8	6.2
.35-.39	10	7.7
>.4	3	2.3
Total	130	100.0

The alcohol problem is further magnified when one considers the fact that approximately 1/2 of the fires were caused by "careless smoking" and that in 2/3 of these fires alcohol was involved. The heavy preponderance of the alcohol cases were males 30 years of age and above.

HYDROGEN CYANIDE

Hydrogen cyanide can be found in the products of combustion of nitrogen containing materials such as wool, nylon, polyurethane, etc. However, very little, if any, consideration has been given to determining whether cyanide was present in the fire victims prior to this study.

The available analytical techniques were examined (Altman, 1976), and the methodology suggested by Valentour et al. (1974) was modified and adapted to this study. This procedure measures cyanogen chloride produced from CN with an electron capture detector and a gas chromatograph. The blood cyanide levels of the fire victims of this study have been measured in this manner since mid 1975 (Caplan, 1977).

NORMAL VALUES

A set of normal values were obtained by measuring blood cyanide levels in 32 living subjects, 64 random postmortem nonfire deaths and 22 impact injury airplane crash victims. The results are shown in Table 3. The range of the readings for the 118 cases was 0.00-0.22 μg/ml. Therefore, values ranging from 0.00-0.25 μg/ml were considered normal for use in further studies.

TABLE 3. NORMAL BLOOD CYANIDE CONCENTRATIONS (μg/ml)

	Number	Mean	Range	S.D.
Random P.M.	64	0.05	0.00-0.22	0.04
Airplane Crash	22	0.06	0.04-0.19	0.03
Living	32	0.07	0.02-0.21	0.05

FIRE VICTIMS

The following material was taken directly from a report prepared by Dr. Y. Caplan (1977) for the APL/JHU project.

Cyanide concentrations were determined in 256 fatalities occurring during the period 1 January 1975 to 28 June 1977. A distribution of the results of these analyses is shown in Table 4. Normal concentrations (0.00-0.25 μg/ml) were found in 30% of cases. Subtoxic concentrations (0.26-1.00 μg/ml) were found in 35% of cases. These concentrations would not be expected to elicit any biological effect although concentrations exceeding 0.26 μg/ml are indicative of the presence of cyanide precursors in building materials. Possible toxic concentrations (1.01-2.00 μg/ml) were found in 26% of cases and probable toxic concentrations (2.01 μg/ml and above) were found in 9% of cases. These concentrations are indicative both of exposure and toxicity. Such concentrations may cause interaction with other toxic substances and possible incapacitation.

TABLE 4. DISTRIBUTION OF BLOOD CYANIDE CONCENTRATIONS IN 256 FIRE FATALITIES OCCURRING IN MARYLAND DURING THE PERIOD 1 JANUARY 1975 - 28 JUNE 1977

Cyanide Concentration (μg/ml)	Number	Percent
0.00-0.25	77	30
0.26-1.00	89	35
1.01-2.00	66	26
2.01 and above	24	9
	256	100

DISCUSSION

The detection of cyanide in concentrations exceeding 0.26 μg/ml in 70% of the fire fatalities is a clear indication of the widespread distribution of nitrogen containing polymeric materials in the average building or vehicle.

A correlation of blood cyanide concentrations with carbon monoxide saturation levels indicates that significant cyanide findings were associated with high carbon monoxide saturations. When cyanide was high, carbon monoxide was high; when cyanide was low, carbon monoxide was low. Cyanide did not apparently explain deaths not otherwise explained. However, cyanide's role as an intermediary agent causing incapacitation and subjecting a victim to a prolonged exposure to carbon monoxide has not been fully elucidated. In one automobile fatality studied, the vehicle contained plastic covered seats filled with urethane foam and padded dashboard. The blood cyanide concentration was 3.13 μg/ml and the blood was 18% saturated with carbon monoxide. Blood alcohol concentration was 0.22% and blood methadone was 0.03 mg/dl. Alcohol and methadone were contributory to the accident. Limited chest and bone injuries and the carbon monoxide were insufficient to cause death. The cyanide concentration was toxic and hence was the primary causative factor. It is anticipated that the study of future cases will reveal similar situations pointing more clearly to the toxic role of cyanide. An additional similar situation has recently been observed in a case in Virginia where a death resulted in a blood carbon monoxide saturation level of 6% and cyanide concentration of 3.1 μg/ml. The fire was of the smoldering type and no other factors were evident.

HEAVY METALS

Scrapings of the soot deposition in the tracheobronchial tree of the victims were collected for analysis for possible heavy metal depositions. The analyses were performed by Mr. D. Shapiro at APL/JHU.

The initial analyses were made on the total samples provided. Each sample had varying amounts of soot, mucosa, blood, etc. The samples were prepared for analysis with a Varian atomic absorption device. Concentrations of heavy metals were determined for 94 cases and the results are shown in Table 5. As can be seen by the data, seemingly significant levels of the metals were obtained even though the sample weight was strongly biased by the items other than soot. Note that Be and Bi were dropped from the analyses after 70 cases were run and Cr and Zn were substituted in the analyses.

TABLE 5. RANGE OF HEAVY METAL CONCENTRATIONS IN INTERNAL SOOT SAMPLES FROM FIRE VICTIMS (NONSEPARATED: 94 CASES)

Metal	Number of Cases Found	Range (μg/g of Sample)
Sb	13	0-1479
Cd	66	0-121
Pb	50	0-767
Mn	8	0-28
Cu	76	0-376
*Bi	0	0
*Be	0	0
**Cr	0	0
**Zn	20	42-444

*Bi & Be - 74 cases analyzed.
**Cr & Zn - 20 cases analyzed.

A technique was developed to separate the soot from the rest of the debris in the samples. Thus, a more accurate determination of the heavy metal deposition on the soot could be made. The results of 8 cases analyzed in this manner are shown in Table 6. One can readily see that the upper limit on several of the metals increased significantly.

TABLE 6. RANGE OF HEAVY METAL CONCENTRATIONS IN INTERNAL SOOT SAMPLES FROM FIRE VICTIMS (SEPARATED: 8 CASES)

Metal	Number of Cases Found	Range (μg/g of Sample)
Sb	1	0-779
Cd	8	0-474
Pb	6	0-3980
Mn	1	0-19
Cu	7	0-1214
Zn	8	0-8855
Cr	0	0

DISCUSSION

There is no doubt that heavy metals are being introduced into the bodies of people exposed to the toxic atmosphere of fires. The major question is what does this finding mean toxicologically? The belief is that there were no acute results apparent in the victims studied, but these were short-term deaths.

Nothing could be done during this study period to determine possible chronic effects of exposures by obtaining samples from survivors of the same fires. This question will hopefully be examined in future work.

Therefore, the results as presented must be considered by appropriate experts to determine what then happens as a result of the insult. The soots are taken into the respiratory and digestive tracts so consideration of possible damage in either mode must be evaluated.

CONCLUSIONS

The fire atmosphere constitutes a serious threat to human life from a toxicological view. The gases that are present are at sufficient levels to cause death. The particulate matter is the transport agent for other toxic and irritating substances.

Carbon monoxide is considered as the primary lethal agent in a fire.

The role of hydrogen cyanide is not clear, but the presence of the gas should be examined to determine what sublethal amounts within the human body will do. The interactions with other toxicants and the mechanisms involved need to be explained.

Heavy metals are being transported into the body but their role in either acute or chronic results is not understood.

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PAPER NO. 7

THE ROLE OF ASPHYXIA IN THE INVESTIGATION OF ANIMAL RESPONSES TO FIRE COMBUSTION PRODUCTS

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INTRODUCTION

Major effort in this laboratory has been directed toward the development and evaluation of approaches to the assessment of the toxicological effects resulting from exposure of rats to smoke produced from burning materials. Special emphasis has been placed on utilization of only those physiological and behavioral methods which are judged to be most meaningful in view of the objectives of a first-tier screening protocol. Previous work led to the choice of incapacitation (as determined by loss of the leg-flexion conditioned avoidance response) and blood chemistry (COHb, O_2HB, pH, Pa_{CO_2}, and Pa_{O_2}) as significant and relevant parameters in such a screening protocol (Hartzell et al., 1976). Since death is the classical observation in the field of toxicology, the mortality resulting from exposure of subjects was also selected, along with gross pathological examination of exposed animals upon either death resulting from the exposure or sacrifice after fourteen days.

The combustion furnace and accompanying test conditions chosen for this work were those reported by Potts and Lederer (1977). The furnace is basically an electrically heated, closed-end vertical tube which supplies conductive heat to the sample. Combustion modes employed with this furnace may be either flaming or nonflaming. The nonflaming mode is defined as the maximum temperature at which a material can be heated without autoignition occurring. The flaming mode is defined as the minimum temperature at which flaming combustion can be initiated within 10 seconds and maintained in the presence of a spark ignition source. These two conditions represent those most likely to produce the maximum toxicological effects. It is inherent in this method that materials are

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compared neither at equivalent heat fluxes nor at equivalent temperatures. Materials are compared at their conditions of maximum expected toxicity. Whether or not these conditions are actually encountered in a real fire must be further evaluated in assessing the overall hazard of a particular burning material. Additional indepth rationale for these combustion methods and conditions are discussed by Potts and Lederer (1977).

THE EXPERIMENTAL PROTOCOL

Combustion of Materials

The conductive heat furnace described by Potts and Lederer is utilized (1977). Materials are burned in both the nonflaming and flaming modes as defined previously. Sample weights are chosen such that nominal smoke concentrations, when contained in a 60-liter chamber, are 2.5, 5, 10, 20, 30 and 40 grams/cubic meter. Smoke concentrations are determined from the weight of sample consumed during the combustion.

The Exposure Chamber

A 60-liter hexagonal acrylic chamber equipped with multiple ports for exposure of rats in the head-only position is employed. The furnace is attached to the bottom plate of the exposure chamber, with the mouth of the quartz combustion beaker essentially flush with the bottom of the chamber. A Teflon®-coated aluminum cone is positioned approximately 2 inches above the mouth of the combustion beaker to shield exposed rats from direct flame radiation and to aid in convection current mixing of combustion gases and smoke. Exposed surfaces within the chamber are either acrylic or Teflon®. Temperature monitoring and sampling of the atmosphere for analyses are accomplished at positions in the chamber representative of those occupied by the rats.

Combustion Atmosphere Monitoring

Combustion product atmospheres are sampled at 5-minute intervals and analyzed by gas chromatographic methods for O_2, CO_2, and CO. Gas chromatographic analysis and/or specific detectors are also employed as appropriate for determination of HCN, HCl, acrolein, other aldehydes, and combustible hydrocarbons.

The temperatures to which the rats are exposed are monitored continuously. Chamber temperatures at the position of the rats rise to about 34 C by the end of a 30-minute exposure in the nonflaming mode. Chamber temperatures during a flaming combustion can rise briefly to above 50 C during the period of flaming, but decrease quickly to approximately 35 C upon cessation of the combustion. Depending upon the nature of the material and the sample size, the time during which rats are exposed to excessive temperatures is normally no greater than 1-2 minutes.

Exposure of Animals

Four male Long-Evans rats weighing from 390 to 420 grams are placed in tubular restrainers and exposed in the head-only mode to combustion product atmospheres for 30 minutes. Two of the four rats are equipped with arterial cannulas to allow for the sampling of blood during the exposure. All four rats are fitted with apparatus for monitoring of the conditioned avoidance response. The rats undergo at least 15 minutes of conditioned avoidance response training prior to the exposure. Up to 24 exposure experiments are conducted in the course of the evaluation of a material, up to 6 concentrations each in the flaming and the nonflaming modes, with each exposure being duplicated.

Physiological/Behavioral Monitoring of Animals

During exposure, the rats are monitored for performance of the leg-flexion conditioned avoidance response. The percent of total exposed animals from duplicate runs which lose the response during the 30-minute exposure is observed at each smoke concentration. The time when each animal loses avoidance is recorded and assessed relative to its blood chemistry at the time of the loss. These data aid in evaluation of the causality of the incapacitation. Times to loss of avoidance appear to have differing causalities and are not used directly in the relative evaluation of material toxicity. Variations in the times to loss of avoidance have been observed previously in rats exposed to irritants and other common combustion products (Hartzell et al., 1976).

The percent of total exposed animals from duplicate runs that die as a result of the exposure is observed. The time at which mortality occurs up to 14 days postexposure is noted.

Blood samples are taken from cannulated rats prior to the exposure in order to establish control values of blood parameters and to assess the health of the animals. Samples are then obtained at the time of loss of the avoidance response and at the time of death. Cannulated rats that survive the 30-minute exposure are also sampled upon termination of the exposure. Blood samples are obtained by cardiac puncture from noncannulated rats that die during the exposure. Blood samples are analyzed for COHb, pH, P_{aCO_2}, and P_{aO_2} content. Results aid in assessing the causality of both avoidance loss and death.

Upon termination of each exposure, surviving rats are subjected to a gross neurological/behavioral examination (e.g., righting reflex, startle response, etc.), observed for 14 days, and then sacrificed. Necropsy is performed on all rats upon death or sacrifice.

Data Analysis

Smoke concentration versus percent response for both the avoidance response and mortality are plotted according to the logarithmic-probability method of Miller and Tainter (1944). Values for the EC_{50} (concentration resulting in loss of the conditioned avoidance response of 50% of the subjects) and the LC_{50} (concentration resulting in death of 50% of the exposed rats) are estimated graphically for both the flaming and the nonflaming modes of combustion, along with calculation of standard errors for each value.

Assessments of causality for both avoidance loss and mortality are made for examinatin of blood gas data, pathologic findings, and combustion atmosphere compositions.

This protocol has been used for the evaluation of six materials, including Douglas fir. It has been successful in differentiating between materials and combustion conditions exhibiting varying degrees of toxicity, some materials indicating a high relative degree of toxicity, and others exhibiting minimal relative toxicologic problems. Furthermore, a basis has been obtained from which the suspected causality can be assessed.

The Role of Asphyxia

The syndrome of asphyxia is essentially characterized by an inadequate supply of oxygen to tissues, accompanied by elevated partial pressures of carbon dioxide (Pa_{CO_2}) in the blood (hypercapnia)(Best and Taylor, 1973). The

somewhat broader term of hypoxia, if further expanded to include histotoxic anoxia (the inability of cells to utilize oxygen), is suggested by data obtained thus far as the principal causality of both incapacitation and death of subjects upon exposure to fire combustion products.

Mortality data on a total of 46 rats exposed to a wide range of smoke concentrations from a variety of materials are shown in Table 1. The mean carboxyhemoglobin (COHb) saturation levels of the subjects at death is consistently in the range of 60-90% for all the materials which do not form HCN upon combustion. Thus, anemic hypoxia is strongly suggested as the causality of death for these materials. In contrast, mean COHb saturation levels at death as a result of exposure to rigid polyurethane foams are much lower at 40%. The low COHb saturation levels, along with the presence of as much as 200 ppm HCN in the combustion atmospheres, are suggestive of histotoxic anoxia as contributing to the cause of death, along with anemic hypoxia. With some materials, postexposure deaths due to arterial hypoxia, presumably from edema, are also observed. In the materials studied thus far, no evidence has been seen for causalities of death other than the various forms of hypoxia.

TABLE 1. ANIMAL MORTALITY

Material	Mode	N	% COHb At Death
Douglas Fir	Nonflaming	6	84.0 ± 4.2
Douglas Fir	Flaming	6	91.6 ± 1.6
Polystyrene Foam	Flaming	7	75.2 ± 2.4
Polystyrene Foam w/FR	Flaming	5	65.9 ± 2.9
Rigid Polyurethane Foam	Flaming	12	40.9 ± 9.2
Rigid Polyurethane Foam w/FR	Flaming	8	43.0 ± 3.7
Flexible Polyurethane Foam	Flaming	2	64.2 ± 0.2

Arterial blood chemistry data obtained at the time of loss of the conditioned avoidance response for 135 rats are shown in Tables 2 and 3. The exposures resulted from the combustion of a variety of materials over a wide range of smoke concentrations. The data show a consistent pattern of relatively low COHb saturation, lowered O_2Hb levels, decreased Pa_{O_2}, increased Pa_{CO_2} and low pH levels. These observations are strongly suggestive of arterial hypoxia.

TABLE 2. LOSS OF CONDITIONED AVOIDANCE RESPONSE (NONFLAMING MODE)

Material	N	%COHb	$\%O_2Hb$	pH	$\%\Delta P_{O_2}$	$\%\Delta P_{CO_2}$
Douglas Fir	15	14.9±20.5	55.5±11.1	7.24±.06	-25.4	+26.2
Polystyrene Foam	8	6.6± 5.2	76.2±13.6	7.34±.07	- 3.1	+10.9
Polystryene Foam w/FR	7	4.1±5.9	63.2±13.4	7.26±.05	-23.4	+33.6
Rigid Polyurethane Foam	15	6.0± 8.2	59.8± 8.8	7.26±.05	-19.9	+36.3
Rigid Polyurethane Foam w/FR	11	6.4±10.8	56.9± 9.5	7.29±.07	-23.4	+25.7
Flexible Polyurethane Foam	11	14.4±11.8	54.0±10.7	7.26±.08	-25.0	+39.7

TABLE 3. LOSS OF CONDITIONED AVOIDANCE RESPONSE (FLAMING MODE)

Material	N	%COHb	$\%O_2Hb$	pH	$\%\Delta P_{O_2}$	$\%\Delta P_{CO_2}$
Douglas Fir	9	32.7±24.6	60.1±22.0	7.26±.06	- 7.2	+26.2
Polystyrene Foam	8	31.6± 9.0	51.9±13.2	7.18±.13	-23.5	+35.0
Polystyrene Foam w/FR	10	35.8±20.4	42.3±13.4	7.13±.10	-37.6	+49.0
Rigid Polyurethane Foam	16	18.0±13.4	55.2±10.4	7.13±.14	-14.6	+41.1
Rigid Polyurethane Foam w/FR	11	11.5± 8.3	57.6± 8.5	7.30±.11	-17.8	+17.0
Flexible Polyurethane Foam	14	11.1±11.1	54.8±14.0	7.26±.11	-23.6	+38.3

A noteworthy exception to the pattern is seen in the case of polystyrene foam in the nonflaming mode. With this material, a normal level of Pa_{O_2} and only a slight increase in Pa_{CO_2} suggest that asphyxia may not be implicated. Incapacitation may thus be due to other causes in this particular case. It should be added, however, that the smoke concentrations required to effect incapacitation with polystyrene foam are quite high compared with other materials in this study and that this material therefore exhibits a relatively low order of toxicity.

Loss of the conditioned avoidance response in the case of rigid polyurethane foam in the flaming mode is accompanied by somewhat smaller than the usually observed decreases in Pa_{O_2}. Histotoxic anoxia, in which tissues cannot utilize oxygen, is therefore suspected. The observation of significant HCN concentrations produced from flaming combustion of rigid polyurethane foam supports this assessment.

The protocol used for assessment of the relative toxicological hazards of burning materials has thus yielded considerable data of value in suggesting causality for both incapacitation and death. Blood chemistry data are strongly indicative of the implication of a variety of forms of hypoxia in both effects. Moreover, such information along with appropriate atmosphere analytical data, is capable of suggesting situations in which hypoxia may not be involved and therefore alternate causality may need to be sought.

ACKNOWLEDGEMENTS

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PAPER NO. 8

THE EFFECT OF TEST CONDITIONS ON RELATIVE TOXICITY TEST RESULTS AND RANKINGS

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INTRODUCTION

The relative toxicity rankings of materials, as measured by the time it takes for animals to manifest overt toxic symptoms, tend to be dependent on the test conditions. This was shown by earlier work with four synthetic polymers (Hilado et al., 1977). Certain materials, however, tend to rank consistently among the most toxic or least toxic over a range of test conditions. The extent to which consistent effects can be found over ranges of test conditions is of considerable interest, because such information would permit a reduction in the number of experiments necessary and hence the amount of work required to evaluate a specific material.

A screening test method for relative toxicity of emitted pyrolysis gases was developed at the University of San Francisco. This involves the exposure of four freely moving Swiss-Webster male mice in a 4.2 liter hemispherical chamber to pyrolysis gases generated by pyrolyzing a one gram sample under different test conditions. Over 270 materials have been evaluated by this test method to date (Hilado and Cumming, 1977c). This paper presents test data for a select variety of materials to demonstrate the effect of test conditions on test results and rankings.

APPARATUS

The animal exposure chamber (Figure 1) is of a design developed and patented by NASA and is made of clear polymethyl methacrylate so that continuous observation of the animals is facilitated. The activity of the free moving mice in the chamber allows observation of natural, unrestrained behavior which can be recorded by the average layman. This spontaneous activity results in fairly uniform distribution of the gases throughout the chamber volume.

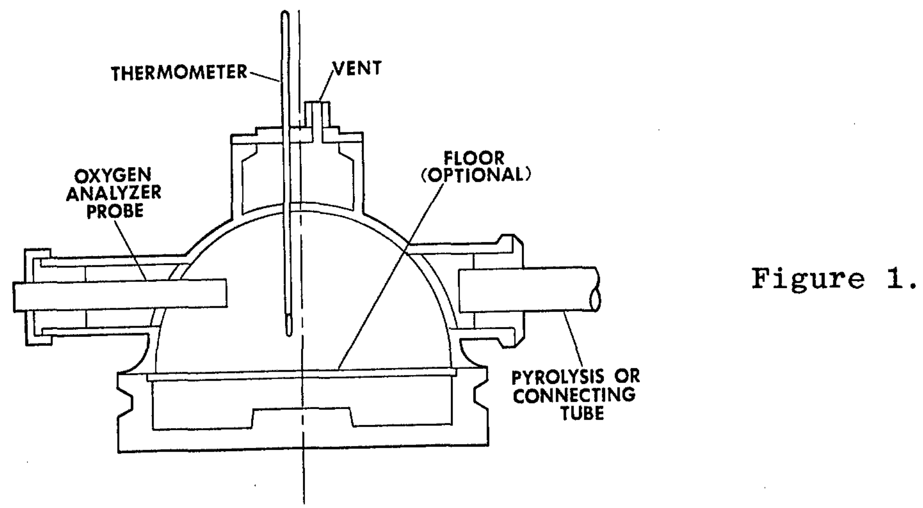

Figure 1.

The polymethyl methacrylate is superior to glass in ease of fabrication, light weight, resistance to shock, and inertness to hydrogen fluoride, which is a pyrolysis effluent from some synthetic polymers. The chamber has a total free volume of 4.2 liters, and is made of an upper dome section and a lower base section, both with a diameter of 203 mm (8 in).

The upper dome section is removable, and is connected to the base section by means of a conventional toggle snap ring; the joint is sealed by an O-ring. Access to the chamber is provided by two horizontal cylinders of different diameter mounted on the dome section. The larger horizontal cylinder, having a diameter of 59 mm (2.38 in), is fitted with an adapter to accommodate the open end of the pyrolysis tube. The smaller horizontal cylinder, having a diameter of 39 mm (1.56 in), is fitted with an adapter to accommodate the probe of a Beckman process oxygen analyzer, and serves also as the entry port for the test animals. A perforated polymethyl methacrylate plate across the larger horizontal cylinder prevents movement of the mice into the pyrolysis tube.

The upper end of the dome section is provided with apertures and a clear polymethyl methacrylate cylinder having a cover plate; the cover plate is connected to a bubbler to permit venting of pressure exceeding one inch of water and prevent entry of fresh air, and is provided with fittings for a thermometer and for gas sampling.

A Lindberg horizontal tube furnace is used for pyrolysis. The sample material is pyrolyzed in a quartz boat center in a quartz tube, closed at one end with a cap and connected at the open end to the animal exposure chamber. For tests with forced air flow, the cap at the normally closed end is replaced by a fitting into which air is pumped in a continuous flow by either a Hush I aquarium pump (nominal 16 ml/sec) or a Metbel MB-21 pump (nominal 48 ml/sec).

PROCEDURE

The pyrolysis tube, pyrolysis boat, animal exposure chamber, and all fittings and adapters are thoroughly cleaned and dried before each test. The pyrolysis boat is weighed without and with the sample under test. Quartz boats and tubes are normally used; where possible, monel or platinum tubes are used when hydrogen fluoride is expected to be present in the pyrolysis effluents.

The test animals are received in plastic cages, with each test group in its own cage. Each animal is removed, inspected for freedom from abnormalities, weighed, and marked on some part of the body with different colors of ink for identification. Four Swiss-Webster male mice, 25 to 35 gram body weight, are used for each test. Four appears to be the optimum number of mice which can be used for each test without excessive oxygen consumption during the test period, as well as the largest number which can be satisfactorily observed by a single operator.

Each experiment is repeated two or three times. This replication provides measures of variation between test animals and between experiments.

The mice are placed in the animal exposure chamber and given a minimum of 5 minutes to accustom themselves to their surroundings. The entire system is sealed (except for the safety vent) and all joints are checked for proper seating. The pyrolysis tube containing the sample is introduced into the furnace, which is preheated to 200 C in the case of rising temperature programs, or 600 C or 800 C in the case of fixed temperature programs. In the case of rising temperature programs, the furnace is turned on at the start of the test at the predetermined heating rate of 40 C/minute; when the furnace approaches or reaches the upper limit temperature (600 C or 800 C), this temperature is maintained by either automatic or manual control until the end of the test. The test period is 30 minutes, unless 100% mortality occurs earlier; the test is terminated upon the death of the last surviving animal.

Time to first sign of incapacitation is judged as the time to the first observation of either loss of equilibrium (staggering), prostration, collapse, or convulsions of any animal. Time to death is judged as the time to cessation of movement and respiration.

After the test is terminated and the animals are removed from the chamber, the pyrolysis boat containing the sample is removed and weighed to permit calculation, by difference, of the weight of sample pyrolyzed. Surviving animals are observed daily for a 14-day period after the test, and any significant changes from normal appearance, behavior, or weight are noted.

Temperatures and oxygen concentrations in the animal exposure chamber are recorded at 1 minute intervals throughout the entire test period.

DATA AND DISCUSSION

The effect of air flow rate on time to first sign of incapacitation and time to death, using the rising temperature program from 200 to 800 C, is shown in Figures 2 and 3, respectively. The materials are flexible polyurethane foam, neoprene foam, and wool fabric. Increasing the air flow rate from 0 to 16 ml/second resulted in a decrease in both time to first sign of incapacitation and time to death; the observed effect is attributed largely to more rapid delivery of toxicants to the animals; other factors may be increased transport of solid and liquid particles, and a higher degree of oxidation. This trend continued with increased air flow to 48 ml/second in the case of flexible polyurethane foam. Increasing air flow from 16 to 48 ml/second in the case of neoprene foam and wool fabric resulted in longer times to first sign of incapacitation and times to death; this reversal may be due to dilution of the toxicants by the continuing flow of air.

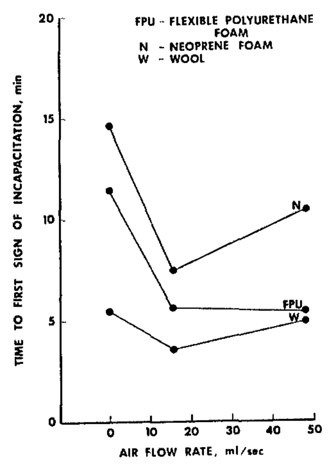

Figure 2. Effect of air flow rate on time to first sign of incapacitation: rising temperature, 200-800 C.

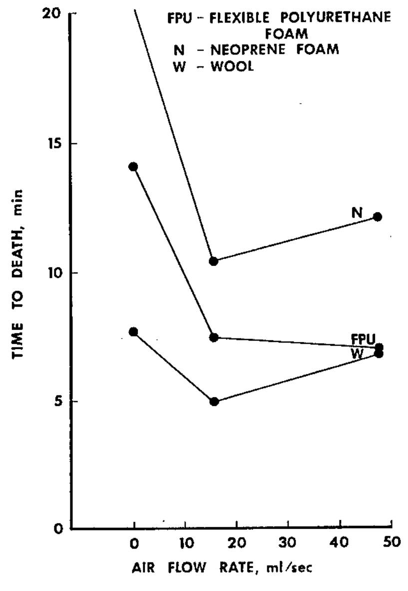

Figure 3. Effect of air flow on time to death: rising temperature, 200-800 C.

The effect of heating rate and air flow rate on time to first sign of incapacitation and time to death, for ABS, polycarbonate, and rigid polyurethane foam, is shown in Figures 4 and 5, respectively. Changing from the rising temperature program (40 C/min) to the fixed temperature program (immediate exposure to 800 C) resulted in markedly reduced times to first sign of incapacitation and times to death; this effect is attributed to more rapid generation of toxicants. With the fixed temperature program at 800 C, increasing the air flow rate from 0 to 16 ml/second resulted in a decrease in both time to first sign of incapacitation and time to death; this effect is attributed to more rapid delivery of gaseous toxicants, increased transport of solid and liquid particles, and perhaps a higher degree of oxidation. The apparent reversal in ranking between polycarbonate and rigid polyurethane foam may be statistically significant but may have no practical significance. Further increases in air flow rate to 48 ml/second seemed to have no significant effect on time to first sign of incapacitation, but air dilution may have had some effect on times to death with polycarbonate and rigid polyurethane foam.

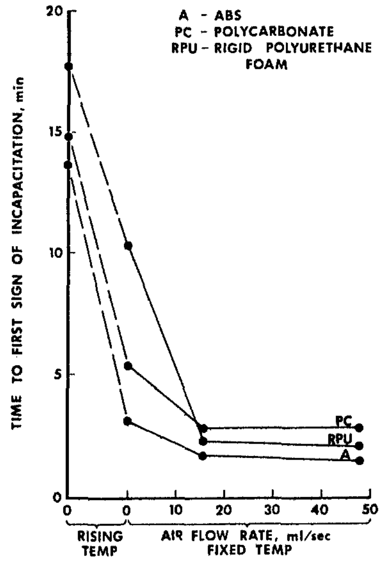

Figure 4. Effect of heating rate and air flow rate on time to first sign of incapacitation: rising temperature and fixed temperature, 800 C.

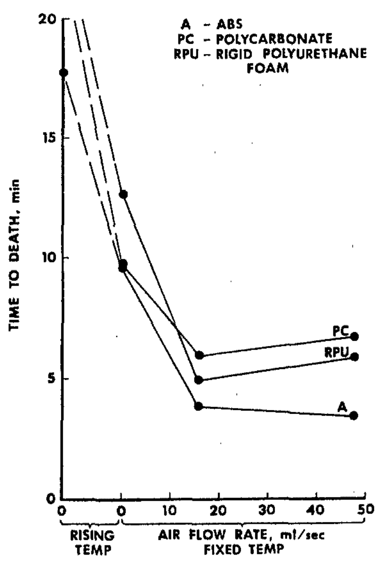

Figure 5. Effect of heating rate and air flow rate on time to death: rising temperature and fixed temperature, 800 C.

The effect of heating rate and air flow rate on time to first sign of incapacitation and time to death, for six upholstery fabrics, is shown in Figures 6 and 7, respectively. The observed effects were similar to those found for ABS, polycarbonate, and rigid polyurethane foam. With the fixed temperature program at 800 C, times to first sign of incapacitation were all less than 5 minutes, and the observed changes and apparent reversals may have no practical significance.

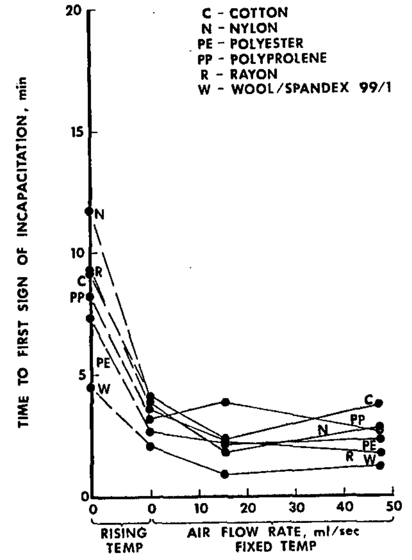

Figure 6. Effect of heating rate and air flow rate on time to first sign of incapacitation: rising temperature and fixed temperature, 800 C.

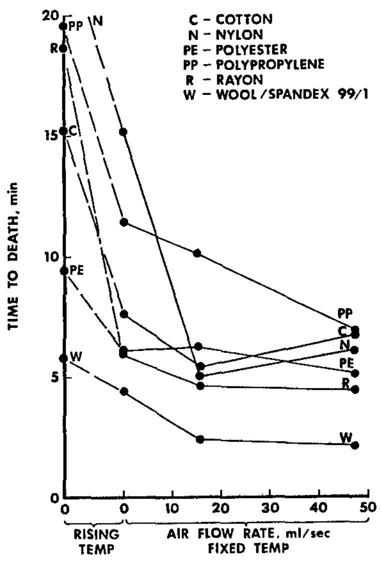

Figure 7. Effect of heating rate and air flow rate on time to death: rising temperature and fixed temperature, 800 C.

Some consistent differences between materials were observed despite all changes in test conditions. On the basis of animal response times, the wool fabric was consistently more toxic then the nylon and polypropylene fabrics. The relative ranking of wool fabric, flexible polyurethane foam, and neoprene foam, was consistent through three sets of test conditions.

Because air flow affects the supply of available oxygen, the effect of test conditions on oxygen concentration should be considered. The oxygen in the animal exposure chamber is depleted primarily by the oxygen consumption of the test animals. The average oxygen concentrations in control tests, with the animals in the chamber but without any sample in the pyrolysis boat, are shown by the solid lines in Figure 8. Oxygen depletion would appear to be significant only in the absence of air flow. Earlier work (Hilado and Cumming, 1977b) indicated that oxygen replenishment had an effect on survival in the last 5 minutes of the 30-minute exposure, when using no forced air flow.

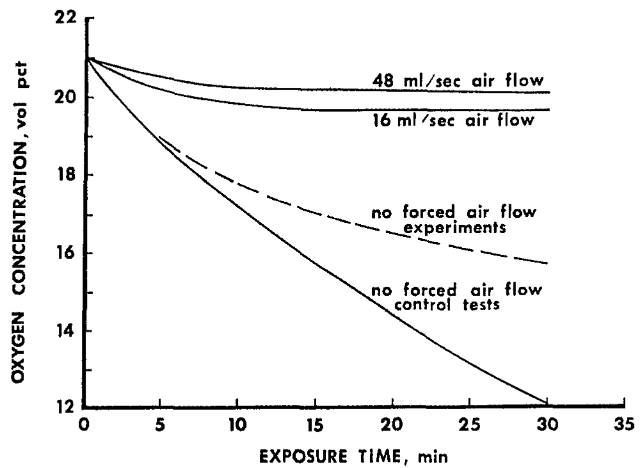

Figure 8. Effect of exposure time and air flow rate on oxygen concentration.

The average oxygen concentrations in actual experiments, shown by the broken line in Figure 8, tended to be considerably higher than the levels expected on the basis of the control tests, although displacement of oxygen by expanding pyrolysis gases might have led one to anticipate even lower concentrations. This phenomenon is attributed to inhibition of respiration by sensory irritants, a subject investigated by Alarie et al. (1977), and reduced oxygen consumption by animals that have been incapacitated.

The displacement of oxygen by nontoxic pyrolysis gases does not appear to be a significant factor. Earlier studies (Hilado et al., 1977) have shown that the generation of enough hydrocarbon gases to produce a 1% concentration in the chamber tends to be accompanied by the generation of about 10,000 ppm of carbon monoxide. The displacement of 0.2 volume percent of oxygen by these hydrocarbon gases is insignificant compared to the fact that 10,000 ppm of carbon monoxide is lethal to Swiss albino mice in 5 minutes (Hilado and Cumming, 1977a).

CONCLUSIONS

Changes in test conditions can significantly affect toxicity test results. In most cases, however, the relative rank order of materials tends to remain unchanged despite changes in test conditions.

The use of air flow permits maintenance of higher oxygen levels in the animal exposure chamber.

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PAPER NO. 9

COMBUSTION PRODUCT TOXICOLOGY

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Text of this presentation is not available for publication.

DR. CAVENDER (Becton, Dickinson and Company): I would like to ask all of the panel who would care to comment on this to do so. One thing I am very distressed with in the presentations this afternoon is the use of a single animal species with each test system discussed. One of the correlating facts that Dr. Halpin referred to was that there were some unexplained deaths due to laryngeal spasms, and sensory irritants can, indeed, induce laryngeal spasm in the guinea pig. Dr. Mary Amder at Harvard has shown that there are certain supersensitive individuals in the guinea pig population that will respond at a very high level. That is true perhaps in picking one nonsensitive species such as a rat or going to an isolated test system such as Mallory's mouse system. If you are going to come up with a standard protocol, it seems to me that the best toxicological data that can be derived will use at least two species of different sensitivities to make a proper evaluation.

DR. WINSTEAD (National Academy of Sciences): I would like to reiterate a comment made in my talk. I was describing guidelines for a very simple screening test to set priorities for further testing and that this is equivalent to determining an LD_{50}. I don't think there are multiple species used in most screening procedures. Obviously if a material is going to be put into widespread use, you have to use more definitive experiments and investigate it more thoroughly.

DR. BIRKEY (National Bureau of Standards): I'll try to comment in terms of the philosophy of the approach we've taken. I would not want to negate the point you've made except to say that our first step is to try to get a fix on the major causes of fire fatalities. The laryngeal spasm that was shown on the slide was probably the result of direct exposure to flames. In terms of fatality, that is not a significant fact at this point. It may explain 3% or less of total fire fatalities. The major cause of fire deaths is the inhalation of chemical toxicants and not the response of a few select people that are supersensitive. A bigger factor than that yet, it seems to me, is the cardiovascular disease problem that Dr. Halpin also alluded to and that probably explains a larger percentage of fire deaths than laryngeal spasm. We are not ignoring this problem. The first step is to try and get a fix on major causes of fatalities.

DR. HARTZELL (University of Utah): I've got to agree with that position. The state of the art of fire toxicity has not advanced very far. We're still looking at some of the basic phenomena that are involved. After we get this thing fine-tuned, I'd be inclined to agree with you that we ought to go to other species.

DR. BIRKEY: Let me just comment further on that. There are other researchers with programs going on that are using different animal species. There's a rather extensive program at the University of South Paris using rabbits. There's work going on in England using rats and also some primates. There are other species being used in fire toxicity research programs. Those studies, however, go beyond the purpose of screening tests for comparative toxicity and begin to evaluate toxic mechanisms of toxicity.

DR. HILADO (University of San Francisco): From the viewpoint of the test methodology, the differences that you see are probably not species differences but are most likely differences in the method of combustion or pyrolysis. We have done some collaborative work with Dr. Packham where we adapted the behavioral response system which he developed at the University of Utah to the University of San Francisco (USF) small chamber. In our initial experiments, we found a 1-to-1 relationship for time to incapacitation between free moving mice observed visually and inability to avoid a shock using Dr. Packham's system with all the electronic relays. I think that might have been fortuitous. This approach may be perfectly all right for carbon monoxide intoxication. For wool combustion products, the correlation was not 1-to-1. It was close, but not 1-to-1. We have also worked with the Civil Aeromedical Institute (CAMI) where they used rats in a tumbling cage. Again, comparing what they call incapacitation and what we call incapacitation and times to death, for example, at USF and at CAMI, their definition of incapacitation corresponded to that last and final observation of collapse in the USF method. I think that most of the differences observed in fire toxicity measurements probably are other than species differences.

DR. MAC FARLAND (Gulf Science and Technology Company): I would like to ask a question about human fire fatalities. Can anyone tell me the proportion of the deaths that occur during or within a very short period after the fire as opposed to delayed deaths that occur 24 or more hours later?

DR. STEMMER (University of Cincinnati, Kettering Laboratory): I can give you some figures on the Southgate fire in Kentucky. Approximately 159 people died immediately in the fire. An additional 7 or 8 people died during a period of up to three weeks after the fire. The cause of death in those that had medical care and died has been described. They actually developed within 24 hours after the exposure marked pulmonary edema with pneumonia superimposed on that. Most of them died because of the ensuing pneumonia and heart failure.

DR. BIRKEY: I'd like to comment just briefly on Dr. MacFarland's question. You really have to separate fire fatalities into two categories. Some people will die of thermal burns and we want to separate those deaths from the ones who die from inhalation toxicity. In regard to pulmonary edema, we know that's a predominant problem when we have fires involving vinyl materials. It may also be a problem that we have when we burn wood or have a conventional wood fire but we don't have the extensive data that we've been able to accumulate on the electrical insulation fires that we've had in various locations around the country. There was some vinyl material but there was also quite a bit of cellulosic material in the Southgate, Kentucky fire. So it's going to be a little difficult to evaluate toxicological responses. I don't know if autopsies were performed on those fire victims, but if antimony was discovered in a significant quantity in the Southgate fire victims, then of course that would be a clue to the fact that indeed quite a bit of PVC was involved in that fire. We have done material identification in that fire, but I don't know what percentage of cellulosics versus vinyls versus other materials was involved in that fire.

DR. MAC FARLAND: Thank you both very much for what you've told me. I'll tell you what was in the back of my mind. You have the acute deaths that occurred at the time of the fire and when you look at the various types of toxicants that are being considered here today, the things that you might think of are carbon monoxide and cyanide because if a person survives an exposure to carbon monoxide, once they get out of the air containing the carbon monoxide, they are essentially starting to recover because they are blowing it off. So if they are going to be killed by carbon monoxide, it's going to have to happen while they are being exposed, essentially. The same thing is true about cyanide as an acute poison. The thing that I was interested in was the heavy metals, and I was interested in the remarks about the effects of antimony on the heart. I was concerned about

cadmium. As you know, cadmium, particularly in the form of a freshly formed fume which occurs with heating of cadmium compounds at elevated temperatures, is extremely toxic. It's so toxic, in fact, that its use as a chemical warfare agent was once considered. The lethal dose in man is something on the order of 2400 mg minutes per cubic meter. The tissue concentration in a man receiving a lethal exposure is of the order of about 2 micrograms per gram of lung tissue. I don't know what human lungs weigh, but I think that if you multiply that thing out, the quantity of the concentration of cadmium that you found in some of those lungs, it seems to me, must be rather close to this. The important thing about cadmium fume poisoning is that it is not immediately lethal. Death ensues from the pulmonary edema usually 12 to 48 hours afterwards, and this is the point that I was trying to make.

DR. BIRKEY: Those characteristics are quite similar to what we've seen with exposure to PVC materials.

DR. STEMMER: I would like to make one other comment about cadmium and other toxicants that come into play after the survival of the immediate effects of fire. We did some studies of the smoke in regard to chelating capacity. In the combustion of urethane, there is very strong binding of copper and zinc components of the smoke. We know that cadmium is an antagonist of copper and if you give suboptimal copper dietary intake to animals and expose them to 15 or 20 ppm of dietary cadmium that they develop heart lesions and heart failure over a period of a few months. There may be a similar mechanism involved in people like firefighters who are repeatedly exposed to the other compounds in smoke than just carbon monoxide or cyanide. As mentioned, cadmium and chelating agents in that smoke may be of great importance.

DR. BIRKEY: I'd like to comment on that firefighter comment. The predominant cause of death among firefighters is heart failure. It may or may not be related to what you are suggesting. Certainly we are concerned about the question of stress on the firefighter and what that may be doing to cardiac function. I don't know whether it's related to the comments you made or not. It could be.

DR. MC GRATH (General Motors Research): I am quite interested in what kind of experience you've had in using spectrophotometric techniques, especially the CO-Oximeter in measuring blood carboxyhemoglobin levels. In our hands, we find there can be many interfering substances such as anticoagulants or just handling and processing the blood. I wonder if you can comment on this.

DR. HARTZELL: I know there have been considerable difficulties in our laboratories with this instrument. I am not the one who has been involved with this problem in our laboratory but I am aware of the problems and the laboratory analysts have them pretty well under control now. It is difficult calibrating the instrument for rat blood, for example. There are certain interferences. If you want more details, I'll give you a name to communicate with who can tell you the details of how they were able to get around these kinds of things. Dr. Birkey, do you have any comment on this?

DR. BIRKEY: I don't believe the problem with analyzing rat blood with the CO-Oximeter wasn't simple chemical interference. It was the result of blood coagulating very rapidly in the technique that was being used. There is a lot of concern and in fact, the manufacturer has voiced a lot of those concerns about its use for a precise measurement of carboxyhemoglobin.

LCDR ANDERSEN (Naval Medical Research Institute): Dr. Rodkey is in the midst of working with a few of these problems such as measuring CO with the CO-Oximeter and with Dr. Radford's technique. Perhaps he would speak on this point.

DR. RODKEY (Naval Medical Research Institute): Indeed, all measurements for carboxyhemoglobin that are currently being done in a routine manner are spectrophotometric. The CO-Oximeter 182, which is the old model, is the one most frequently used and is calibrated to the spectrophotometric constants for the human species. This is one of the problems. The other problem is that on that particular instrument, very small blood clots are extremely detrimental. Another problem with that particular instrument is that the calibration does not include that fraction of the total hemoglobin which is present as methemoglobin. Therefore, if there is methemoglobin present as there is in most post mortem cases, the measurements are inaccurate. My feeling for the spectrophotometric methods of carboxyhemoglobin analysis are quite adequate for most purposes. That is, it will tell you fairly well whether the carboxyhemoglobin concentration is elevated but small differences in values may not be reliable or reproducible. It's quite adequate for its intended purposes. The new CO-Oximeter now on the market, the IL282, is capable of being calibrated for any species that you want to determine the coefficients for and does take into account methemoglobin, oxyhemoglobin, reduced hemoglobin, CO hemoglobin, and deoxyhemoglobin - all species which are normally present.

I would like to make one other comment if I might. When you induce some methemoglobinemia in a baboon, you can have fantastically high cyanide concentration with absolutely no indication of toxicity. We injected anesthetized baboons with cyanide at levels up to 50 times the lethal dose after inducing a mild methemoglobinemia without producing symptoms or signs of cyanide toxicity. They had sufficient methemoglobin to combine with all that cyanide and at the conclusion of the experiment simply woke up from the anesthetic and walked away. Therefore, I think that the fatal levels in cyanide could very well be related to the existing methemoglobin levels within an animal at the time of exposure. When you have enough methemoglobin to bind all the cyanide, then you don't have free cyanide in the plasma which then gets into the respiratory cells which are the primary point of attack.

DR. KRIVANEK (E. I. duPont de Nemours and Company, Inc.): I'm a bit concerned about the type of test model for evaluating incapacitation, particularly the animal model. There are several types of models which are available; the tumble cage, decrease in respiration rate, the rotating rod, the shuttle box avoidance test. Different animals perform differently in these tests. So I'd like to hear comments from the panel on the advantages of the different tests so we can get a better idea of which ones might be most useful to test the toxicity of compounds with.

DR. BIRKEY: Well, I don't profess to be a behavioral toxicologist and I don't certainly want to be thought of as that. I can only speak from what experience we've had and what data I've seen. The models that I've seen data on are the leg flexion model that Dr. Hartzell can talk about, the tumble cage that's been used for evaluation of 75 aircraft cabin materials, the rotor rod that is being used at the University of Michigan, and the Alarie model for respiratory rate measurements. Exclusive of the Alarie method, I have not seen a great deal of difference between the various behavioral tests in ranking of toxicity from combustion products. The tumble cage seems to give a little earlier indication of incapacitation than the Utah model. I do not have that type of comparison with the rotor rod but I suspect from what I've seen, that that is going to give an incapacitation time very similar and possibly just a little earlier than the tumble cage. The Alarie model, which only ranks sensory irritants, takes a much lower concentration of combustion products to measure effects than any of the other three models. That certainly gives an earlier indicator of a problem. With respect to the advantages and disadvantages,

the Utah model is certainly more convenient in terms of monitoring the animal, taking blood samples and measuring respiratory rate. I think the major limitation of the tumble cage is not being able to see the animals well during the exposure. Now that's not true of all exposures. The FAA says that they don't have any problem seeing it, but we tried it on some large scale fires and we totally lost sight of the animals. That is, we built room fires, transferred the products to the animals to measure time to incapacitation, and we just couldn't see the animals at all under any circumstances. So we tried to do the correlation of our laboratory experiments with large scale fires using that model and we just couldn't observe the animals to determine the time to incapacitation. So I would say as far as my experience has been, the tumble cage is a difficult one to see the animals.

DR. HARTZELL: I've got to give essentially the same answer. We have looked rather extensively at comparing the leg flex avoidance response with the tumble cage in the case of carbon monoxide alone with air. The comparison is very good. They are very reproducible. I don't see any problems with either one. The differences, as Dr. Birkey just said, come in seeing the animal. If you will recall the picture I showed of polystyrene smoke in the flaming mode, I don't really see how you can possibly see the animals in there if you have a heavy smoke concentration of that type. In our laboratory, we also like to get blood samples during the course of the experiment and that's kind of difficult in the case of the tumble cage. Therefore, we have chosen the leg flexion avoidance test. I haven't really seen enough data obtained from the rotating rod or shuttle box to make any comparisons. I have the impression that the tumble cage requires somewhat more training than we would like to give the animals. We would like to have some type of device which requires a minimum amount of training. We are talking about testing quite a large number of animals and if you get some kind of behavioral experiment like bar press or some kind of experiment like that which may require extensive training, it just takes too much time and manpower.

DR. HILADO: I disagree with some of the earlier comments. In the process of comparing test methods, we have run the tumble cage. When you use the tumble cage in the CAMI Laboratory configuration, there is a very close clearance between the wall of the chamber and the animals. There is also a lamp which back lights the tumble cage at an angle. Visual observation is a function of obscuration, which is not a problem with the particular apparatus used at CAMI. Visibility is really no problem because you don't have much smoke between you and the animal. Now I can see where you would have a problem with a bigger apparatus because you would have twice as much, or maybe five times as much thickness of smoke

between you and the animal. I don't think we should be critical of the tumble cage for what is really a case of engineering design.

DR. BIRKEY: I would agree with that but I have another major concern about those three models that has not been brought out. I think perhaps the shuttle cage and maybe some others would resolve my concern but we don't have very much data on some of these models. That concern is simply that in all three of these models incapacitation is not very far away from death and is very close in terms of the hind leg flexure test.

CDR JENKINS (Naval Medical Research Institute): I'd like to comment on that point. I think that a lot of the problem with behavioral paradigms, Dr. Birkey, has to do with the degree of negative reinforcement and the motivation, therefore, for the animal to perform the task that he's supposed to perform; the shock or the discomfort or the pain is a very motivating factor. They perform just as long as they can and make superrodent efforts to perform because it's a very distasteful situation if they don't. On the other hand, the ability for a rodent to find his way through a maze with a positive reward at the end is not as strong a motivator as a negative reward. The point being that there are a lot of behavioral tests and different degrees of motivation. We are using right now the tumble cage or the rotating wheel and have found it to be at least reproducible while we have not found a practical way to use such tests as the rat maze in a smoke toxicity chamber. There are techniques that can be used with the rotor rod wherein there is an automatic counter on a shock grid which might work in a chamber with smoke obscuration. If you have an automatic counting device on the bottom of the cage, the rat shuts off his own clock when he falls. That might be a useful technique in a situation where you have dense smoke.

DR. HARTZELL: I could add something to this regarding the restraining device used for leg flex avoidance. There has been some question as to whether a restrained animal is under a different degree of stress than an unrestrained, running animal. The only comparison I have with this is with a study that Dr. Potts did at Dow on the same Douglas fir material that we tested at Utah. We used the same combustion furnace, except that his chamber was larger than ours so that the absolute quantity of material burned was greater. He was using free running rats and looking only at mortality. He had no way of looking at incapacitation. So we're looking only at mortality comparing restrained rats against free running rats. In determining the LC_{50} for the fir in the nonflaming mode, we found it to be 21 grams per

cubic meter and he obtained a result of about 19 grams which is very close. In the flaming mode, the two results were 23 and 24 grams per cubic meter. As far as mortality was concerned, the results obtained were identical whether the rats were restrained or not restrained.

MR. FARRAR (University of Utah): I would just like to expand on your comment, Dr. Birkey, about the fact that the loss of avoidance or the loss of behavioral responses is very close to death. It all depends on what material produced the primary insult. If you have carbon monoxide intoxication, then presumably anoxia or hypoxia is going to be the major cause of the loss of the behavioral response which is probably close to death. In the case of polystyrene, however, where there is a very low carbon monoxide production, you have loss of behavioral response at a very low dose compared to that dose which would cause death. So I think one of the advantages of the behavioral system is that it will be influenced by a number of different factors; lack of oxygen, an anesthetic effect, or even a direct neurotoxic effect whereas other responses may be specifically related, say to an irritant effect in the respiratory tract.

DR. BIRKEY: I think that points out a very important issue that there is probably no single animal behavioral model that is going to address the toxicity of the complexity of products that we are talking about in fires. I gave you an example of some data that I saw on a vinyl material in which the animals were never incapacitated during the exposure but 100% of them died within 14 days. There is a serious problem, I think, in terms of utilizing the animal incapacitation model as a means of ranking or rejection of materials from a toxic hazard viewpoint.

DR. KRIVANEK: Dr. Terrill at Haskell Laboratory has done a considerable amount of work in this area. A major problem we've had with the tumble cage is that the result varies from observer to observer. One observer may call a misstep incapacitation where another one may just call it a normal misstep of the rat going around in the cage. We had difficulty in getting people to agree exactly on what they were calling an error in the walking ability of the rat. With things such as condition avoidance like the shuttle box and operant conditioning, these are easily automated and you can get objective type of data. We have done very little testing on these methods but they do allow you to make a more objective evaluation of the situation and it may approach the real life situation. This is why we think test methods like the shuttle box may have a little more use than leg flexion tests, although as you mentioned there may not be any difference between a

hindered animal and an animal that is free. We just think that animals that are allowed to walk around mimic the human situation a bit more closely than the restrained animal as we are in the process of trying to evaluate the different types of test procedures.

DR. WINSTEAD: I think discussion to this point has certainly illustrated the fact that there is no one best method for measuring incapacitation. It is my understanding that Dr. Cornish at the University of Michigan currently is conducting a program comparing the rotor rod, the shuttle box and the rotating wheel. These studies are not complete yet.

SESSION III

BASIC MECHANISMS IN TOXICOLOGY

Chairman

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PAPER NO. 10

GENETIC DIFFERENCES IN THE TOXICITY OF DRUGS AND CHEMICAL CARCINOGENS

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INTRODUCTION

To study the genetic control of drug metabolism is often called pharmacogenetics. The term "ecogenetics" might be used to study the genetic control of metabolism of all environmental chemicals. In a single sentence, "pharmacogenetics" may be defined as the attempt to understand why the same dose of the same drug given to two different individuals (with the possible exception of identical twins) may cause widely varying responses. These "responses" include therapeutic effects of a drug, e.g. anticoagulation, improvement in myocardial contraction, or control of seizures, but also may include unwanted deleterious effects such as cancer, toxicity, or birth defects. The experimental system to be examined in detail represents principally a genetic difference in receptor concentration; because of this defect there are large genetic differences in the biotransformation and pharmacokinetics of certain drugs and other environmental pollutants, resulting in important differences in risk toward cancer and drug toxicity.

The general characteristics of the P-450-mediated monooxygenases and their coordinated enzymes are first described. Secondly, the genetic differences in this model system in mice are examined. How these differences are associated with increased risk toward cancer are then shown as an example. Numerous other conditions in mice associated with this genetic system are also listed. Lastly, current evidence for this genetic difference in man is analyzed.

CYTOCHROME P-450 MONOOXYGENASES AND COORDINATED ENZYMES

Many environmental pollutants and other foreign compounds are chemicals that are so hydrophobic they would remain in the body indefinitely were it not for the metabolism resulting in more polar derivatives. These drug-metabolizing enzyme systems,

which are localized principally in the liver, are usually divided into two groups: Phase I and Phase II (Williams, 1959). During Phase I metabolism, one or more polar groups (such as hydroxyl) are introduced into the hydrophobic parent molecule, thus allowing a "handle," or position, for the Phase II conjugating enzymes (such as UDP glucurosyltransferase) to attack. The conjugated products are sufficiently polar, so that these "detoxified" chemicals are now excreted from the cell and from the body.

One of the most interesting of the Phase I enzyme systems is a group of enzymes known collectively as the cytochrome P-450-mediated* monooxygenases (Conney, 1967; Thorgeirsson and Nebert, 1977; Kouri and Nebert, 1977). These membrane-bound enzyme systems are known to metabolize: polycyclic aromatic hydrocarbons such as BP** (ubiquitous in city smog, cigarette

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*The nomenclature for various forms of cytochrome P-450 is currently inadequate, in view of the six or more forms distinguishable by electrophoretic (Haugen et al., 1976) or immunochemical (Thomas et al., 1976) techniques. An eventual better understanding of chemical and catalytic properties (Ryan et al., 1975; Haugen et al., 1975; Haugen and Coon, 1976) should permit in time a more suitable nomenclature to be devised. In this paper, "P-450" is defined as those forms of CO-binding hemoprotein not induced by polycyclic aromatic compounds or, in the more general sense, all forms of CO-binding hemoproteins associated with membrane-bound NADPH-dependent monooxygenase activities. We define "P_1-450" as those forms of CO-binding hemoprotein which increase during polycyclic aromatic inducer treatment. It is not yet known whether cytochromes P_1-450 in liver are electrophoretically or catalytically identical to corresponding cytochromes in any nonhepatic tissue.

**Abbreviations used include: BP, benzo(a)pyrene; MC, 3-methylcholanthrene; AHH, aryl hydrocarbon [benzo(a)pyrene] hydroxylase (EC 1.14.14.2); TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; GSH, reduced gluthathione; B6, the C57BL/6N, C57BL/6J, or C57BL/6Cum inbred mouse strain used interchangeably unless otherwise specified; D2, the DBA/2N, DBA/2J, or DBA/2Cum inbred mouse strain used interchangeably; and C3, the C3H/HeN, C3H/HeJ, or C3H/fCum inbred mouse strain used interchangeably.

smoke and charcoal-cooked foods) and biphenyl; halogenated hydrocarbons such as polychlorinated and polybrominated biphenyls, insecticides, and ingredients in soaps and deoderants; strong mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine and nitrosamines; aminoazo dyes and diazo compounds; N-acetylarylamines and nitrofurans; numerous aromatic amines, such as those found in hair dyes; nitro aromatics, and heterocyclics; wood terpenes; epoxides; carbamates; alkyl halides; safrole derivatives; certain fungal toxins and antibiotics; many of the chemotherapeutic agents used to treat human cancer; most drugs; small chemicals such as benzene or ethanol; both endogenous and synthetic steroids; and other endogenous compounds such as biogenic amines, indoles, thyroxine, and fatty acids.

Evidence is growing (Thorgeirsson and Nebert, 1977; Kouri and Nebert, 1977; Nebert et al., 1977) that metabolism to reactive intermediates by cytochrome P-450-mediated monooxygenases appears to be a prerequisite for mutagenesis, carcinogenesis, and toxicity caused by numerous drugs, polycyclic hydrocarbons, and other environmental pollutants. These reactive intermediates probably bind covalently to numerous cellular macromolecules. Most of this binding is probably random, but some may be nonrandom, i.e. specific binding dependent upon the chemical structures of the reactive intermediate and the cellular macromolecule. Among these various types of covalent binding, there probably exists a very small amount of important binding of the ultimate carcinogen to its critical subcellular target, thereby initiating tumorigenesis.

The steady-state levels of these reactive electrophilic intermediates and, consequently, the rates at which they interact with the critical nucleophilic target(s) are dependent upon a delicate balance between their generation and detoxification (Figure 1). The balance of these enzymes obviously may vary, depending upon different tissues, strains, and species. Age, genetic expression, nutrition, hormone concentration, diurnal rhythm, pH, saturating versus nonsaturating conditions of the substrate, K_m and V_{max} for each enzyme, subcellular compartmentalization of each enzyme, efficiency of DNA repair, and the immunological competence of the animal--may all be important factors contributing to an individual's susceptibility to tumors or drug toxicity. Induction or suppression of one or more of these enzymes by chemicals also may affect the incidence of tumorigenesis or toxicity. We will show that a very small number of genes controls the formation of particular forms of P-450--termed cytochrome P_1-450. We therefore can examine, among individuals in the same uterus or siblings in the same family, genetic differences in P_1-450 content and the associated high or low steady-state level of

reactive intermediates. It is thus possible to determine whether a genetically-controlled high steady-state level of reactive intermediate is detrimental or advantageous to the individual, with respect to cancer, mutation, drug toxicity, or birth defects.

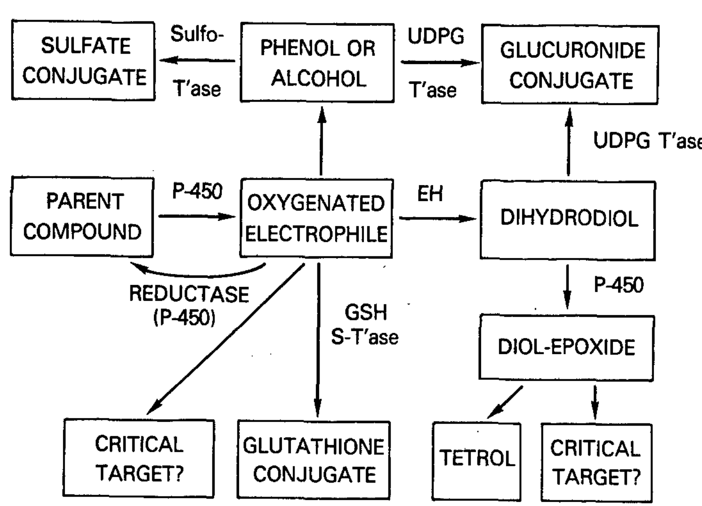

Figure 1. Scheme depicting the oxygenation of various drugs, polycyclic hydrocarbons, and other environmental pollutants by cytochrome P-450-mediated monooxygenases and the further metabolism of the oxygenated electrophilic intermediate by other enzymes (Nebert, 1978). One additional class of compounds not shown is the N-acetylarylamines, the formation of N-hydroxy derivatives from which, like the phenol or alcohol, can be conjugated with sulfate by sulfotransferase(s) (Sulfo-T'ase) or with glucuronide by UDP glucuronosyltransferase(s) (UDPG T'ase); potent electrophiles of N-hydroxy-N-acetylarylamines can also be formed by deacetylase(s) and acetyltransferase(s) (Thorgeirsson and Nebert, 1977). Evidence for sulfotransferase (Cohen et al., 1977), UDP glucuronsyltransferase (Dutton et al., 1976), epoxide hydrase (EH) (Oesch, 1972), glutathione S-transferase (GSH S-T'ase) (Jerina and Bend, 1977), arene oxide P-450-dependent oxygen-sensitive reductase (Booth et al., 1975), and participation of various forms of P-450 (Thorgeirsson and Nebert, 1977; Kouri and Nebert, 1977; Nebert et al., 1977) is described or reviewed in the references cited. (Reproduced with permission from Plenum Press Corporation.)

THE Ah LOCUS IN THE MOUSE

Evidence from this laboratory (Nebert and Gielen, 1972) was presented about seven years ago for a single gene difference between B6 and D2 inbred mouse strains in the induction of a hepatic monooxygenase activity, AHH, and cytochrome P_1-450 by MC treatment. With BP as the substrate in vitro, "AHH activity" is equated with the rate of formation of 3-hydroxybenzo(a)pyrene and probably other phenols (such as

[9-hydroxybenzo(a)pyrene] having similar wavelengths of fluorescent activation and emission (Nebert et al., 1975; Nebert et al., 1978). AHH activity and P_1-450 are induced by polycyclic aromatic compounds in the B6 and in other responsive mouse strains, but the induction of this enzyme and form(s) of cytochrome by polycyclic aromatic compounds is absent in liver and markedly decreased in lung, bowel, kidney, lymph nodes, skin, bone marrow, retinal pigmented epithelium of the eye, ovary, and mammary gland in the D2 and other nonresponsive mouse strains (Thorgeirsson and Nebert, 1977; Kouri and Nebert, 1977; Nebert et al., 1977; Nebert et al., 1975; Nebert et al., 1978). This "responsiveness" to aromatic hydrocarbons was designated the Ah locus: the allele Ah^b denotes the B6 and Ah^d the D2 inbred strain.

Numerous studies (Thorgeirsson and Nebert, 1977; Kouri and Nebert, 1977; Nebert et al., 1977; Nebert et al., 1975; Nebert et al., 1978) indicate that an important product of the Ah (regulatory) locus in mice is a cytosolic receptor (Poland et al., 1976) capable of binding to certain polycyclic aromatic inducers (Figure 2). Such an inducer-receptor complex in some manner activates structural gene(s), thereby leading to increases in enzymes which metabolize these inducers (and metabolize other polycyclic aromatic noninducing compounds as well). In addition to innocuous products, reactive metabolites may also be generated, thereby leading to an increased risk for cancer, mutation, toxicity, and teratogenesis.

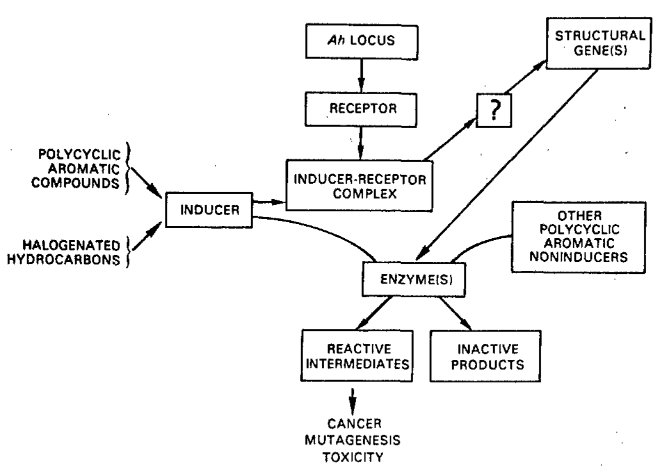

Figure 2. Simplified scheme demonstrating the relationship of the Ah locus in the mouse with cancer, mutagenesis, toxicity, and teratogenesis (Kouri and Nebert, 1977). (Reproduced with permission from Cold Spring Harbor Press.)

Using AHH induction as an indicator of phenotype at the Ah locus, several laboratories have found that about half, or slightly more than half, of all inbred mouse strains examined are responsive (as are wild mice, randombred mice, and about 20 inbred strains of rats tested (unpublished data), and the remaining mouse strains are nonresponsive (Kouri and Nebert, 1977). There has evolved during the past 60 or 70 years of developing these inbred mouse strains, therefore, a stable mutation whereby certain strains lack (either quantitatively or qualitatively) the gene product of the Ah locus, the cytosolic receptor molecule (Nebert et al., 1975; Poland et al., 1976; Guenthner and Nebert, 1977).

DIFFERENT TYPES OF GENETIC EXPRESSION AT THE Ah LOCUS

Induction of AHH activity and cytochrome P_1-450 by MC is expressed almost exclusively as an autosomal dominant trait among offspring of the appropriate crosses between B6 and D2 inbred strains. Besides the B6 x D2 genetic cross, at least 11 other crosses between responsive and nonresponsive strains exhibit similar autosomal dominant (or almost exclusively dominant) expression of AHH inducibility by polycyclic aromatic compounds (Kouri and Nebert, 1977; Nebert et al., 1978). In crosses between C3 and D2, MC-inducible AHH activity in the F_1 is intermediate between the responsive C3 parent and the nonresponsive parent, and there is a 1:2:1 distribution in the F_2 population: (one-fourth nonresponsive, one-half intermediate, and one-fourth highly responsive). This type of inheritance is called additive, or gene-dose. A third type of inheritance is observed in which the lack of AHH induction by MC is dominant when B6 and AKR mice (both from the NIH mouse colony) are crossed. Thus, the F_1 population and three-fourths of the F_2 population are nonresponsive. This finding suggests there may exist an interstrain-specific suppressor gene. However, AHH induction is expressed as a dominant trait in crosses between C57BL/6N and AKR/J, C57Bl/6J and AKR/N, or C57Bl/6J and AKR/J (Nebert et al., 1975; Nebert et al., 1978). During the past four years, this gene controlling the lack of hydroxylase induction by MC has apparently been lost from the NIH animal colony, although we have about nine recombinant inbred sublines (approaching the F_{20} generation) possessing this genetic information. Further genetic studies are being carried out.

The simplest genetic model to accommodate all existing data in the mouse is a minimum of six alleles and two (regulatory) loci (Thorgeirsson and Nebert, 1977; Nebert et al., 1975; Nebert et al., 1978). How the gene products of these

Ah loci are regulatory is unknown, but it is believed that one of the gene products is the cytosol receptor (Thorgeirsson and Nebert, 1977; Poland et al., 1976; Guenthner and Nebert, 1977) which can bind specific aromatic hydrocarbon inducers. It appears that the mutation in nonresponsive mice involves a defective cytosol protein receptor (either a decreased number of binding "sites" and/or decreased affinity for inducers) and that nonresponsive mice have the necessary structural genes for induction of AHH activity and its associated cytochrome(s) by very potent polycyclic aromatic inducers.

In studies involving the association of the Ah locus with cancer, mutation, or toxicity, the routine use of offspring from appropriate crosses between B6 and D2 parent strains (Figure 3) is ideal, because expression of AHH induction by MC most closely approximates a single-gene difference: Ah^b is the dominant allele for responsiveness; Ah^d is the recessive alleles, the Ah^d/Ah^d animal being genetically nonresponsive. One therefore can determine whether this single allelic difference is advantageous or disadvantageous with respect to risk for cancer or toxicity when all individuals receive the same dose of the same drug. We can thus evaluate the possible importance of steady-state levels of reactive intermediates in the mechanism of chemically induced carcinogenesis, mutagenesis, or toxicity among individuals in the same family or among siblings sharing the same uterus. This genetic probe is a particularly powerful experimental model system in the research areas of pharmacology, toxicology, teratology, and chemical carcinogenesis, because the test compounds studied often cause undesirable side effects (e.g. sedation, diarrhea, malnutrition, hormonal imbalance, etc.) that are hard to distinguish from specific pharmacologic, toxicologic, or carcinogenic effects of the compounds.

F_1	Ah^b/Ah^b X Ah^d/Ah^d ↓ Ah^b/Ah^d	Ah^b/Ah^d X Ah^b/Ah^b ↓ Ah^b/Ah^b:Ah^b/Ah^d
F_2	Ah^b/Ah^d X Ah^b/Ah^d ↓ Ah^b/Ah^b:Ah^b/Ah^d:Ah^b/Ah^d:Ah^d/Ah^d	Ah^b/Ah^d X Ah^d/Ah^d ↓ Ah^b/Ah^d:Ah^d/Ah^d

Figure 3. Simplified genetic scheme for aromatic hydrocarbon "responsiveness" in the mouse (Nebert and Felton, 1975). (Reproduced with permission from Plenum Press Corporation.)

INDUCIBLE ENZYME "ACTIVITIES" AND METABOLITES ASSOCIATED WITH THE Ah LOCUS

We have now found that the induction of at least 20 monooxygenase "activities" is closely associated with the Ah^b allele (Nebert et al., 1978). Cytochrome P-450-mediated monooxygenases not with the Ah locus include the induction of: aminopyrine N-demethylase, d-benzphetamine N-demethylase, diphenylhydantoin hydroxylase, hexobarbital monooxygenase, aniline hydroxylase, benzenesulfonanilide hydroxylase, chlorcyclizine N-demethylase, ethylmorphine N-demethylase, pentobarbital hydroxylase, and testosterone 7α-, 16α-, and 6β-hydroxylases. Also not associated with the Ah locus is the induction of NADPH-cytochrome c reductase and NADPH-cytochrome P-450 reductase, epoxide hydrase, and GSH S-transferase (Nebert et al., 1978). Other inducible enzymes that are not monooxygenases but that appear to be associated with the Ah^b allele (and therefore may require the cytosolic receptor protein) include microsomal UDP glucuronosyltransferase (Owens, 1977), cytosolic reduced NAD(P):menadione oxidoreductase (Kumaki et al., 1977), and cytosolic ornithine decarboxylase (Nebert and Oka, 1976). This concept of a class of inducers binding to a cytosolic receptor with very great affinity, thereby evoking a "pleiotypic response," has been discussed elsewhere in greater detail (Nebert et al., 1978; Kumaki et al., 1977).

Several lines of evidence indicate that at least two different AHH activities exist (Nebert et al., 1975) and are associated with different forms of P-450: the enzyme from MC-treated responsive mice associated with cytochrome P_1-450; and the enzyme from control or phenobarbital-treated responsive and nonresponsive mice, or from MC-treated nonresponsive mice, associated principally with some form(s) other than P_1-450. This finding is extremely important because it is now apparent that different forms of P-450 may generate different ratios of metabolites from the same substrate. Comparing MC versus phenobarbital as the inducer in rat liver, for example (Figure 4), various groups have shown that hydroxylations may occur predominantly in different chemical positions on the molecule for such substrates as biphenyl, testosterone, 2-acetylaminofluorene, bromobenzene, n-hexane, and benzo(a)pyrene. Such differences in the metabolite profile of a polycyclic hydrocarbon or other foreign chemical reflect presumed differences in the nature of the intermediates formed; differences in the reactivity of these intermediates might result in marked dissimilarities in the toxicity or carcinogenicity of a given compound.

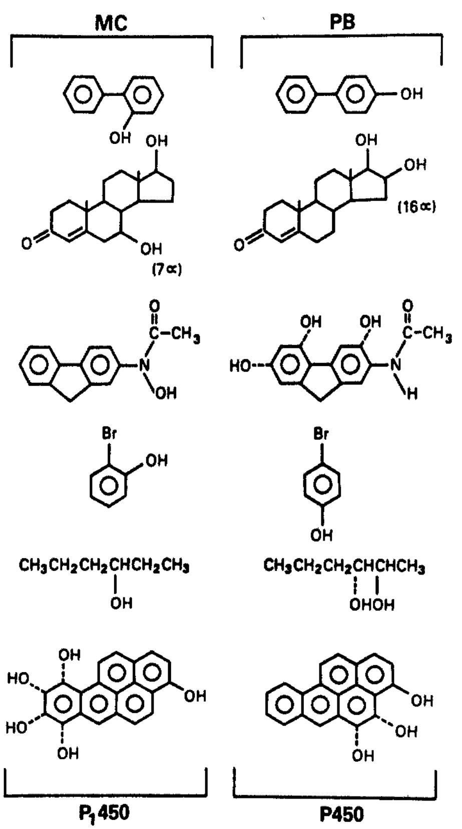

Figure 4. Chemical structures of known differences in metabolite formation when each of these six substrates is oxygenated in vitro with liver microsomes from MC- or phenobarbital-(PB) treated rats (Thorgeirsson and Nebert, 1977). These products are not formed exclusively by one or another form of cytochrome, but there is overlapping of substrate specificity, i.e. an increased appearance of these products when MC treatment is compared with PB treatment, or when microsomes from MC-treated rats are compared with microsomes from control rats. Similar differences in metabolite profile exist in mice for biphenyl, 2-acetylaminofluorene, and BP, but not for testosterone or bromobenzene (Thorgeirsson and Nebert, 1977; Nebert et al., 1975). To our knowledge n-hexane metabolites have not been examined in MC- and PB-treated mice. (Reproduced with permission from Academic Press, Inc.)

A responsive polycyclic hydrocarbon-treated mouse is therefore subject to both quantitative and qualitative increases in the steady-state levels of certain reactive intermediates, because of both an increase in cytochrome(s) P_1-450 content and an increased P_1-450/P-450 ratio in numerous tissues. This relative content of P_1-450, compared with other forms of P-450, may be especially large (e.g. ratios of 10:1 or 50:1) in tissues such as skin and lung but never reaches even a 1:1 ratio in liver (Kahl et al., 1976). This relatively large change in the profile of extrahepatic cytochrome(s) P-450, compared with smaller increments of change in epoxide hydrase (Oesch, 1976), and GSH S-transferase (Jerina and Bend, 1977) activities with BP as substrate, might be a factor in explaining why polycyclic hydrocarbons cause tumors in skin and lung but rarely in liver. This marked increase in the extrahepatic P_1-450/P-450 ratio might also be important in explaining the dramatic genetic differences that we observe in bone marrow toxicity and cataract formation (Nebert et al., 1977).

Fibrosarcomas initiated by subcutaneously administered MC are associated with genetically aromatic hydrocarbon responsiveness among 14 inbred strains of mice (Figure 5). Table 1 demonstrates that the carcinogenic index for subcutaneous MC in offspring from crosses involving the B6 and D2 inbred parental strains is, in fact, associated with the Ah^b allele: the carcinogenic index is greater than 42 in all responsive phenotype groups and less than 12 in all nonresponsive phenotype groups.

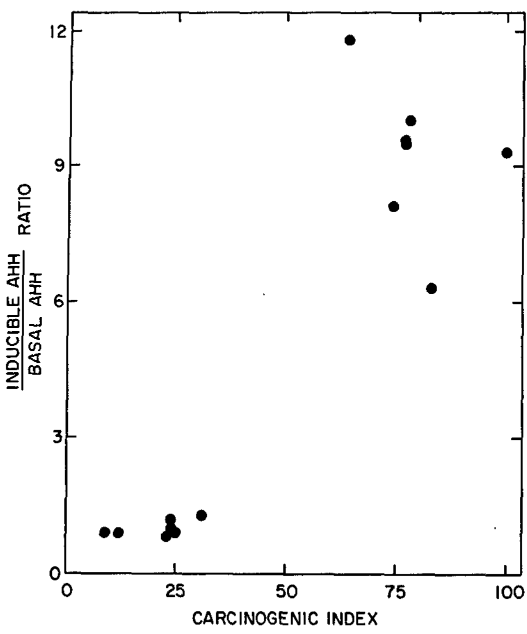

Figure 5. Relationship between the carcinogenic index (Iball, 1939) for subcutaneous MC and the genetically mediated induction of AHH activity by MC for each of 14 inbred mouse strains (Nebert et al., 1974). The correlation coefficient is 0.90 ($P<0.001$). Each closed circle represents the average result from a group of 30 inbred mice of a certain strain. The carcinogenic index was evaluated after a subcutaneous dose of 150 µg of MC had been given to a minimum of 30 weanling mice of each strain. The "inducible AHH/basal AHH ratio" reflects the mean of hepatic AHH activity in MC-treated mice divided by the mean hepatic enzyme activity in control mice ($N \geq 5$ for each of the two groups). Whether the MC-inducible AHH activity in the nonhepatic tissues appears to segregate as a single gene with the inducible hepatic AHH activity has not been examined for all of these strains. (Reproduced with permission from Marcel-Dekker, Inc.)

TABLE 1. RELATIONSHIP BETWEEN AROMATIC HYDROCARBON RESPONSIVENESS AND SUSCEPTIBILITY TO SUBCUTANEOUS MC- AND BP-INITIATED TUMORS AMONG OFFSPRING FROM APPROPRIATE CROSSES INVOLVING THE B6, C3 AND D2 STRAINS OF MICE.

Animals received as weanlings 150 μg of MC or BP in trioctanoin subcutaneously, and the carcinogenic index was determined over an 8-month period (Kouri and Nebert, 1977). The carcinogenic index is defined (Iball, 1939) as the percent incidence of subcutaneous fibrosarcomas, divided by the average latency in days, times 100. Further experimental details are described elsewhere (Kouri et al., 1974; Kouri, 1976). (Reproduced with permission from Cold Spring Harbor Press.)

Strain or Offspring	Expression at Ah locus[a]	Carcinogenic index for MC	Strain or Offspring	Expression at Ah locus[a]	Carcinogenic Index for: MC	Carcinogenic Index for: BP
B6	++	61	C3	++	73	56
D2	0	11	D2	0	10	4
$B6D2F_1$	++	43	$C3D2F_1$	+	37	19
F_1 x B6	++	58	F_1 x C3	++	74	27
				+	60	24
F_1 x D2	++	54	F_1 x D2	+	46	1
	0	8		0	9	1
F_2	++	63				
	0	6	F_2	++	69	31
				+	61	7
				0	17	2

[a]The phenotypic expression at the Ah locus is ranked as: ++ = fully responsive, 0 = nonresponsive, + = -ntermediate responsive, as judged by the data illustrated in Figure 2.

With respect to the carcinogenic index for subcutaneous MC in offspring from crosses involving the C3 and D2 lines, however, unexpected values in Table 1 can be seen. Although the intermediate phenotype has intermediate carcinogenic index values among $(C3D2)F_1$ individuals and among offspring from the $(C3D2)F_1$ x D2 backcross (37 and 46, respectively), the values among progency of the $(C3D2)F_1$ x C3 backcross and the $(C3D2)F_2$ generation are more susceptible to MC-initiated tumors than can be accounted for by their inducible AHH activity alone (carcinogenic indices of 60 and 61, respectively). There was also nearly a doubling (carcinogenic index of 17) in nonresponsive F_2 individuals. We conclude that there probably exist other genes carried by the C3 mouse that make this strain particularly sensitive to MC tumorigenesis.

The carcinogenic index for subcutaneous BP (far right of Table 1) is disproportionately low among $(C3D2)F_1$ progeny (value of 19) and among all offspring from both backcrosses and the $F_1 \times F_1$ intercross. The intermediate phenotype of the $(C3D2)F_1 \times D2$ backcross is particularly resistant to BP tumorigenesis, having a carcinogenic index (value of 1) lower than that for the D2 parent. It seems likely that the D2 strain carries other genes that confer even a higher resistance to BP-induced tumors than would be expected from their AHH content alone. Nonetheless, the Ah locus still plays a major role in the susceptibility of these animals to BP tumorigenesis, because both the $(C3D2)F_1 \times C3$ progeny and the $(C3D2)F_2$ generation demonstrate a close association between tumor susceptibility caused by BP and inducible AHH activity. Thus, though some other genes may also influence susceptibility to BP- and/or MC-initiated tumors, the primary determinant for cancer susceptibility is the allele(s) regulating inducible AHH activity in numerous tissues of the mouse. Among recombinant inbred sublines having C57BL/6N and AKR/N as the progenitor strains, in which the lack of induction is expressed as an autosomal dominant trait, susceptibility to MC-initiated tumors remains linked with inducible AHH activity (Nebert et al., 1978).

A statistically significant ($p<0.01$) correlation between lung tumors produced by intratracheal MC and the Ah^b allele was recently demonstrated (Kouri et al., 1977). This correlation is most clearly seen in offspring from the $(B6D2)F_1 \times D2$ backcross, in which the responsive individuals were observed to be more than three times more susceptible to lung cancer than the nonresponsive individuals. Again, some contribution of genes other than the Ah locus seem to be responsible for the increased susceptibility to MC-initiated pulmonary tumors found in the F_1 and F_2 offspring and the progeny from both backcrosses (Kouri and Nebert, 1977). Most likely genes controlling DNA repair, susceptibility to oncogenic virus infection, or immunological surveillance (e.g. differences in the H-2 locus) may be important in the overall susceptibility of certain tissues of an individual to chemically-induced cancer (Figure 6). Although these other genes may also influence susceptibility to BP- and/or MC-initiated tumors, however, one primary determinant for cancer susceptibility is the Ah^b allele regulating inducible AHH activity in numerous tissues of the mouse.

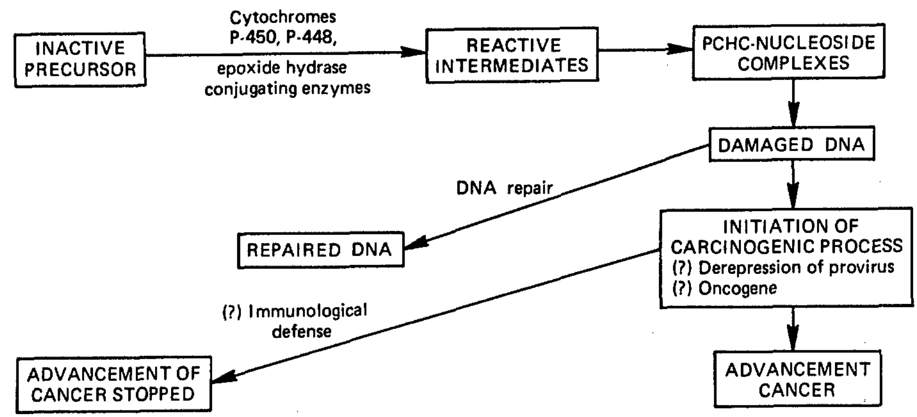

Figure 6. Hypothetical scheme by which polycyclic (PCHC) may initiate tumorigenesis (Nebert et al., 1977). Following metabolic activation by the various drug-metabolizing enzymes, interaction of reactive intermediates with nucleic acid may damage the DNA. The advancement of cancer, however, most likely can still be prevented by the host's mechanisms of DNA repair and immunological competence. (Reproduced with permission from Plenum Press Corporation.)

Hepatic and nonhepatic AHH activity and its associated cytochrome P_1-450 can be stimulated in nonresponsive inbred strains by the potent inducer TCDD to levels just as high as those in responsive strains; however the ED_{50} is approximately 15 times higher in nonresponsive strains than in responsive strains (Nebert et al., 1978). The carcinogenic index for subcutaneous MC is increased in TCDD-treated nonresponsive D2 mice to more than half of that of B6 mice in the presence or absence of TCDD (Kouri et al., 1978). We believe the most likely explanation for this effect is that TCDD acts as a cocarcinogen by inducing P_1-450 in the nonresponsive mouse. The newly induced cytochrome is now capable of metabolizing MC to the ultimate carcinogen more readily (Kouri et al., 1978).

In summary, MC and BP are either metabolized to higher steady-state levels of a proximal or ultimate carcinogenic intermediate(s) in the subcutaneous connective tissue or lung of responsive mice because of increased P_1-450 content in responsive mice, compared with nonresponsive mice, or are predominantly metabolized to a particular proximal or ultimate carcinogen(s) because of a marked change in the P_1-450/P-450 ratio.

THE Ah LOCUS AND GENETIC DIFFERENCES IN DRUG TOXICITY IN THE MOUSE

In addition to tumors, polycyclic hydrocarbons and other chemicals and drugs cause toxicity under varying experimental conditions, presumably related to metabolic potentiation by cytochrome(s) P_1-450 controlled by the Ah locus (Table 2). 7,12-Dimethylbenz(a)anthracene causes skin ulcers more readily in responsive strains than in nonresponsive strains; the offspring of the backcrosses and intercross were never studied (Trell et al., 1976), but most likely this condition is associated with the responsive allele at the Ah locus. Shortened survival time after large intraperitoneal doses of polycyclic hydrocarbons and birth defects likewise appear to be associated with the responsive allele at the Ah locus and are presumably due to increases steady-state levels of toxic polycyclic hydrocarbon reactive intermediates. On the other hand, increased metabolism, associated with the Ah^b allele, is responsible for detoxification in the experimental conditions of: shortening the effect of zoxazolamine, protecting the animal from acute lindane toxicity, preventing aplastic anemia caused by daily ingestion of BP and preventing leukemia caused by topical MC (Duran-Reynals et al., 1978) or oral BP (Nebert and Gordon, in preparation). Metabolic potentiation of acetaminophen, associated with an increase in N-hydroxylation by cytochrome(s) P_1-450, leads to glutathione depletion, increased covalent binding of reactive intermediates, hepatotoxicity, and cataractogenesis.

Specific increases in the mutagenicity of MC, 6-aminochrysene, and 2-acetylaminofluorene in vitro are associated with cytochrome(s) P_1-450 and the Ah^b allele. Although the mutagenicity of BP in TA1535, TA1537, and TA1538 bacterial tester strains was not found (Nebert and Felton, 1975) to be associated with the Ah^b allele, more recent data (Levvit et al., 1977) with TA98 demonstrate such a correlation.

BP metabolite-nucleoside complexes formed by incubating radioactive BP with liver or skin microsomes and DAN in vitro can be separated by column chromatography and, in some cases, partially identified; increases in eight out of nine such peaks are associated with cytochrome(s) P_1-450 and the Ah^b allele (Nebert et al., 1977). If one compares the responsive C3 with the responsive B6 inbred strain, there is a 5- to 6-fold higher carcinogenic index for subcutaneous BP in the C3 (Table 1). This finding in vivo is not demonstrable in vitro by either the mutagenesis assay or the binding of BP metabolites to DNA nucleosides (Nebert et al., 1977), indicating the difficulty that still exists in attempting to predict in vivo carcinogenicity of a compound by in vitro test systems.

TABLE 2. TUMORIGENIC OR TOXICOLOGIC PHENOMENA ASSOCIATED WITH AROMATIC HYDROCARBON RESPONSIVENESS IN MICE

	References
A. Increased susceptibility to:	
a. MC-initiated subcutaneous sarcomas	Kouri et al., 1974
b. Squamous cell carcinoma of the lung produced by intratracheal instillation of MC	Kouri et al., 1977
c. BP-initiated subcutaneous sarcomas	Kouri, 1976
d. Hepatic necrosis due to acetaminophen	Thorgeirsson et al., 1975
e. Skin inflammation due to 7,12-dimethylbenz(a)anthracene	Thomas et al., 1973
f. Fetal toxicity and malformations due to administration of MC, 7,12-dimethylbenz(a)anthracene, or BP to the mother	Shum et al., 1977
g. Cataractogenesis caused by acetaminophen	Shichi et al., in preparation
h. Primordial oocyte toxicity due to MC	Mattison and Thorgeirsson, 1977
B. Shortened survival time following:	
a. Intraperitoneal administration of polycyclic aromatic hydrocarbons or polychlorinated biphenyls	Robinson et al., 1975
b. Oral administration of polychlorinated biphenyls	" "
C. Increased resistance to:	
a. Intraperitoneal administration of lindane	" "
b. Oral administration of polycyclic aromatic hydrocarbons or lindane	" "
c. Bone marrow toxicity produced by oral BP	Nebert et al., 1977; Robinson et al., 1975
d. Paralysis produced by zoxazolamine	Robinson and Nebert, 1974
e. Leukemia produced by topical MC	Duran-Reynals et al., 1978

TABLE 2. (CONTINUED)

	References
D. Increased mutagenicity in vitro by:	
a. MC, 6-aminochrysens, and 2-acetylaminofluorene	Nebert and Felton, 1975
b. BP	Levvit et al., 1977
E. Increased binding of specific metabolites to DNA:	
a. BP	Nebert et al., 1977
b. MC, 7,12-dimethylbenz(a)anthracene, dibenz(a,h)-anthracene, 2-acetylaminifluorene, benzidine, and estrone	Pelkonen et al., 1978

EVIDENCE FOR THE Ah LOCUS IN MAN

As repeatedly emphasized, the mouse genetic model of cytochrome P_1-450 inducibility has been an extraordinarily useful tool in elucidating the functions and properties of the mammalian monooxygenase system, especially with regard to the mechanisms of drug toxicity and chemical carcinogenesis. Another ramification of the model, however, is that it points up the likelihood of genetic heterogeneity among other mammals, including man.

In 1973 two published reports (Kellermann et al., 1973a; Kellermann et al., 1973b) gave initial hope to clinical geneticists and oncologists. The extent of AHH induction in cultured mitogen-activated lymphocytes by MC was examined in 353 healthy subjects, ranging in age from 2 to 89 years old and including 67 families with 165 children (Kellermann et al., 1973a). The distribution of inducibilities in the patients tested in the Houston area was trimodal, the groups being designated "low," "intermediate," and "high" inducible. The data were consistent with a hypothesis of two alleles at a single locus, and gave an excellent fit to the Hardy-Weinberg equilibrium, with a frequency of 0.717 for the "low-inducibility" allele and 0.283 for the "high-inducibility" allele (although the sample is biased by including parents and siblings. Fifty patients with bronchogenic carcinoma were then compared with 46 patients having other types of tumors and with 85 healthy controls (Kellermann et al., 1973b).

The authors concluded that a person having the "intermediate" phenotype has a 16 times increased risk, and a person having the "high" phenotype has a 36 times increased risk, of developing bronchogenic carcinoma, compared with persons having the "low" inducibility phenotype.

Because of the day-to-day variability of the lymphocyte AHH assay, several laboratories (Kouri and Nebert, 1977; Nebert et al., 1978) have experienced difficulty in confirming the reproducibility of the assay and the trimodal distribution. In spite of this, however, there have been recent claims of laryngeal carcinoma (Trell et al., 1976) and bronchogenic carcinoma (Guirgis et al., 1976) associated with the high inducibility phenotype. The former report (Trell et al., 1976) suffered from a major shortcoming: the values for patients free of cancer were not done under the same conditions as the patients with cancer. In fact, control cancer-free patients in Lund, Sweden, were not studied at all, but rather the incidence of AHH inducibility phenotypes was taken directly from the studies of Kellermann and coworkers (1973b) in Houston, Texas. In view of the large assay-to-assay variation in AHH Levels observed even under exactly the same culture conditions (Kouri et al., 1974; Gurtoo et al., 1975; Atlas et al., 1976; McLemore et al., 1977), the comparison of data from different laboratories under different culture conditions, lots of fetal calf serum, etc., is quite unrealistic. Other laboratories have failed to observe an association between an increased risk for bronchogenic carcinoma and AHH inducibility in cultured mitogen-activated lymphocytes (Paigen et al., 1977) or in pulmonary macrophages (McLemore et al., 1977). In spite of numerous difficulties with the cultured lymphocyte model system (Kouri and Nebert, 1977; Nebert et al., 1978), however, consistent differences between two individuals can be found if the samples are always carried together through the same experiment; this fact probably explains the recent demonstration (Figure 7) of a heritable component of AHH inducibility in cultured lymphocytes of monozygotic and dizygotic twins. The successful development of an assay for determining the human phenotype at the Ah locus would be the first biochemical marker of its kind, because of its potential utility in predicting increased susceptibility to certain types of environmentally caused cancers in man.

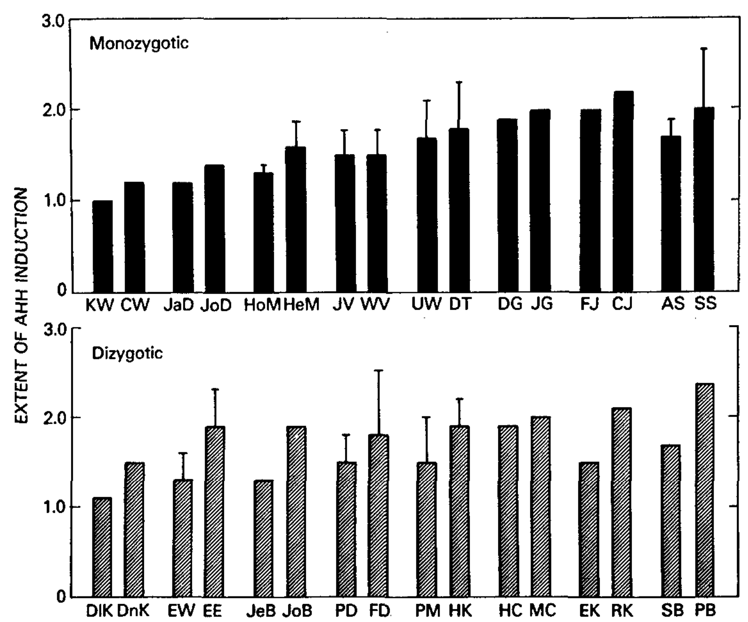

Figure 7. Variance in the extent of AHH induction in cultured mitogen-activated lymphocytes from identical and fraternal twins (Atlas et al., 1976). Vertical brackets represent standard deviations in those pairs whose lymphocytes were tested in at least three separate experiments. A single experiment originated from a single drawing of blood, and two to five determinations of AHH activity were each carried out on MC- and control cultures. Each twin pair was studied simultaneously under identical conditions. The AHH assay was performed at pH 8.5. (Reproduced with permission from The Williams and Wilkins Company.)

In a recent study (Brown et al., 1976) in which benz(a)-anthracene or benz(a)anthracene plus aminophylline were used as inducers, the gene controlling AHH induction was assigned to human chromosome 2. Yet, it has also been reported (Huberman et al., 1976) that two distinctly different forms of the hydroxylase activity -- one induced by benz(a)anthracene and one induced by dibutyryl cyclic AMP plus theophylline --are under independent genetic regulation. Furthermore, there is no way of telling in this study (Brown et al., 1976) whether the regulatory gene for the human cytosolic receptor on chromosome 2, for example, "derepressed" the hamster structural gene for P_1-450. In somatic-cell hybrids formed by Sendai-virus-augmented fusion of "responsive" mouse 3T3 fibroblasts (in which AHH is inducible by benz(a)anthracene and of "nonresponsive" rat hepatoma cells (in which AHH is not induced), the induced hydroxylase levels ranged from the same as, to more than 20-fold greater than, inducible AHH in the 3T3 parent (Benedict et al., 1972). The most likely interpretation of these data (Benedict et al., 1972) is that mouse gene(s) "depressed" the rat hepatoma structural gene for P_1-450, because the induced specific AHH activity in some of the somatic-cell hybrids was more than 6 times greater than that ever seen

in any nonhepatic tissue in culture. Although "mouse 3T3 fibroblast AHH" could not be distinguished from "rat hepatoma AHH" at that time (Benedict et al., 1972), recent advances in electrophoretic techniques will allow us to test this hypothesis within the year. If the regulatory gene for AHH induction is part of the "structural gene cluster" for AHH, as has been shown (Paigen et al., 1975) to be the case for the "β-glucuronidase cluster" in mice, it might be argued (Brown et al., 1976) that the regulatory and structural genes for AHH activity both reside on human chromosome 2. In view of the trans genetic regulation of the Ah locus evoking increases in numerous structural gene products (as discussed in previous sections of this paper) in the mouse, however, it is certainly too premature to suggest that the regulatory Ah gene and the AHH structural gene (i.e. gene for P_1-450 apoprotein) are closely linked--in either the mouse or the human.

In conclusion, there exists sufficient evidence that heritable variation of AHH inducibility occurs in man. Experimental difficulties, however, make it impossible at this point in time to be certain of whether AHH induction is controlled by a single genetic locus or by two or more loci (i.e. polygenic).

In view of the high ratio of P_1-450 in all likelihood in extrahepatic tissues in vivo--just as appears to be the case in cultured lymphocytes, monocytes, pulmonary macrophages, or even skin fibroblasts--further studies of AHH induction in such cultures or other human cells are indicated. For example, although one's risk for bronchogenic carcinoma might not be singularly correlated with the Ah locus, an association between the Ah locus and other types of cancer (e.g. laryngeal or oral or colon carcinoma) or toxicity (e.g. retinitis pigmentosa, idiopathic aplastic anemia or leukemia) might be demonstrable. Improvements in the in vitro or biological assay are still required for attaining reproducibility in determining the AHH phenotype. Until one can increase the range of fold inducibility of AHH activity and/or decrease the amount of experimental "noise" (i.e. nonspecific factors contributing to artifactual induction of "control" AHH activity, making it difficult to determine accurately the true basal enzyme activity), however, AHH inducibility in cultured mitogen-activated lymphocytes or any other similar test system cannot be used as a biochemical marker for determining who is at risk for bronchogenic carcinoma or other cancers.

SUMMARY

The Ah locus controls the induction of at least 20 monooxygenase "activities" and associated cytochrome(s) P_1-450 by MC and numerous other polycyclic aromatic compounds. AHH induction is associated with P_1-450 induction. One product of the regulatory Ah gene is believed to be a cytosolic receptor protein, which has a high affinity (apparent $K_d \cong 1$ nM) for polycyclic aromatic inducers and which appears to be defective (i.e. diminished affinity) in nonresponsive mice. Other induced macromolecules that appear to be under the same regulatory control include microsomal UDP glucuronsyltransferase, cytosolic reduced NAD(P):menadione oxidoreductase, and cytosolic ornithine decarboxylase.

Regulation of responsiveness probably involves several alleles at more than one locus, but differences between C57BL/6 (responsive, Ah^b) and DBA/2 (nonresponsive, Ah^d) mice can be almost completely explained by the difference at the Ah locus. Heterozygotes (Ah^b/Ah^d) are responsive, but other genetic crosses between appropriate inbred mouse strains can result in the expression of additive inheritance or a situation in which the lack of responsiveness is dominant. Responsiveness occurs not only in liver but also in numerous nonhepatic tissues such as lung, kidney, bowel, skin, lymph nodes, pigmented epithelium of the retina, bone marrow, ovary, and mammary gland. Compared with Ah^d/Ah^d mice, Ah^b/Ah^b and Ah^b/Ah^d individuals have: a high inflammatory response to topical application of 7,12-dimethylbenz(a)anthracene; a high susceptibility to MC- and BP-induced subcutaneous sarcomas and MC-induced lung tumors; an increased resistance to zoxazolamine-induced paralysis, lindane toxicity, and polycyclic hydrocarbon-induced bone marrow toxicity and leukemia; and an increased susceptibility to acetaminophen-induced hepatic necrosis and cataract formation and to polycyclic hydrocarbon-induced birth defects, stillborns, resorptions, decreased birth weight, and ovarian primordial oocyte depletion. The Ah^b allele is associated in vitro with a high mutational rate in <u>Salmonella</u> by metabolic activation of several chemical carcinogens and increases in numerous specific metabolites of chemical carcinogens bound to DNA nucleosides.

There exists sufficient evidence that heritable variation of AHH inducibility occurs in man. Experimental difficulties in the day-to-day variability of the AHH assay with cultured lymphocytes or monocytes, however, make it impossible at this time to be certain of whether this induction process is controlled principally by a single gene. It therefore remains to be determined whether this genotype can ever be used as a biochemical marker for predicting increased susceptibility to certain types of environmentally caused cancers or toxicity in man.

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PAPER NO. 11

MOLECULAR TOXICOLOGY OF FLUOROCITRATE

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INTRODUCTION

The pioneering work of Peters and his school (Pattison and Peters, 1966) established that the neurotoxic agent fluorocitrate, which is one of the most widely used rodenticides, exerts its toxic action by being converted in the animal body to fluorocitric acid. This discovery was made at a time when the recognition of the citric acid (Krebs) cycle in biochemistry had its greatest impact on biology. The Krebs cycle was correctly seen as the system which in enzymological terms can explain the interconversion of carbohydrates, fatty acids and amino acids. In the enthusiasm of their success proponents of the Krebs cycle proclaimed its capability of also predicting cellular regulation of metabolic and bioenergetic events. Once this seemingly convincing notion had been accepted, the idea was automatically replicated in textbooks of biochemistry and the matter assumed an axiomatic character, and thus dipped below the horizon of scientific problem searching. A further, apparently compelling, argument emerged with the discovery that fluorocitric acid (obtained by Peters as a rather complex mixture of isomers) actually inhibited an enzymatic component of the citric acid cycle, aconitase (Morrison and Peters, 1954). This effect provided a plausible explanation of the mode of action of fluorocitrate which was immediately accepted because of its timeliness. The multienzymatic kinetics of the "metabolic interconversion" system of mitochondria, the Krebs cycle, is an unsolved problem (Kun, 1969), and its elusiveness still puzzles biophysicists who hope-perhaps in vain--to explain cell physiology in relatively simple enzymological terms. At the time of Peters' discovery the precise chemistry of fluorocitric acids, the enzymology of aconitase, and the distributive nature of the Krebs cycle were poorly understood. The relatively simplified notion of envisioning the Krebs cycle as a linear multienzymatic pathway would imply that inhibition of any enzymatic component necessarily results in an obligatory cessation of the pathway, and thus would predict cell death as a result of deprivation of ATP. It would follow that fluorocitrate should be a universal metabolic

poison acting indiscriminately on any cellular system which contains mitochondria, the well established site of the Krebs cycle. Since fluorocitrate does induce citrate augmentation as a symptom of "inhibition of the Krebs cycle," a further argument was at hand which reinforced the aconitase inhibition related mode of toxic action of fluorocitrate.

This investigator's interest in the regulatory mechanisms of cellular functions arose from direct experiences in the field of enzyme chemistry. The reasonably clearcut catalytic events promoted by metabolic enzymes,--components of the citric acid cycle,--and the remarkable excess of most enzymatic components as related to overall metabolic rates (Sols and Gancedo, 1972) seemed to this investigator to be in gross contradiction to the tacit assumption that regulation of cellular metabolism is in quite general terms directly related to the catalytic activity of metabolic enzymes. It has not escaped the attention of specialized biochemists (Sols and Gancedo, 1972) that indeed substrate concentrations, not enzyme content alone, were the real critical rate limiting factors in cell physiology. Ordinarily the molar ratios of substrates to enzyme concentration are much larger numbers in the cell than in the customary in vitro models of "cuvette-enzymology." These apparent minutiae escaped the attention of most textbook writers, and thus simplified views of metabolic biochemistry prevailed and so did the cardinal achievement of biochemical toxicology, which explained the toxic action of fluorocitrate by the inhibition of aconitase.

Pharmacologists never quite accepted this enzymologic dogma. The primary experience of pharmacologists and toxicologists is that of a high degree of specificity (e.g. neurotoxicity), not compatible with universal metabolic events which seemed much too monotonous to explain cell or organ function. The scientifically more comprehensible and experimentally straightforward field of biochemistry with its technically persuasive powers remained in apparent conflict with the complex phenomenology of pharmacology, and the intellectual rift between these traditional disciplines sustained a feeling of dissatisfaction among those who wished for rational answers in complex fields. The establishment of the journal Molecular Pharmacology by the Publications Committee of the American Society for Pharmacology and Experimental Therapeutics expressed a conscious attempt to bridge the gap between molecular sciences and the vastly complex and challenging phenomenology of drug actions.

Early Experimental Approaches

It is a remarkably unrewarding situation to be confronted with conventionalism of biochemical knowledge on one hand and confusing phenomenology of toxicology on the other. Since the specific example, the scientific status of the biochemistry of fluorocitric acid in the early 1960's--to the taste of this investigator--was quite unsatisfactory, work had to be started at the basic chemical level.

According to enzyme chemistry the condensing enzyme can synthesize fluorocitric acid either from fluoro-acetyl-CoA + oxalacetic acid or from fluorooxalacetic acid and acetyl CoA. It was shown in our laboratory that the fluorocitric acid isomer, which was synthesized from fluoroacetyl CoA + OAA, was the only toxic species (Fanshier et al., 1962; Fanshier et al., 1964), corresponding to (-)erythrofluorocitric acid as identified by chemical synthesis and stereochemical resolution (Dummel and Kun, 1969) as well as x-ray diffraction analysis (Carrell et al., 1970; Carrell and Glusker, 1973). Solution of the next question, the enzymologic action of (-)erythrofluorocitrate, was undertaken in several laboratories including ours (Kun, 1976) and with purified aconitase under proper conditions a strictly reversible linearly competitive inhibitory mechanism was established with K_i values between 60-260 μM, with respect to citrate. This inhibitory constant implies that inhibition of aconitase should be reversed by citrate; therefore, the well known phenomenon of citrate accumulation during fluorocitrate poisoning should reverse toxicity if inhibition of aconitase is the molecular cause of toxicity. This prediction is clearly wrong since no reversal of fluorocitrate toxicity has even been observed; furthermore, no quantitative correlation between K_i (aconitase) and LD_{50} values could be established, the latter estimated to be 4-6 orders of magnitude lower than predicted from K_i. We have performed another critical experiment. Assuming that inhibition of aconitase is a lethal mechanism, it was equally plausible to postulate that inhibition of another member of the Krebs cycle enzymes should result in a fatal outcome in vivo. Monofluoro-oxalacetic acid inhibits malate dehydrogenase competitively with a K_i of 0.1 μM (Kun, 1976), and thus should be 10^4 more toxic than (-)erythrofluorocitric acid. In vivo administration of fluoro-oxalacetic acid to mice in doses which inhibited 99% of malate dehydrogenase had no visible toxic consequences, although hyperglycemia and other metabolic perturbations clearly indicated that the inhibitor reached malate dehydrogenase sites in tissues. Important work of Gal (1972) and Morselli et al. (1968) disclosed that the mitochondrial concentration of fluorocitrate in lethally poisoned rats had to be 10^{-8}M or smaller; therefore, a mitochondrial toxic site of fluorocitrate had to be postulated which was sensitive to fluorocitrate at a level of about 50 pmoles of fluorocitrate/mg mitochondrial protein.

Identification of the Mitochondrial Site of Action of (-)Erythrofluorocitrate

It was found in our laboratory in 1970-1972 that preincubation of isolated mitochondria with 30 to 60 pmoles of (-)erythrofluorocitrate per mg mitochondrial protein for 5 to 10 minutes resulted in an almost complete inhibition of isocitrate efflux from mitochondria when mitochondria were subsequently exposed to citrate or cis-aconitate (Kun, 1972; Eanes et al., 1972; Kun, 1976). These results indicated that influx of these tricarboxylic acids was blocked by preincubation with minute amounts of (-)erythrofluorocitric acid. As found subsequently (Kirsten et al., in press) the similar behavior of citrate and cis-aconitate was due to contamination of mitochondria with cytoplasmic aconitase (Eanes and Kun, 1971) and after removal of this contaminant the inhibition became absolutely specific for citric acid. When the stringent conditions of preincubation with fluorocitrate were not followed and citrate flux was monitored for short periods only, inhibition by fluorocitrate could not be observed (Brandt et al., 1973). Because of faulty kinetic analyses (Kun, 1976) the inhibitory effect of fluorocitrate on mitochondria was ascribed to aconitase, and contradictory views remained unsettled (Brandt et al., 1973)

The problem of the site of action of (-)erythrofluorocitric acid on mitochondria was finally clarified in 1975-1977. One of the critical technical advances which promoted this work was related to the development of a technique of isolation of mitochondria which assured the stability of the inner membrane against decay by lysosomal enzymes (Kun, 1976; Kun et al., 1978). Another technical advancement was made by developing kinetic assays for citrate influx and efflux in mitochondria under conditions which closely resemble the intracellular physiological situation. Influx was monitored in maximally phosphorylating mitochondria by measuring citrate dependent ATP synthesis. Efflux was determined by coupling intramitochondrially generated citrate to the cytoplasmic fatty acid synthesizing system (Kirsten et al., 1971). As shown in Table 1, preincubation of mitochondria (1 mg protein) with 30 pmoles (-)erythrofluorocitric acid equally inhibited both citrate influx and efflux. The important aspect of these results is that the concentration of (-)erythrofluorocitrate used was exactly in the same order of magnitude as found in mitochondria of lethally poisoned rats (Gal, 1972; Morselli et al., 1968), thereby ruling out the ever prevailing difficulty of artificiality so often encountered in the study of molecular pharmacology. That both influx and efflux were equally inhibited rules out the possible involvement of aconitase because mitochondrial citrate dependent fatty acid biosynthesis is independent from aconitase in terms of metabolic pathways.

TABLE 1. INHIBITION OF MITOCHONDRIAL CITRATE ENTRY AND EXIT BY (-)ERYTHROFLUOROCITRATE (50 PMOLES F-CITRATE/MG PROTEIN, PREINCUBATION AT 30° FOR 15 MIN)

Citrate Entry Into Liver Mitochondria (nmoles/mg protein/min)		Citrate Exit from Mitochondria (pmoles/mg protein/min)	
No substrate	2.2	Control (liver)	87
+ Citrate	7.1	+ F-citrate (liver)	21
+ F-malate	2.2	Control (kidney)	63
Citrate + F-malate	40.0	+ F-citrate (kidney)	17
Citrate + F-malate + F-citrate	5.5		

The absolute specificity of inhibition restricted to citrate only is illustrated in Figure 1. It is also apparent that heart mitochondria do not transport citrate inward, but only citrate export function is maintained in cardiac mitochondria. It is noteworthy that we employed the metabolically inert (-)erythrofluoromalate as a cofactor of citrate influx instead of the physiological activator L-malate.

The next advancement was made possible by the stereospecific enzymatic synthesis of ^{14}C(3-6) (-)erythrofluorocitric acid in pure form, with a specific radioactivity of 50 mCi/mmole (Kirsten et al., in press). It was readily shown with labeled fluorocitrate that covalently bound, protein associated fluorocitrate accumulated in the inner membrane of mitochondria under conditions when irreversible inhibition of citrate transport occurred following preincubation of mitochondria with fluorocitrate. The quantity of protein-bound fluorocitrate varied between 6 and 18 pmoles per mg inner membrane protein.

The nature of the fluorocitrate protein bond was investigated. The bond resisted precipitation by trichloroacetic acid, redissolution in 0.1 N NaOH and reprecipitation, but was specifically cleaved by 0.4 M neutral hydroxylamine. Blocking of protein-thiol groups by mersalyl (an organo-mercurial) inhibited the binding of fluorocitrate to inner membrane proteins.

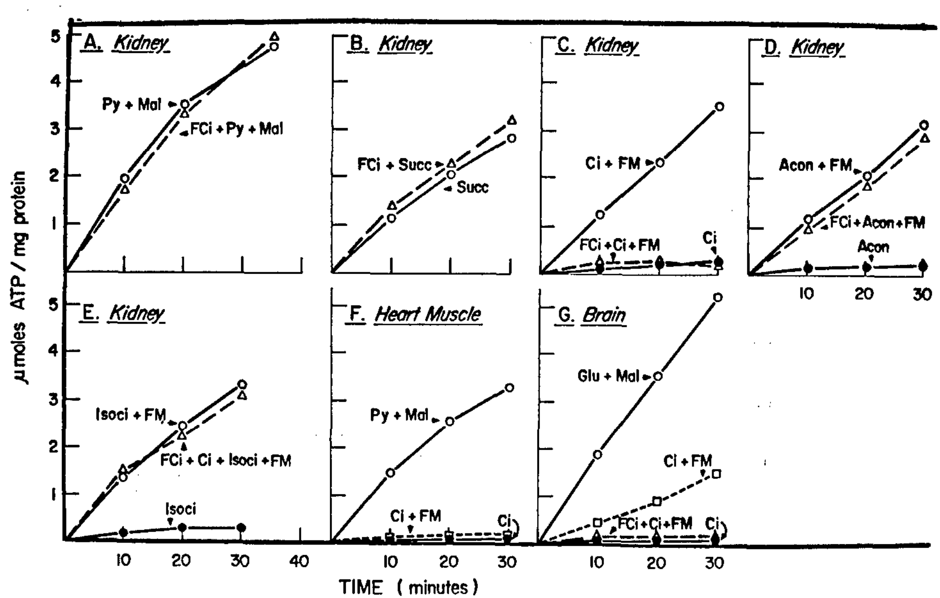


Figure 1. Substrate specificity of the inhibitory effect of (-)erythrofluorocitrate. Mitochondria of kidney, heart and brain were preincubated with 2 mM ADP and 50 pmol (-)erythrofluorocitrate per mg protein for 15 min at 30° prior to measurement of ATP synthesis with various oxidizable substrates, using 0.5 mM (-)erythrofluoromalate as activator of tricarboxylic acid influx as described in Methods (Kirsten et al., in press). The concentration of substrates was 5 mM. Mitochondria from kidney and brain were isolated as described in Methods (Kirsten et al., in press). For the isolation of heart mitochondria, the procedure was modified as follows. The finely chopped rat hearts were ground in a glass-teflon homogenizer with a clearance of 0.15 mm in 8 volumes of medium per g tissue at 0° and coarse particles sedimented at 2700 rpm (Sorvall rotor SS 34) for 1 min. The nuclear pellet was re-extracted once. The combined supernatants were processed further as described in Methods (Kirsten et al., in press). The rate of substrate dependent ATP synthesis was measured ad described in Methods (Kirsten et al., in press. FCi = (-)erythrofluorocitric acid.

The isolation of protein bound fluorocitrate by molecular filtration in 8 M guanidine is illustrated in Figure 2.

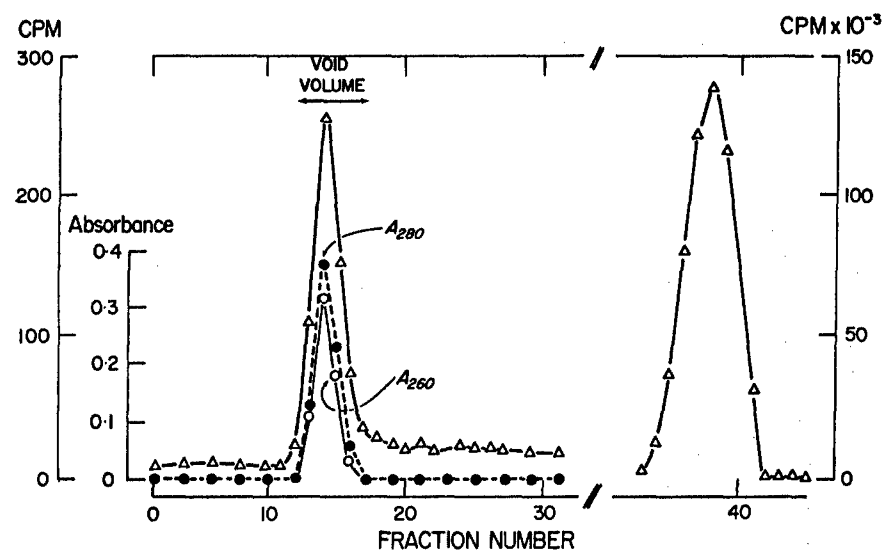


Figure 2. Gel filtration of inner membrane proteins labeled with fluorocitrate in the presence of guanidine hydrochloride. Membrane vesicles (2 mg protein) were incubated with ^{14}C-fluorocitrate (10 μM, total volume 200 μl) for 60 min at 30° in 50 mM tris-HCl buffer, pH 7.2. An aliquot of the incubation mixture (1 mg protein) was solubilized in 8 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.2, and applied to a Sephadex G-50 (medium) column (0.95 x 50 cm), equilibrated and developed at room temperature with the same solvent. Fractions (1.6 ml) were collected at a flow rate of 6.5 ml per h. Absorbances were recorded at 280 nm (...●...●...) and at 260 nm (...O...O...). Radioactivity (cpm) was plotted after subtracting the background (...Δ...Δ...). Gel filtration of macromolecules lasted 14 h. The first radioactive peak contains fluorocitrate bound to protein (void peak, CPM = left ordinate); the second large radioactive fraction is unreacted fluorocitrate (CPM = right ordinate). It was found in control experiments that there was no measurable nonspecific covalent binding of fluorocitrate to nonmitochondrial proteins (e.g., bovine serum albumin) as tested by molecular filtration or acid precipitation (Kirsten et al., in press).

The isolation of fluorocitric acid mono-hydroxamate by high voltage electrophoresis is shown in Figure 3. Isolation of the hydroxamate was performed on a large scale preparation of protein-fluorocitric acid adduct from inner membrane vesicles prepared by molecular filtration without guanidine HCl,

but in the presence of the detergent Brij 56. Proteinase K, a powerful proteolytic enzyme, liberated free fluorocitric acid from the protein-fluorocitrate adduct, indicating that the inherent esterase activity of the protease cleaved the fluorocitrate-protein bond, which therefore had to be an ester (Kirsten et al., in press).

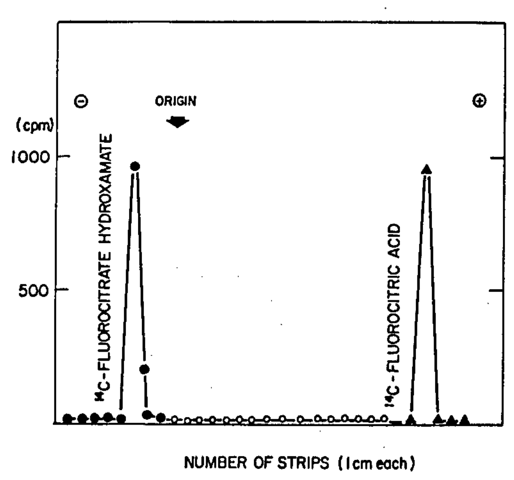


Figure 3. Electrophoretic separation at pH 1.85 of an aliquot of (-)erythrofluorocitric acid and its α-hydroxamate derivative isolated from the fluorocitrate-protein adduct. Time of electrophoresis = 150 min. The technique of electrophoresis is described in Methods (Kirsten et al., in press) and details of isolation in Results (Kirsten et al., in press). The amounts of fluorocitrate and its hydroxamate corresponded to 113 pmol (1,000 CMP). The fluorocitrate α-hydroxamate migrated slowly towards the cathode, whereas fluorocitric acid migrated towards the anode.

The evidence obtained points to the existence of an important novel bond formed between the α-COOH group of fluorocitrate (activated by F-C) and a thiol group of membrane proteins, identifying this bond as a macromolecular thiolester. This is the first time that this macromolecular chemical bond has been observed. As shown later, identification of this bond provided significant clues for the development of an antidote to fluoroacetate poisoning. Fluorocitric acid forms thiolesters with two mitoplast proteins belonging to either 171,000 dalton or 71,000 dalton molecular species. This is shown in Figure 4.

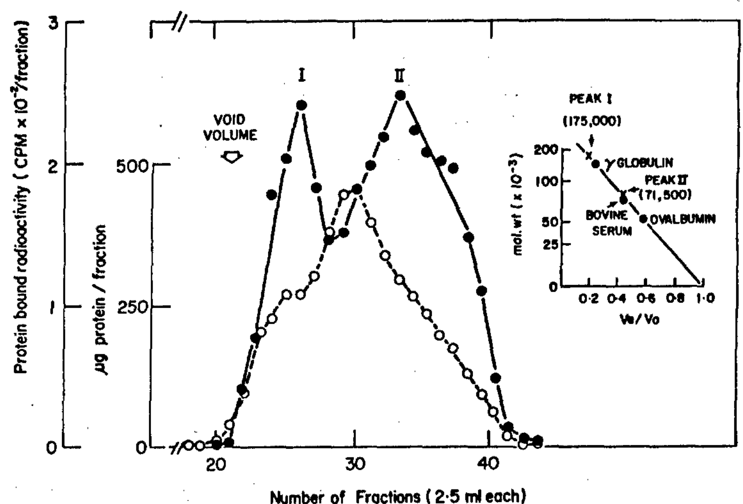


Figure 4. Molecular filtration of protein-bound fluorocitrate on Sephadex G-200 in the absence of detergent. An extract of mitoplasts (in the presence of Brij-56) was concentrated by cone filtration and incubated in a volume of 300 μl (4.8 mg protein) for 60 min with 100 μM labeled fluorocitrate at 30°, pH 7.39. The solution was directly applied to a Sephadex G-200 column (see Methods, Kirsten et al., in press) and developed at 4° for 12 h with 50 mM Tris-Hepes buffer at pH 7.4. Total protein (...O...O...) and radioactive fluorocitrate-containing protein (___●___●___) were determined in each fraction. Protein-bound fluorocitrate was assayed as described in the legend to Figure 2. The molecular weight of radioactive fluorocitrate containing proteins were estimated from a semilogarithmic plot (inset to Figure 4) by comparison with standards.

The mechanism of macromolecular association of fluorocitrate with two mitoplast proteins is illustrated in Figure 5. It became evident that the binding process was enzymatic and was activated by Mn^{2+}. This was a highly significant observation which indicated that fluorocitric acid enters into an enzymatic reaction most probably designed to accommodate the physiological counterpart citrate. Since the only reaction in mitochondria which was inhibited by minute amounts of fluorocitrate--under conditions when binding of fluorocitrate to protein could be measured--was citrate transport, it was evident that the enzymatic process leading to the binding of fluorocitric acid to proteins had to be concerned with the physiological pathway of citrate translocation through the inner mitochondrial membrane. A survey of all carboxylic acid substrates of the Krebs

cycle enzymes revealed that none of them formed covalent adducts with membrane proteins. It was therefore concluded that fluorocitric acid, because of its uniquely activated carboxyl group, forms a covalent bond with an active enzymatic site normally involved in forming a Michaelis-Menten complex with citric acid.

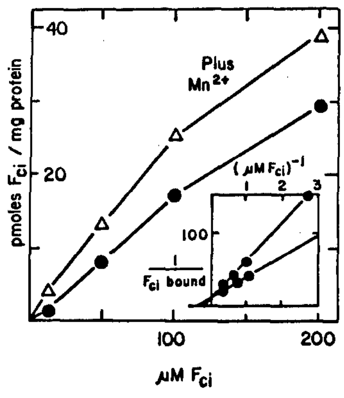


Figure 5. Kinetics of binding of fluorocitrate to proteins. The binding of varying concentrations of labeled fluorocitrate (between 0 and 200 μM) was determined by incubation of 0.7 mg membrane protein with fluorocitrate at 30° for 80 min in 50 mM Tris-Hepes buffer at pH 7.39 in the absence or presence of 0.5 mM $MnCl_2$ in a final volume of 50 μl. The reaction was stopped with 2 ml 10% trichloroacetic acid (at 0°) and the precipitated protein, after centrifugation was redissolved in 0.2 ml 0.1 N NaOH. After 5 min at 4° the protein-bound fluorocitrate was reprecipitated from 0.1N NaOH by 20 ml 5% trichloracetic acid, the finely dispersed protein precipitate isolated by glass fiber filtration, washing with 30 ml 5% trichloracetic acid and after drying, analyzed for fluorocitrate by scintillation spectrometry (see Methods, Kirsten et al., in press). In the inset of Figure 5 (double reciprocal plot) 1/bound fluorocitrate is expressed as pmol fluorocitrate/mg protein (ordinate) and fluorocitrate concentration as 1/μM fluorocitrate (abscissa). The lower curve in the inset represents results in the presence of 0.5 mM Mn^{2+}.

Catalytic Mechanism of Citrate Transport in Mitochondria

A unique observation guided us to the correct mechanism. It was found that reduced glutathione (GSH) when added together with fluorocitric acid augmented the inhibitory action of fluorocitrate on citrate influx, seemingly promoting the binding of fluorocitrate to proteins. No information existed in the literature which suggested the participation of GSH in mitochondrial metabolism; therefore, it was necessary to undertake unconventional experimental approaches to clarify this question. It became apparent that incubation of ^{14}C-citrate with GSSG (the reduced form was participatory only after its oxidation) in the presence of an extract of mitoplasts (with Brij 56) for a few minutes resulted in the formation of a ^{14}C-labeled substance which was quantitatively

adsorbed to Dowex 50 H^+ at pH 2.0 and eluted by alkali. These ionic properties identified the citrate derivative as a substance which contained NH_3^+, obviously derived from GSSG. Double labeling (^{14}C in citrate and ^{35}S in GSSG) and isolation of the adduct (see Figure 6) followed by subsequent chemical identification of the GS-citric acid thiolester as the citric-hydroxamate derivative (after hydroxylaminolysis) revealed a novel enzymatic reaction: the formation of citryl-glutathione thiolester (Kun et al., in press). The compound on the left end of Figure 6 contains GS + citric acid (^{14}C and ^{35}S) in 1:1 molar ratios and was identified as the citryl-S-G thiolester. Extracts of mitoplasts contained not only the citryl-SG thiolester synthetase but also a hydrolytic enzyme which cleaved the thiolester to citrate + GSH. The biphasic time course of the reaction illustrates the two sequentially coupled enzymatic reactions (Figure 7).

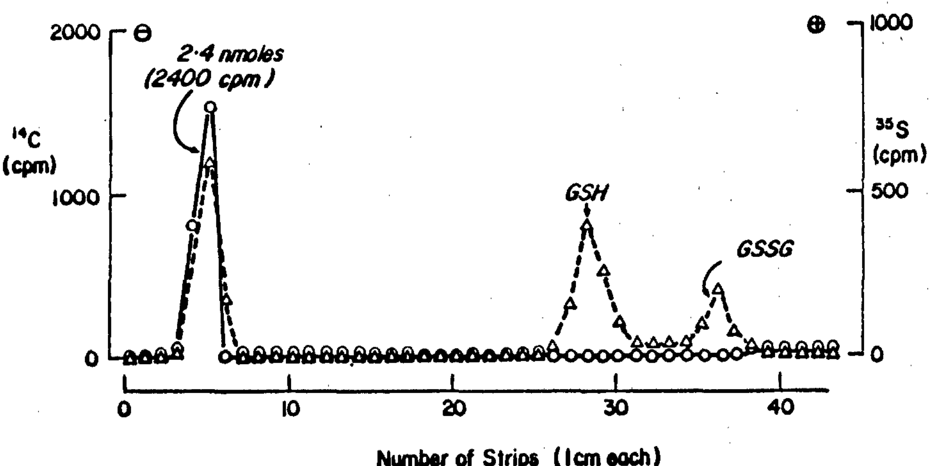


Figure 6. Electrophoretic isolation of the citric acid glutathione adduct containing either ^{14}C or ^{35}S as radioactive label (see Methods, Kirsten et al., in press). Unreacted citric acid was removed in the preceding step (Dowex 50-H^+, see Methods, Kirsten et al., in press). Citric acid placed on the paper migrated quantitatively into the buffer reservoir at the anode at pH 3.5 in 150 minutes. Left ordinate CPM of ^{14}C, right ordinate CPM of ^{35}S. (__0__0__ = ^{14}C; __Δ__Δ__ = ^{35}S).

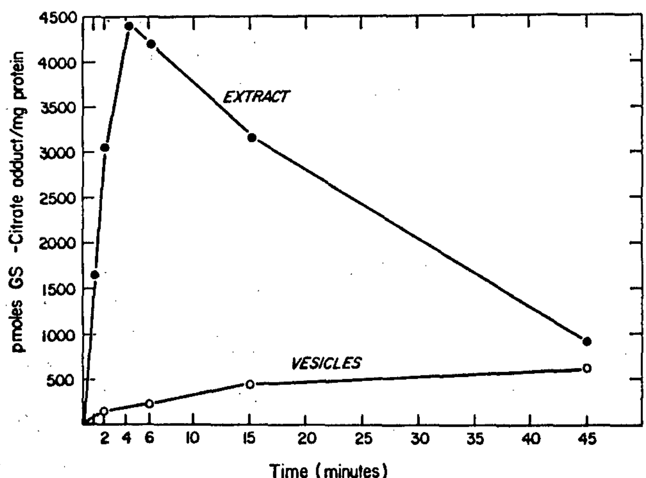


Figure 7. <u>Time course of the enzymatic formation of GS-citryl thiolester catalyzed by an extract of mitoplast and by inner membrane vesicles.</u> The amount of protein/test = 0.7 mg; GSH = 10 mM, GSSG = 10 mM, citrate = 100 μM, t = 30°, time = abscissa, activity = ordinate, pH = 7.4, total volume = 100 μl. Small nonenzymatic rates were always subtracted. GS-citrate = citryl glutathione thiolester (Kun et al., in press).

The synthetase and hydrolase enzymes were readily separated by molecular filtration by exactly the same procedure illustrated in Figure 4 for the isolation of the two fluorocitrate-protein thiolesters. Coincidence of the synthetase with the larger protein molecule (171,000 daltons) and the esterase with the smaller protein (71,000 daltons) and coincidence of these enzymes with the specific macromolecular fluorocitryl-protein thiolesters provided direct evidence for the identity of the enzymes with the two binding proteins of fluorocitrate in mitoplasts.

The enzymology of the GS-citryl thiolester synthetase was further clarified by the identification of the sulfinic acid oxidation product of GSH formed in mole/mole ratios with the thiolester. The enzymatic reaction can be described by the following equation:

$$\text{citr} + \text{GSSG} = 1/2\ O_2 \xrightarrow{Mn^{2+} - E} G - S - \overset{\overset{\displaystyle O}{\|}}{C} - \text{citr} + \overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{GS}}$$

identifying an entirely new process: the oxidative formation of a thiolester, a potentially highly significant mitochondrial process which would not have been even thought of without the search for the mode of action of fluorocitric acid. The involvement of a Mn^{2+} activated enzyme in citryl-S-G synthesis is demonstrated in Figure 8.

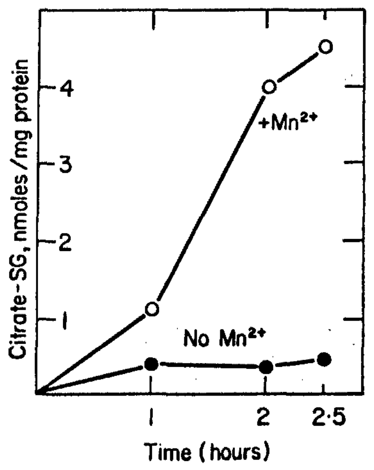


Figure 8. The effect of 0.5 mM $MnCl_2$ on the rates of GS-citryl thiolester formation by a protein fraction (mol. wt. 171,000) of the extract of mitoplasts obtained by molecular filtration on Sephadex G-200, 0.35 mg protein/test system. Conditions were the same as described in the legend of Figure 7. Citrate - SG = citryl glutathione thiolester (Kun et al., in press).

Michaelis-Menten kinetics with respect to both substances, GSSG and citrate, was readily established as shown in Figures 9 and 10.

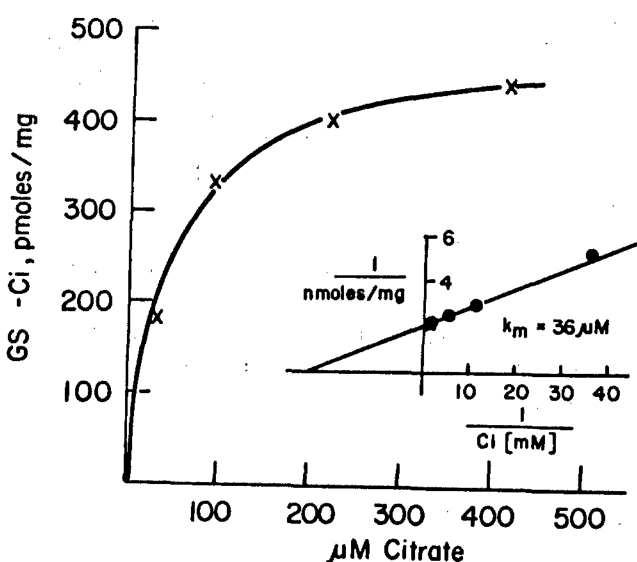


Figure 9.
The effect of increasing concentrations of citrate on the rates (5 minutes) or GS-citryl thiolester formation catalyzed by an $(NH_4)_2SO_4$ fraction (0 to 35%) of the extract. GSSG = 10 mM, $MnCl_2$ = 0.5 mM, 0.3 mg protein/test system. Other conditions were the same as described in the legend of Figure 7, except no GSH was present. GS - Ci = citryl glutathione thiolester (Kun et al., in press).

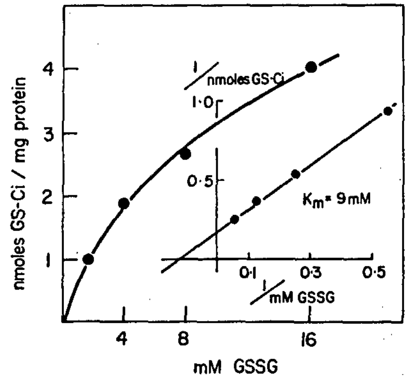


Figure 10. The effects of increasing concentrations of GSSG on the rate (5 minutes) of GS-citryl thiolester formation by an extract of mitoplasts. Citrate = 100 μM, $MnCl_2$ = 0.5 mM, protein = 0.2 mg/test system. Other conditions were the same as described in the legend of Figure 9. GS - Ci = citryl glutathione thiolester (Kun et al., in press).

The identity of the citryl-S-G synthetase reaction by a second chromatographic method suitable for large scale preparation of GS-carboxylic acid thiolesters is illustrated in Figure 11. Separation of all components, citric acid, GSH, GSSG and the GS-citryl-thiolester was readily accomplished on Dowex 1 by gradient elution with dilute formic acid.

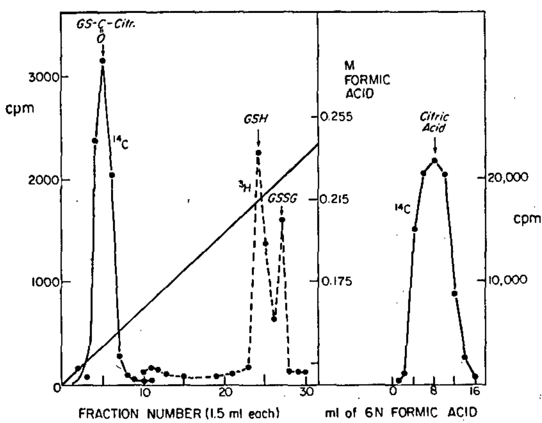
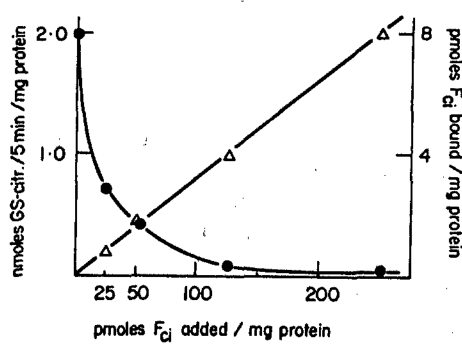


Figure 11. Separation of GS-citrate from GSH + GSSG on Dowex 1 H^+. 1 mg (protein) of liver mitoplast extract was preincubated at pH 7.4 with 20 mM ^3H-labeled GSH (spec. activity 27000 cpm/ μmole) for 0.5 mM $MnCl_2$ for 30 minutes at 30 C (total volume 90 μl). After chilling to 0 C 20 mM GSSG and 100 μM ^{14}C-labeled citric acid (spec. act. 15.5 x 10^6 cpm/μ mole) were added and the GS-citrate synthesis reaction was allowed to proceed for 10 minutes at 30 C. The reaction was stopped by adding 300 μl of 10% TCA. The TCA supernatant was neutralized to pH 7 and applied to a column (0.9 x 11 cm) of Dowex 1 x 4, 200-400 mesh, formate, equilibrated with 0.125 N formic acid. Elution was performed at room temperature with a linear formic acid gradient (consisting of 80 ml 0.125 N and 80 ml of 0.4 N formic acid) at a flow rate of 20 ml/hr. After elution of GS-citrate and GSH + GSSG unreacted citric acid was eluted with 6 N formic acid.

The definitive identification of the molecular site of action of fluorocitrate was accomplished by the inhibition of citryl-SG synthetase by fluorocitrate coincidental with protein-fluorocitryl thiolester formation. This is shown in Figure 12. The powerful specific inhibitory effect of fluorocitrate is illustreated by the fact that 50% inhibition occurred when 0.8 pmoles fluorocitrate was covalently bound to 1 mg protein. This inhibition occurred after 10 minutes preincubation with 25 pmoles fluorocitrate/mg protein, in excellent agreement with values obtained with intact mitochondria measuring citrate influx or efflux. The quantity of covalently bound fluorocitric acid to protein increased linearly even after complete inhibition of the citryl-S-G thiolester synthetase because the extract of mitoplasts contained also the thiolester hydrolase (about 3-fold excess over synthetase) and this enzyme also binds fluorocitrate as a protein-thiolester. Maximal binding in mitoplast extracts corresponds to 80 pmoles/mg protein and assuming a mole to mole binding of fluorocitrate to enzymes the synthetase is 1.2% of the protein fraction of 171,000 daltons, whereas 0.6% of the protein fraction of 71,000 daltons is the esterase. This information is a valuable guideline for further experiments concerned with the isolation of the enzyme in a molecularly homogeneous form.



Correlation between the inhibitory effect of (-)erythrofluorocitrate on rates of GS-citrate thiolester formation and the covalent binding (Carrell et al., 1970) of fluorocitrate to protein. (a) Left ordinate shows the synthetase activity (5 minutes) after preincubation of 0.9 mg (protein) extract of mitoplasts for 10 minutes with 10 mM MSG + 10 mM GSSG and varying concentrations of fluorocitrate (-●-●-). (b) Right ordinate indicates the quantity of protein bound fluorocitrate (-Δ-Δ-) determined under comparable conditions by incubating the extract with the same concentration of fluorocitrate. Protein bound fluorocitrate was determined by precipitation of proteins with 10% TCA, redissolution of precipitate in 0.1 N NaOH, reprecipitation with 10% TCA and glass fiber filtration (Carrell et al., 1970; Gal, 1972) followed by scintillation spectrometry. Conditions for (a) were the same as described in the legend of Figure 2. GS - Citr. = citryl glutathione thiolester. F_{Ci} = (-)erythrofluorocitric acid.

Identification of two specific membrane associated enzyme proteins as the target site of binding of fluorocitrate and demonstration of these two enzymes as being involved in the catalytic process of citrate transport through the inner mitochondrial membrane provides new information related to the hitherto unknown mechanism of carboxylic acid transport. The clarification of the mechanism of the citryl-S-G-synthetase reaction is a major scientific challenge. Regulators of this reaction are listed in Table 2 (Kun et al., in press).

TABLE 2. THE EFFECTS OF ACTIVATORS AND INHIBITORS ON THE ENZYMATIC FORMATION OF GS-CITRYL THIOLESTER

Inhibitors or Activators	pmoles GS-Thiolester Formed per mg Protein in 5 Minutes
None	2180
0.5 mM Mn^{2+}	4260
1 mM Mg^{2+}	3770
1 mM Ca^{2+}	1660
10 mM Ca^{2+}	80
1 mM phenazine methosulfate	362
200 mM KCl	471
0.5 mM mersalyl (preincubation)	104
1 mM 1,2,3-propane tricarboxylate	408

An extract of mitoplast (0.4 mg protein) was incubated with 10 mM GSH + 10 mM GSSG and C-labeled citrate (final molarity of 100 M) for 5 minutes in the presence of the agents listed in Column 1 of Table 2. Quantitative assay for GS-citryl thiolester is described in Methods (Kirsten et al., in press). The effect of mersalyl (0.5 mM) was tested by preincubation for 5 minutes prior to the enzymatic assay (Kun et al., in press).

Activation by Mn^{2+} is better than by Mg^{2+}, whereas Ca^{2+} is inhibitory. KCl at concentrations not uncommon in mitochondria exerts an inhibitory, probably regulatory effect. The free radical trapping agent phenazine methosulfate and the -SH binding reagent mersalyl are powerful inhibitors. At this time relatively less is known about the thiolester hydrolase because its study requires the availability of larger, substrate quantities of enzymatically synthesized citryl-S-G. After recognizing the specific enzymatic site of action of fluorocitrate we attempted to test the

possibility of preventing the inhibitory effect of fluorocitric acid by reagents which may cleave the thiolester bond between fluorocitrate and a thiol group of the protein. Results are summarized in Table 3.

TABLE 3. EFFECT OF DTT, BAL, GSSG AND OXIDIZED CYSTEAMINE ON THE INHIBITION OF GS-CITRATE SYNTHESIS BY (-)ERYTHROFLUOROCITRATE

Preincubation F-Citrate (pmoles/mg)	Addition	Reaction nmoles GS-Citrate per mg Protein	% Inhibition
0	0	2.52	
1.5	0	1.27	50
3	0	0.56	78
6	0	0.32	87
0	DTT	3.71	
1.5	DTT	2.78	25
3	DTT	2.48	33
0	BAL	3.26	
3	BAL	2.53	23
0	GSSG	2.63	
1.5	GSSG	2.17	13
3	GSSG	2.49	5
0	Cysteamine (ox.)	1.05	
3	Cysteamine (ox.)	0.47	59

1 mg (protein) of liver mitoplast extract was preincubated at pH 7.4 with 10 mM GSH in the presence or absence of DTT (10 mM), BAL (8 mM), GSSG (20 mM) or oxidized cysteamine (20 mM) and of F-citrate (1.5 to 6 pmoles/mg protein) for 60 minutes at 30 C in a total volume of 90 μl. After chilling to 0 C 100 μM ^{14}C-labeled citric acid and (where not present) 20 mM GSSG was added and the GS-citrate synthesis reaction was allowed to proceed for 5 minutes at 30 C. The reaction was stopped by adding 300 μl of 10% TCA. GS-citrate was measured in the TCA supernatant.

When the period of incubation of the enzyme with fluorocitrate is increased to 1 h (controls are incubated simultaneously without fluorocitrate) the effectivity of fluorocitrate is greatly increased and 6 pmoles of added fluorocitric acid per mg protein is 87% inhibitory (compare with 50-80 pmoles if incubation lasts only for 10 minutes). Dithiothreitol, BAL and GSSG are powerful protecting agents, GSSG being most effective. These results predict that antidotes to fluoroacetate poisoning can be found among various -SH and -SS-containing substances, and the search to achieve this goal had already begun.

SUMMARY AND PERSPECTIVES

The experimental evidence obtained points to a novel site of action of fluorocitrate by covalent binding to two catalytic components of mitoplasts apparently involved in citrate transport. This at first sight nonselective mechanism--in terms of ubiquity of citrate transport--is capable of offering a plausible explanation for the neurotoxicity of fluorocitrate. It is well known (Kirsten et al., in press) that acetylcholine biosynthesis critically depends on citrate export from mitochondria because acetyl-CoA formed from citrate in the cytoplasm is the immediate acetylating agent of choline. Clearly inhibition of fatty acid synthesis that is dependent on mitochondrially generated citric acid is not likely to result in a fatal pathology of a vital organ, but inhibition of the biosynthesis of the neurotransmitter acetylcholine in the central nervous system can critically interfere with the function of cholinergic centers. This question now is open to direct experimental tests. A further consequence of this probable mechanism of neurotoxicity is that unexplained neuropathological phenomena may find their causative origin in defective mitochondrial citrate export, exemplifying an unusually simple intracellular molecular information transfer mediated by citrate to the neurotransmitter acetylcholine.

The basic membrane function of the inner mitochondrial membrane specialized in citrate transport has been resolved into two coupled enzymatic reactions capable of providing a simple catalytic model for translocation. This hypothetical model is illustrated in Figure 13, which is a schematic representation of one half of a protein channel containing the two enzymes, synthetase and hydrolase. According to this mechanism transport is a two-enzymic coupled process. It should be remembered that the enzymatic mechanism does not fully explain the known phenomenon of exchange diffusion, of carboxylic acids in mitochondria, but provides hitherto missing chemical intermediates. The entire process of anion transfer in mitochondria depends on both electron and cation transport coupling, a problem which now can be approached by new avenues. The fate of glutathione sulfinic acid is of considerable interest. This substance appears in the in vitro enzyme system in equimolar quantity to the thiolester. The sulfinic acid is, however, not found in mitochondria because a number of reducing systems apparently reconvert it to GSSG and GSH, providing the as yet unexplored possibility of coupling of transport to an electron transfer process.

CITRATE TRANSPORT

Figure 13.

The question justifiably emerges: is the enzymatic mechanism of citrate transport unique, or are these types of reactions common to all carboxylic acids which need to penetrate the inner mitochondrial membrane? Results indicate the correctness of the second supposition. It is shown in Table 4 that specific malyl and citryl thiolester synthetases exist in mitochondria as discriminated by the selective inhibition of each enzyme by the appropriate fluoro acid.

TABLE 4. EFFECTS OF F-CITRATE AND F-MALATE ON GS THIOLESTER-SYNTHETASE ACTIVITIES OF SUBMITOCHONDRIAL PREPARATIONS WITH CITRATE AND L-MALATE AS SUBSTRATES. RESULTS ARE EXPRESSED AS PMOLES THIOLESTER FORMED IN 5 MINUTES/MG PROTEIN.

Enzyme Preparation and Carboxylic Acid Substrate	Control	Inhibitors: F-Citrate (125 pmoles per mg protein)	Inhibitors: F-Malate (50 pmoles per mg protein)
Extract + Citrate	2770	110	2802
Membrane Vesicles + L-Malate	305	298	0

Conditions for the assay for GS-citrate thiolester formation were the same as described in the legend of Figure 2, except the extract (1 mg protein) was preincubated with or without the inhibitors for 10 minutes at 30 C. The concentration of L-malate (77,000 CPM/nmole) was 68 µM in the GS-malyl thiolester synthetase system; otherwise conditions were the same as for the GS-citryl thiolester forming system (Kun et al., in press).

Isolation of glutaminyl and glutamyl -S-G thiolesters generated from GSSG and the appropriate carboxylic acids by incubation with fragments of inner mitochondrial membranes is shown in Figures 14 and 15.

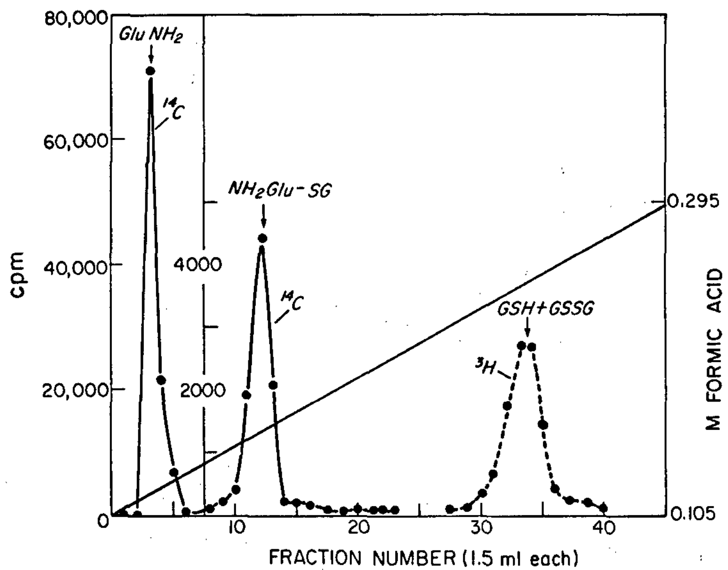


Figure 14. Separation of L-glutamine-SG from L-glutamine and GSH + GSSG. 1 mg (protein) of kidney mitoplast extract and 1 mg (protein) of inner membrane vesicles from kidney mitochondria were preincubated at pH 7.4 with 20 mM ^{3}H-labeled GSH (specific activity 27 x 10^{3} cpm per µmole) and 0.5 mM $MnCl_2$ for 20 minutes at 30 C in total volumes of 90 µl. After chilling to 0 C 200 µM of ^{14}C-labeled L-glutamine (specific activity 11.5 x 10^{6} cpm per µmole) and 20 mM GSSG were added to each sample. After 15 minutes incubation at 30 C the reaction was stopped by adding 300 µl of 10% TCA. The combined TCA supernatants of both samples were neutralized to pH 7.0 and applied to a column (0.9 x 11 cm) of Dowex 1, X4, 200-400 mesh, formate form, equilibrated with 0.105 M formic acid. Elution was performed at room temperature with a linear gradient consisting of 80 ml 0.105 M formic acid and 80 ml 0.4 M formic acid at a flow rate of 20 ml per hour.

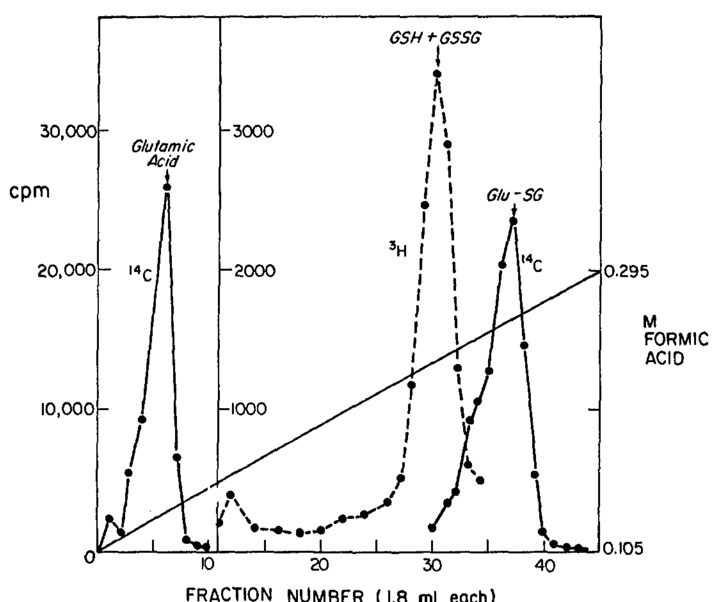


Figure 15. Separation of glutamic acid-SG from GSH + GSSG and glutamic acid. 1 mg (protein) of liver mitoplast extract and 1 mg (protein) of inner membrane vesicles were incubated (separately) at pH 7.4 with 20 mM ^{3}H-labeled GSH (specific activity 27 x 10^3 cpm/μmole) and 0.5 mM $MnCl_2$ for 30 minutes at 30 C in total volumes of 90 μl. After chilling to 0 C 20 mM GSSG and 250 μM of ^{14}C-labeled L-glutamic acid (specific activity 6.3 x 10^6 cpm/μmole) were added. After 5 minutes of incubation at 30 C the reaction was stopped by adding 300 μl of 10% TCA. The combined supernatants were neutralized to pH 7 and applied to a column (0.9 x 11 cm) of Dowex a, X4, 200-400 mesh, formate form, equilibrated with 0.105 M formic acid. Elution was performed at room temperature with a linear gradient consisting of 80 ml 0.105 M formic acid and 80 ml 0.4 M formic acid at a flow rate of 20 ml/hr.

It is, therefore, apparent that an entirely new enzyme system involving all carboxylic acid substrates of the Krebs cycle has been discovered. In all probability these enzymes critically regulate cell function by their controlling role on substrate transport.

ACKNOWLEDGEMENT

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PAPER NO. 12

APPLICATION OF PHARMACOKINETIC PRINCIPLES IN PRACTICE

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INTRODUCTION

The objective of a toxicological study is to elucidate the untoward effects of a chemical, qualitatively and quantitatively. The ultimate goal to be realized from acquisition of this information is to assess the potential hazard incurred by man or animals exposed to specified amounts of the chemical. Since toxicity is elicited through reactions of a chemical with receptors in target tissues, realization of the ultimate goal necessitates resolution of, as a function of time, the absorption, distribution, biotransformation, and excretion of the chemical, i.e., its pharmacokinetics, as well as its toxicity.

The scientific rationale for extrapolation of untoward effects produced by toxic doses of a chemical in one species to predict the toxicity which may be incurred by lower doses in the same species or by equivalent or lower doses in another species is associated inextricably with knowledge of the pharmacokinetics of the chemical. The purpose of this presentation is to illustrate how the pharmacokinetic profile of a chemical can be utilized to assess the hazard incurred via exposure to a chemical.

The simplest, but frequently applicable, profile for describing the concentration of chemicals in a tissue or the amount in the body as a function of time occurs when the rate of change in these parameters is equal to a rate constant times the concentration or amount present, apparent first order kinetics. If there are no apparent differences in the affinity of tissues for the chemical, the mathematical expression for elimination of the chemical, from plasma for example, is the linear differential equation

1) $$\frac{\partial C}{\partial t} = -k_e C,$$

where C is the concentration at time t and k_e is the rate constant for elimination via all routes. Solution of this equation yields

$$\text{2)} \qquad \text{Log } C = \text{Log } C_o - k_e t/2.303,$$

where C_o is the initial concentration present. Thus a plot of the logarithm of the concentration as a function of time will give a straight line with a slope of $k_e/2.303$. The time required for the concentration to be reduced by one-half, $t_{1/2}$ or biological half-life, is $t_{1/2} = 0.693/k_e$. Much more complex linear pharmacokinetic profiles or models are required for some chemicals (Gehring et al., 1976).

In toxicology, the doses of a chemical required to elicit discernible toxicity frequently exceed the capacity for its detoxification via excretion and/or biotransformation. In such cases, nonlinear pharmacokinetic profiles conforming to the Michaelis-Menten Equation are needed to describe the concentration of the chemical in plasma or other tissues as a function of time (Levy and Gibaldi, 1975):

$$\text{3)} \qquad \frac{\partial C}{\partial t} = \frac{V_m C}{K_m + C}.$$

In this equation, V_m is the maximum rate of the process and K_m the Michaelis constant; the other parameters are as defined previously. When the concentration is small compared to K_m, the Michaelis-Menten Equation reduces to a form like that presented previously, equation 1. This is called "apparent" first order kinetics. At high concentrations, the rate of change in concentration as a function of time becomes constant, equal to V_m. This is called zero order kinetics.

Figure 1 depicts a typical tissue or plasma concentration - time curve or an amount in the body - time curve for a chemical whose elimination follows nonlinear or Michaelis-Menten kinetics. As long as the dose incurred via exposure to a chemical results in concentrations or amounts significantly less than K_m, the linear portion of the semilogarithmic plot is applicable. Doses which provide concentrations or amounts approaching or superseding K_m lead to the nonlinear portion of the curve in Figure 1. If this nonlinear portion is plotted using linear coordinates rather than semilogarithmic coordinates, a straight line will result.

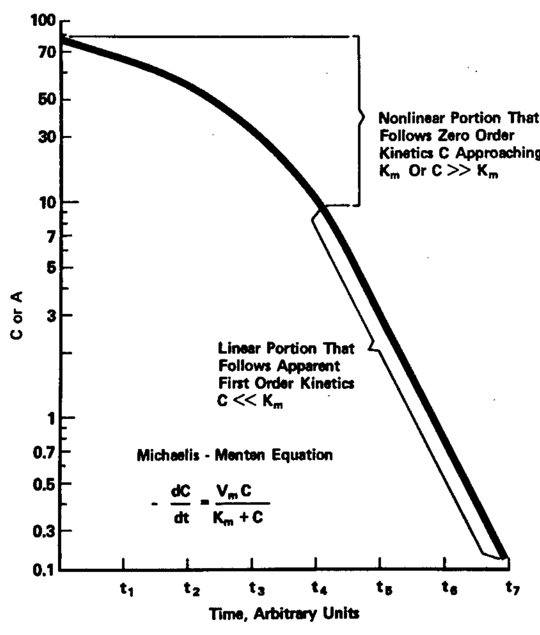

Figure 1. Simulated plasma concentration of a chemical (C) or the amount in the body (A) as a function of time for a chemical which displays dose-dependent or nonlinear pharmacokinetics described by the Michaelis-Menten Equation. $\partial C/\partial t$ is the change in concentration with time. V_m is the maximum rate of the process, and K_m is the Michaelis constant.

Some criteria which serve as indications that nonlinear pharmacokinetics are applicable for describing the elimination of a chemical from the body have been set forth by Levy (1968).

1. Decline of the levels of the chemical in the body is not exponential.

2. The $t_{1/2}$ increases with increasing dose.

3. The area under the plasma concentration versus time curve is not proportional to the dose.

4. The composition of the excretory products may be changed both quantitatively and qualitatively by dose.

5. Competitive inhibition by other chemicals metabolized or actively transported by the same enzyme system is likely.

6. Dose-response curves may show an unusually large increase in responses with increasing dose, starting at the dose level where "saturation" effects become evident.

For the toxicologist, the last criterion is of particular importance. To illustrate the importance of nonlinear pharmacokinetics further, consider the barrels with slits in Figure 2. As increasing amounts of fluid are placed suddenly into the barrels, the outflow (elimination) via the upper slit will become relatively more important. The illustration is particularly useful for portraying the importance of repetitive or continuous exposure when nonlinear pharmacokinetics are applicable to the elimination of a chemical. As the input rate increases more and more of the chemical is eliminated via the process represented by the upper slit. This is a scenario of repetititive exposures to doses of a chemical which saturate primary routes of elimination; such doses are used frequently in carcinogenicity and teratology bioassays. Results obtained from such studies should be interpreted cautiously when assessing the hazard of exposure to small, nonsaturating doses.

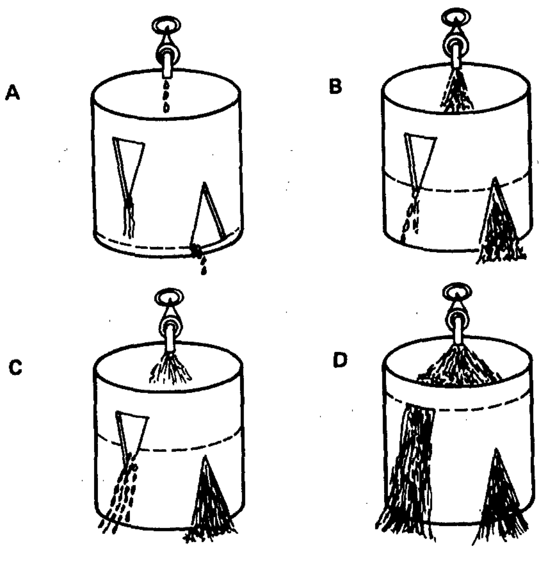

Figure 2. Diagrammatic representation of the elimination of a chemical via a primary saturable pathway and by a secondary pathway whose significance increases upon saturation of the primary pathway.

With respect to carcinogenesis, overwhelming detoxification processes are particularly important because rather than being detoxified, reactive electrophilic intermediates will react with macromolecules such as DNA, a commonly recognized mechanism for carcinogenesis as well as other manifestations of toxicity. Gillette (1974a and 1974b) has given special consideration to the pharmacokinetics of reactive metabolites of foreign compounds that react with macromolecules - DNA, RNA, protein - causing toxicity.

To illustrate the use of pharmacokinetics in assessing the hazard of chemicals, examples using specific chemicals follow.

2,4,5-TRICHLOROPHENOXYACETIC ACID (2,4,5-T), LINEAR AND NONLINEAR PHARMACOKINETICS AND SPECIES DIFFERENCES

2,4,5-T is a herbicide, the safety of which has been questioned because doses of 100 mg/kg/day or more during the period of organogenesis have been reported to be teratogenic, fetotoxic, and embryotoxic (Courtney et al., 1970; Courtney and Moore, 1971; Sparschu et al., 1971; Roll, 1971; and Collins and Williams, 1971). To elucidate the potential hazard of 2,4,5-T, 5 mg/kg of ring-labeled 2,4,5-T was administered orally to rats and dogs (Piper et al., 1973). Plasma concentration-time curves were characterized by apparent first order absorption and clearance (Figure 3). The $t_{1/2}$ values for clearance by rats and dogs were 4.7 and 77.0 hours, respectively. The slower rate of elimination by dogs than by rats correlates with the higher toxicity in dogs for which the single oral LD_{50} is 100 mg/kg compared to 300 mg/kg for rats (Drill and Hiratzka, 1953; Rowe and Hymas, 1954). Generally, the toxicity of an agent to an individual or species is related inversely to the ability of the individual or species to eliminate the compound and directly to the concentrations attained in plasma.

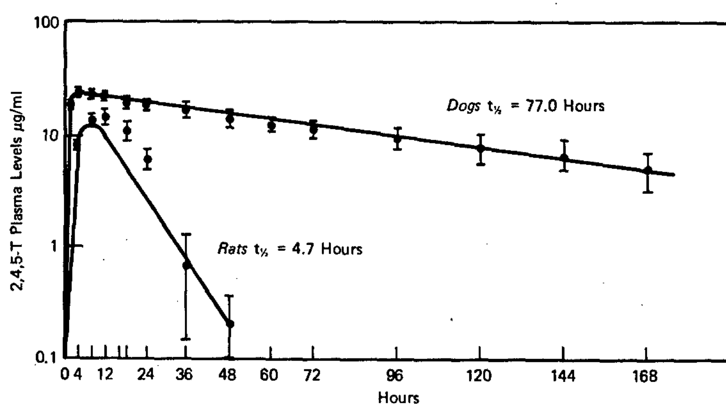

Figure 3. Concentration of ^{14}C-activity expressed as µg equivalents 2,4,5-T/ml plasma in dogs and rats following ^{14}C-2,4,5-T. Each point represents a mean and SE.

Almost all of the 2,4,5-T excreted by rats was via the urine, while approximately 20% of that excreted by dogs was via the feces. Measurable amounts of biotransformation products of 2,4,5-T were not produced by rats given 5 mg/kg while 10% of the ^{14}C-activity excreted in the urine of dogs was attributable to biotransformation products. This illustrates a pertinent principle - if elimination via one route is retarded, in this case via urine, a greater fraction will be eliminated via other routes.

Since 2,4,5-T is an organic acid, it was hypothesized that the poorer ability of the dog than the rat to eliminate 2,4,5-T was associated with the poor organic acid secretory process of the dog kidney. If an active secretory process of the kidney were the primary elimination process, elimination should be a nonlinear process, saturable in the rat by administration of higher doses. Using doses of 5, 50, 100, or 200 mg/kg, clearance from the plasma was found to be nonlinear (Figure 4). Elimination from the body, predominantly via the urine, was slower than the clearance from plasma in rats given 5 or 50 mg/kg but in those given 100 or 200 mg/kg the rate of clearance from plasma approached the rate of elimination via urine. This change was attributable to a saturable renal uptake of 2,4,5-T.

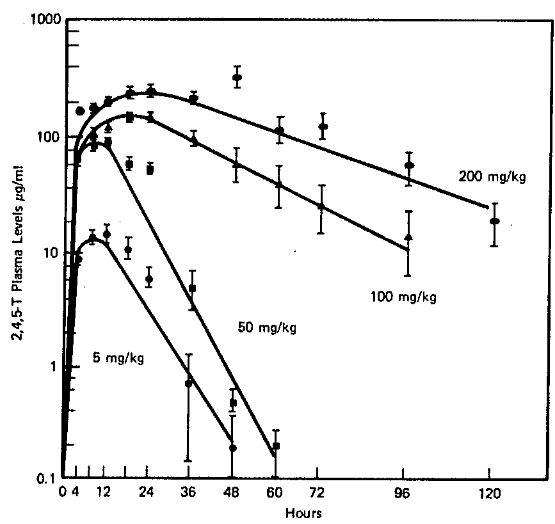

Figure 4. Concentration of ^{14}C-activity expressed as µg equivalents 2,4,5-T/ml plasma in rats following a single-oral dose of 5, 50, 100 or 200 mg/kg ^{14}C-2,4,5-T. Each point represents a mean and SE.

In addition to the lack of superposition of the normalized concentration-time curves for the elimination of 2,4,5-T from plasma with increasing doses, another criterion for nonlinear pharmacokinetics was revealed. A larger percentage of the ^{14}C-activity administered as ^{14}C-2,4,5-T was excreted in the feces as the dose was increased. Furthermore, biotransformation products of 2,4,5-T were identified in the urine of rats given 100 or 200 mg/kg while none was found in the urine of rats given 5 or 50 mg/kg.

In order to characterize better the nonlinear pharmacokinetics of 2,4,5-T, rats were given intravenous doses of 5 or 100 mg/kg (Sauerhoff et al., 1976). Elimination from the plasma of rats given 100 mg/kg (Figure 5) occurred in accordance with the Michaelis-Mention Equation,

$$\frac{\partial C}{\partial t} = \frac{V_m C}{K_m + C}.$$

The values for V_m and K_m were 16.6 $\pm$ 1.8 μg/g/hr and 127.6 $\pm$ 25.9 μg/g, respectively. During the linear phase of excretion, between 36 and 72 hours after administration of 100 mg/kg, the $t_{1/2}$ was 5.3 $\pm$ 1.2 hours. This value is not significantly different than that found for rats given 5 mg/kg.

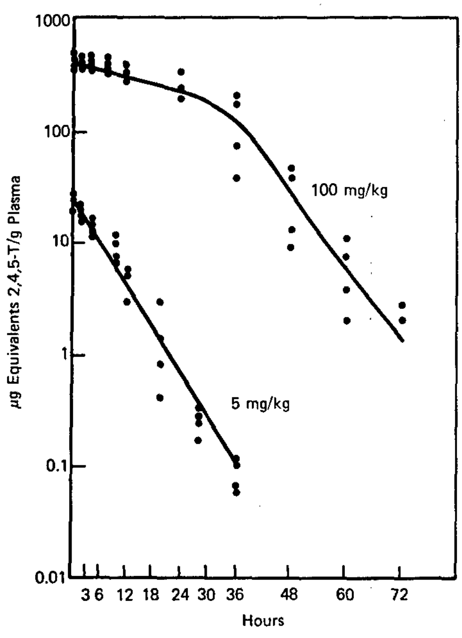

Figure 5. Concentration of ^{14}C-activity expressed as μg equivalents 2,4,5-T/g plasma of rats following a single intravenous dose of 5 and 100 mg/kg ^{14}C-2,4,5-T. The curve for rats receiving the latter dose typifies dose-dependent elimination.

The volume of distribution, V_d, increased from 190 $\pm$ 8 ml/kg to 235 $\pm$ 10 ml/kg for rats receiving the 5 and 100 mg/kg ^{14}C-2,4,5-T, respectively. This increase in V_d indicates that as the dose is increased a disproportionately larger fraction finds its way into tissue and cells. Undoubtedly, the toxicity will also increase disproportionately as a result.

A saturable renal uptake of 2,4,5-T was illustrated vividly by a five-fold smaller renal tissue/plasma ratio 8 hours after a dose of 5 mg/kg than a dose of 100 mg/kg. To elucidate further the saturable active transport of 2,4,5-T by rat and dog kidney, in vitro studies were conducted using renal slices (Hook et al., 1974). These studies demonstrated conclusively: (1) that 2,4,5-T is transported actively by the organic acid excretory mechanism of the kidney, (2) that kidney tissue of dogs and newborn rats has less capacity to transport 2,4,5-T than adult rats, and (3) that large concentrations of 2,4,5-T overwhelm the transport system.

To provide a basis for extrapolation to man, the fate of 2,4,5-T following oral doses of 5 mg/kg was determined in man and compared with rats and dogs (Gehring et al., 1973). Plasma concentration versus time for the respective species together with $t_{1/2}$ values are shown in Figure 6. These data suggest that the toxicity of 2,4,5-T to man will be greater than to rats but less than to dogs. The higher peak plasma levels attained with a dose of 5 mg/kg in man than in either dogs or rats was associated with a greater degree of plasma protein binding in man. In man, the volume of distribution was only 80 mg/kg, attesting to the retention of 2,4,5-T in the vascular compartment.

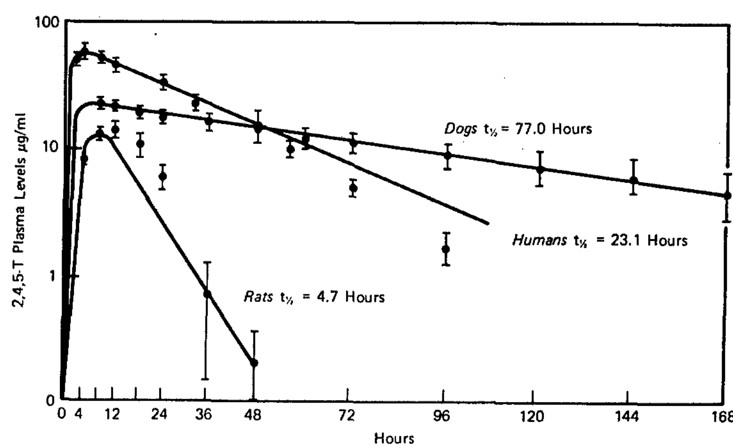

Figure 6. The concentration of 2,4,5-T in blood plasma of humans, dogs, and rats following a single oral dose of 5 mg/kg. Each point represents a mean and SD.

Figure 7 illustrates the simulated levels of 2,4,5-T in the plasma that would be attained in humans with repeated ingestion. If 0.25 mg/kg 2,4,5-T were ingested daily, a level equaling that attained by ingesting a single dose of 5 mg/kg, as in this study, would never be attained. Finally, determination of renal clearance values revealed values exceeding glomerular filtration as was expected from the results of the studies in animals described above.

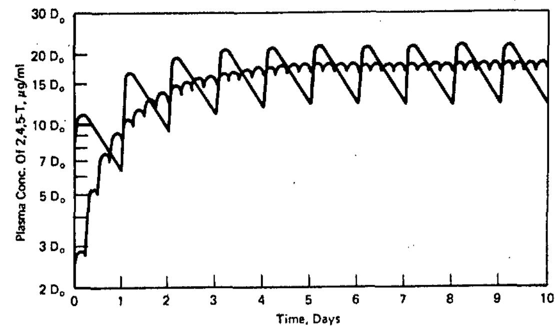

Figure 7. Predicted plasma concentrations of 2,4,5-T in humans ingesting a dose of D_O in units of mg/kg every 24 hours (curve with large excursions) or a dose of 1/4 D_O every 6 hours (curve with small excursions).

These pharmacokinetic studies of 2,4,5-T in man and animals together with the results of toxicological studies in animals support the conclusion that the hazard of exposure to small amounts of 2,4,5-T potentially encountered via its recommended use is nil.

1,4-DIOXANE: SATURATION OF METABOLISM CORRELATED WITH TOXICITY IN RATS

Studies by Kociba et al. (1974) demonstrated a small increased incidence of hepatomas and nasal carcinomas in rats maintained on drinking water containing sufficient dioxane to provide doses exceeding 1000 mg/kg/day. This dose supersedes that necessary to produce death in some rats and marked pathology of the liver and kidneys in all. Hepatic and renal damage but no tumors occurred in rats receiving 100 mg/kg/day. No untoward effects were discernible in rats receiving 10 mg/kg/day. Since humans exposed to the current Threshold Limit Value (TLV) established by the American Conference of Governmental Industrial Hygienists, 1976, of 50 ppm (180 mg/m^3) for 6 hours received a total dose of 5.4 ± 1.1 mg/kg, (Young et al., 1977), the meaningfulness of toxicological data obtained at doses more than one hundred-fold greater was questioned by the results of the pharmacokinetic data.

Figure 8 shows the plasma concentration time-curves for ^{14}C-dioxane in rats given single intravenous doses of ^{14}C-dioxane ranging from 3 to 1000 mg/kg. The clearance of dioxane from plasma is markedly dose-dependent and in accordance with Michaelis-Menten kinetics. The area under the curve increases disproportionately with dose indicating that the elimination of dioxane is a saturable, dose-dependent or nonlinear pharmacokinetic process. The pharmacokinetic model which described best the data was a parallel combination of Michaelis-Menten and first order elimination. Parameters for this combination were $V_d = 301 \pm 41$ ml; $V_m = 13.3 \pm 1.1$ µg/ml/hr; $K_m = 20.9 \pm 2.0$ µg/ml and $k_e = 0.0149 \pm 0.0015$ hr^{-1}. The maximum capacity for elimination of dioxane by the rat is 4003 µg/hr. Thus a dose of 1000 mg/kg to a 250 g rat exceeds 62 times the maximum capability of the rat to eliminate dioxane per hour.

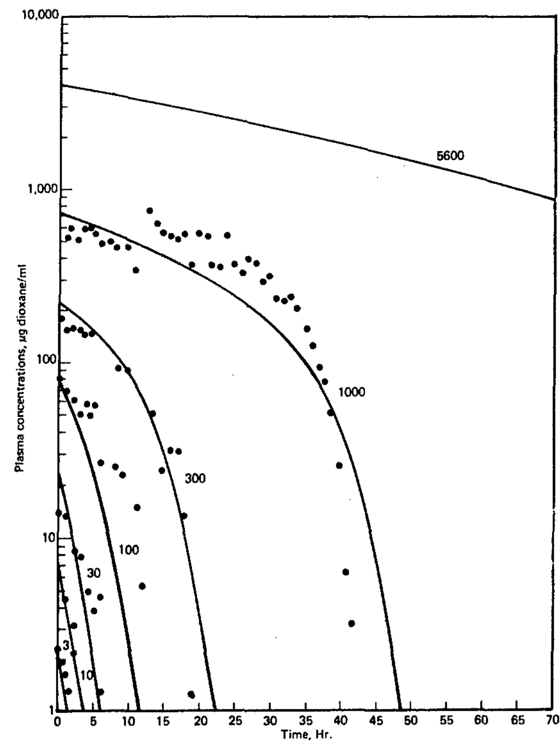

Figure 8. Concentration of dioxane per se in plasma of rats given various intravenous doses of dioxane.

The excretion of ^{14}C-activity by rats given various doses of ^{14}C-dioxane also demonstrated dose-dependent kinetics; as the dose was increased, more dioxane per se was eliminated via exhalation; at low doses essentially all was excreted rapidly as β-hydroxyethoxyacetic acid (HEAA) in the urine. Thus, the biotransformation of 1,4-dioxane to the detoxification product, HEAA, is a saturable process which is overwhelmed by increasing the magnitude of the dose. The marked retention of dioxane with an increasing dose led us to conclude that the metabolism of dioxane must be induced markedly with repeated daily doses.

Figure 9 shows the body burden of radioactivity in rats given repeated daily oral doses of 10 or 1000 mg/kg for 17 days. The body burden was calculated by subtracting the total cumulative excretion by all routes from the total cumulative dose and expressing the results as a percentage of the total cumulative dose. The body burden in rats given 10 mg/kg/day dioxane averaged about 5% and ranged between 2 and 9% with no apparent upward or downward trend. However, a striking decrease occurred in the body burden of rats given 1000 mg/kg/day dioxane during the first four days of administration. This indicates that a dose of 1000 mg/kg/day but not 10 mg/kg/day caused a marked induction of the elimination of dioxane. In

essence, the rats receiving 1000 mg/kg/day dioxane had undergone marked biochemical alterations, their responses to dioxane, toxicological or carcinogenic, are no longer extrapolatable to rats receiving low doses.

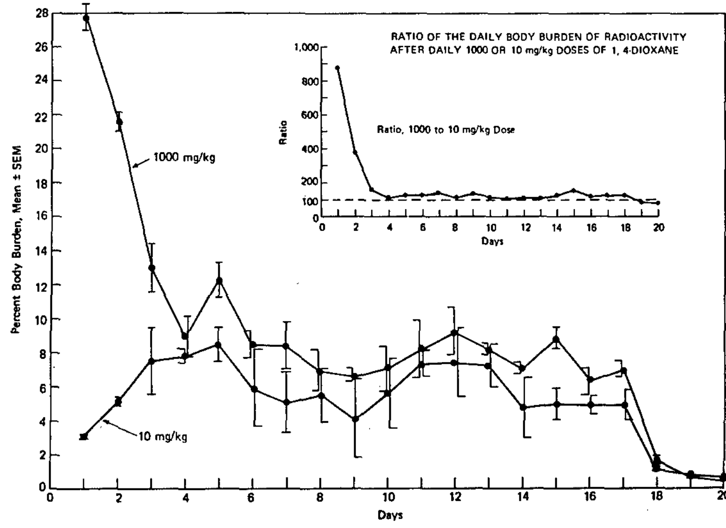

Figure 9. Daily body burden of ^{14}C-radioactivity of rats given oral doses of 10 or 1000 mg dioxane/kg/day. The body burden was calculated by subtracting the total cumulative excretion by all routes from the total cumulative dose and expressing the results as a percentage of the total cumulative dose. Insert - Ratio of the daily body burden of ^{14}C-radioactivity after daily doses of 10 or 1000 mg/kg dioxane.

Metabolic induction itself has been shown to increase tumorigenesis. Peraino et al. (1973a) demonstrated an enhancement of tumorigenesis in mice given 0.05% phenobarbital in their diet, a well known inducer of metabolism. Furthermore, induction of metabolism in rats by phenobarbital enhanced tumor production by 2-acetylaminofluorene (2AAF), a known hepatic carcinogen (Peraino et al., 1973v), suggesting that induction may enhance the expression of tumors by naturally occurring carcinogens.

In order to assess the potential hazard incurred by inhalation of dioxane, rats were exposed to 50 ppm dioxane for 6 hours. After 6 hours of exposure a steady-state level of 7.3 μg/ml had been attained in plasma. Following exposure, the rate of elimination of dioxane from plasma was equivalent to that observed after low intravenous doses of dioxane, $t_{1/2}$ = 1.01 hours. Thus, this level of exposure had not saturated the detoxification mechanism for dioxane.

To assist in extrapolation of toxicological data to man, four human volunteers were exposed to 50 ppm dioxane for 6 hours (Young et al., 1977). The concentration of dioxane and its metabolite HEAA in plasma during the following exposure is shown in Figure 10. During exposure, a steady-state level was obviously attained. Subsequent to exposure, the

elimination of dioxane was apparent first order kinetics having a $t_{1/2}$ of 1.0 hr^{-1}. As in the rat, essentially all of the dioxane inhaled was eliminated as HEAA in the urine. This conclusion was reached because the amounts of HEAA excreted in the urine of the subjects was equivalent to the amount expected if all the dioxane in an assumed tidal volume of 10 ℓ/min had been absorbed. Using the amount of HEAA excreted in the urine to estimate the total dose received, it was further determined that a human exposed to 50 ppm dioxane receives on a per kg basis about one-thirteenth the dose received by a rat exposed to the same concentration. Thus, for a material readily absorbed upon inhalation and metabolized such as dioxane, experiments conducted in rats provide a 13-fold safety factor.

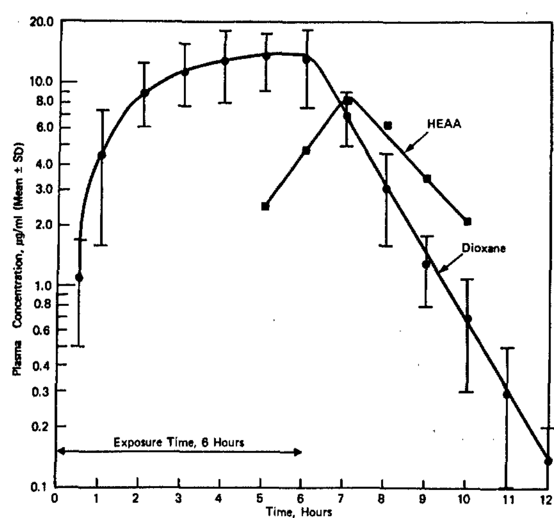

Figure 10. Plasma concentration-time curve for dioxane and HEAA for humans exposed to 50 ppm dioxane vapor for 6 hours. Dioxane concentrations are the mean ± SD, n = 4. HEAA concentrations are averages of 2 to 3 individuals.

The simulated plasma concentration of dioxane versus time is shown in Figure 11 for repeated exposures to 50 ppm dioxane. It is obvious that, upon repeated exposure, levels of dioxane will not be attained which saturate the capability for detoxification and elimination.

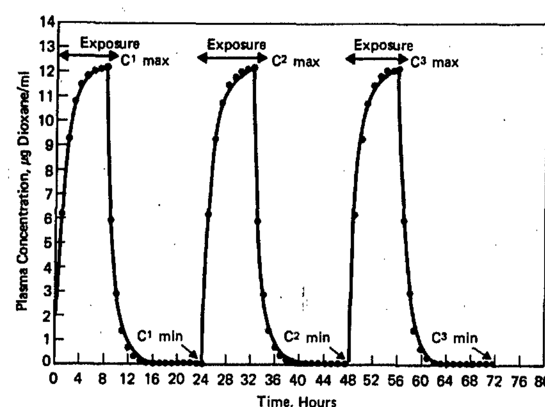

Figure 11. Simulated plasma concentration-time curve for humans exposed to 50 ppm dioxane 8 hours/day. Parameters used for the simulation were obtained in the 6-hour exposure, Figure 10.

Using the pharmacokinetic data together with the toxicological data, it is possible to conclude that in rats adverse effects are encountered only when doses of dioxane supersede those which can be detoxified readily without induction of morphological and biochemical changes. Since people exposed to 50 ppm dioxane readily detoxify dioxane, it is reasonable to conclude that it is highly unlikely that this level of exposure will be associated with untoward effects. It is highly significant that exposure of rats to 111 ppm dioxane, 7 hours/day, 5 days/week for 2 years causes no untoward effects (Torkelson et al., 1974). In rats, this level of exposure provides a daily dose on a per kg basis 30-fold greater than that which will be received by a man exposed continuously to 50 ppm for 6 hours.

2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD), LINEAR PHARMACOKINETICS USED TO ASSESS POTENTIAL FOR BIOACCUMULATION AND INCURRENT TOXIC HAZARD

TCDD is a highly toxic compound formed as an unwanted contaminant in the manufacture of 2,4,5-trichlorophenol (Schwetz et al., 1973). Use of trichlorophenol to manufacture 2,4,5-trichlorophenoxyacetic acid may result in contamination of the latter with TCDD. The physical-chemical properties of TCDD suggest that exposure to small amounts may result in persistent accumulation of the highly toxic material and eventually lead to toxicity. To elucidate the propensity of TCDD to accumulate in the body, a series of pharmacokinetic studies were conducted (Rose et al., 1976). In these studies, one group of rats was given a single-oral dose of 1 μg/kg ^{14}C-TCDD and the excretion of ^{14}C-activity in urine, expired air, and feces was determined. Other groups of rats were given orally 1.0, 0.1 or 0.01 μg ^{14}C-TCDD/kg/day, Monday through Friday, for up to 7 weeks. In addition to determining the amounts of ^{14}C-activity excreted in the urine and feces of these rats, the amounts remaining in the body as a function of time were calculated and the levels of ^{14}C-activity residing in various tissues after 1, 3, and 7 weeks of administration were determined.

The amounts of ^{14}C-activity remaining in the bodies of rats decreased at an apparent first-order kinetics rate following the single-oral dose of 1 μg/kg ^{14}C-TCDD. The $t_{1/2}$ for elimination of ^{14}C-TCDD from the body ranged from 21 to 39 days. All of the ^{14}C-activity was eliminated via the feces.

The concentrations of ^{14}C-activity in the bodies of rats given 1.0 or 0.1 μg/kg/day, Monday through Friday, for 7 weeks as a function of time are shown in Figure 12. The data in this figure show clearly that with repeated exposure the concentration of ^{14}C-activity in the body increases but the rate of increase decreases and the amount in the body begins to plateau even though exposure continues. Similar data could not be obtained for those rats given 0.01 μg/kg/day because the levels of ^{14}C-activity excreted were too low to allow accurate measurement and subsequent calculation of the body burden, a term meaning the amount remaining in the body.

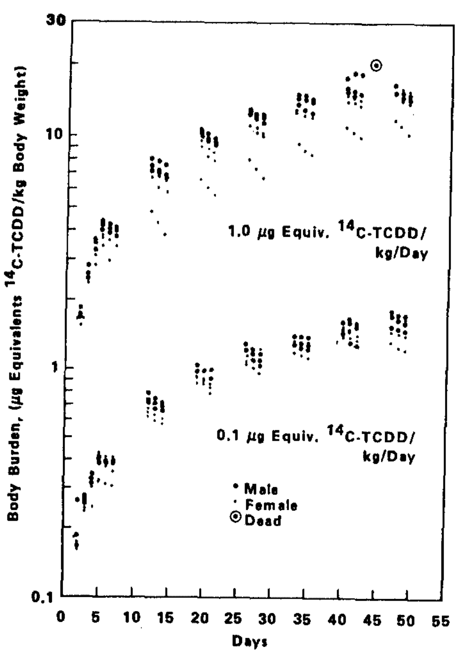

Figure 12. The concentration of microgram equivalents TCDD in the body of rats given oral doses of 0.1 or 1.0 μg/kg/day ^{14}C-TCDD, Monday through Friday, for 7 weeks.

Mathematical analyses of the data presented in Figure 12 revealed a rate constant for excretion of TCDD of 0.0293 $\pm$ 0.0050 day^{-1} which corresponds to a half-life of 23.7 days. The fraction of each dose absorbed was 0.861 $\pm$ 0.078. Using these values, it may be calculated that the ultimate steady-state body burden would be 21.3 D_O for rats given a daily dose of D_O, 5 consecutive days weekly for an infinite number of weeks. If D_O were administered every day for infinite time, the ultimate steady-state body burden would be 29.0 D_O.

Within the 7 weeks of this study, the rats had attained 79.1% of the ultimate steady-state body burden. The time required to reach 90% of the ultimate steady-state body burden would be 78.5 days.

What do the results and calculations mean insofar as assessing hazard of repeated exposure to very small amounts of TCDD? TCDD will not continue to accumulate in the body with repeated prolonged exposure. In rats, 93% of the ultimate steady-state level of TCDD in the body will be attained within 90 days. A toxicological evaluation of TCDD was conducted in rats given doses of 1.0, 0.1, 0.01, or 0.001 μg TCDD/kg/day, Monday through Friday, for 13 weeks (Kociba et al., 1975). Perceptible untoward effects did not develop in rats given 0.001 or 0.01 μg TCDD/kg/day.

Untoward effects including hepatic pathology and functional changes, atrophy of the thymus, and hematological alterations were observed in rats receiving 1.0 or 0.1 μg TCDD/kg/day. Indeed, some rats receiving 1.0 μg TCDD/kg/day died. The results of the studies on the fate and accumulation of TCDD in rats given repeated daily doses show clearly that even with more prolonged exposure those rats which received 0.01 μg TCDD/kg/day would not continue to accumulate TCDD in the body and its tissues leading to toxic manifestations as seen in those rats receiving 1.0 or 0.1 μg/kg/day. Since the levels of TCDD in the tissues had plateaued, essentially within 90 days, more prolonged exposure would not be expected to lead to the attainment of toxic amounts of TCDD in the body or its tissues.

DECABROMODIPHENYL OXIDE (DBDPO) AND OCTOBROMOBIPHENYL (OBBP): LINEAR PHARMACOKINETICS USED TO SELECT A COMPOUND FOR RESEARCH AND DEVELOPMENT

DBDPO and OBBP are potential fire-retardant additives for inclusion in plastics. The similarity of these agents to polychlorinated biphenyls (PCB's) suggested a potential for the bioaccumulation of DBDPO and OBBP and an associated cumulative toxicity with repeated ingestion. To ascertain the potential for bioaccumulation, ^{14}C-labeled OBBP and DBDPO were administered orally to rats and the excretion of ^{14}C-activity in the feces, urine and expired air was monitored (Norris et al., 1973). Figure 13 expresses the percent of a single-oral dose of ^{14}C-DBDPO and ^{14}C-OBBP remaining in the body as a function of time. DBDPO was eliminated rapidly in the feces. Also eliminated in the feces, OBBP eliminated was biphasic, the initial rapid phase very likely representing unabsorbed OBBP while the very slow phase represents material absorbed and subsequently excreted via the bile slowly. The $t_{1/2}$ for the slow phase was approximately 62 days. Using these data, it was suggested that the research and development of DBDPO continue and that OBBP be dropped from further consideration for the prescribed use because of its potential for bioaccumulation and associated cumulative toxicity.

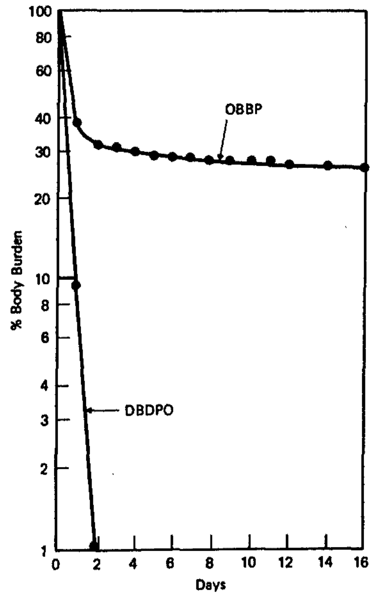

Figure 13. Percent of ^{14}C-activity remaining in the body as a function of time following a single-oral dose of 1 mg/kg ^{14}C-OBBP or ^{14}C-DBDPO in rats.

An additional study had been initiated to evaluate the chronic ingestion toxicity of DBDPO and OBBP. Analysis of the fat for bromine using neutron activation revealed steadily increasing concentrations of OBBP over 180 days in rats receiving 0.1 mg/kg/day (Figure 14). DBDPO did not accumulate. Similar results were obtained by analysis of the liver. These pharmacokinetic data in conjunction with the toxicological data substantiated further the low potential hazard of DBDPO as a result of bioaccumualtion and the high potential hazard of OBBP due to bioaccumulation. This example illustrates a means whereby pharmacokinetic studies can be used not only to assess the hazard of existing materials but also to provide guidance for the prudent use of research and development dollars for new chemicals.

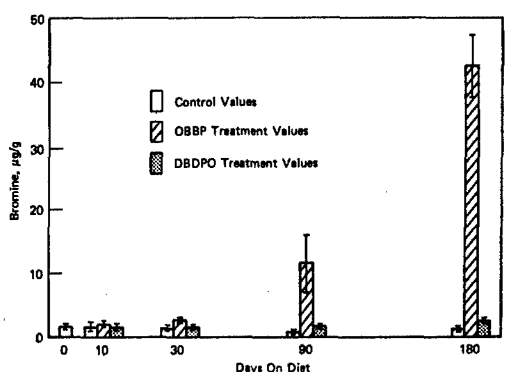

Figure 14. Bromine content of adipose tissue from rats maintained on diets providing a dose of 0.1 mg/kg/day of OBBP or DBDPO. Values are mean $\pm$ SE.

2,2-DIBROMO-3-NITRILOPROPIONAMIDE (DBNPA): RAPID ELIMINATION AND EXTENSIVE DEGRADATION

DBNPA is under development as a slimicide for use in the manufacture of paper and paperboard products. As such, the compound per se or degradation products may be leached to a small degree into food packaged in paper products and subsequently ingested. To elucidate the potential hazard, ^{14}C-DBNPA, 12 mg/kg, was administered orally to rats (LeBeau et al., 1973). Figure 15 shows that the ^{14}C-activity administered as ^{14}C-DBNPA is excreted rapidly. Within 24 hours, 82.4%, 5.0%, and 0.2% had been eliminated in the urine, feces, and expired air, respectively. An additional 5.2% and 0.6% were recovered from urine and feces in the subsequent 6 days giving a recovery of 93.3% of the dose of ^{14}C-activity from excreta. To ascertain whether the remaining 6.7% was retained in the body, the carcass and tissues were analyzed; only 0.6% remained.

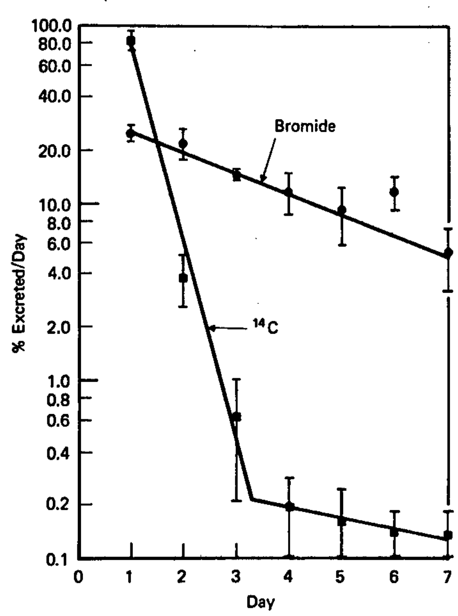

Figure 15. Percent of administered ^{14}C-activity or bromide excreted in the urine from rats following a single-oral dose of 12 mg/kg ^{14}C-DBNPA. Each point represents the mean and SD.

Concurrently, with the determination of ^{14}C-activity in the urine, urinary bromide levels were determined as can be seen in Figure 15. The excretion of bromide was slower having a $t_{1/2}$ of approximately 2.9 days. Since the bromine in the ^{14}C-DBNPA was covalently linked to the ^{14}C-labeled carbon, it was obvious that DBNPA was debrominated and metabolized readily. Subsequent analyses of the urine using thin-layer chromatography and mass spectrometry revealed ^{14}C-cyanoacetamide as the major degradation product as well as much smaller amounts of cyanoacetic acid and oxalic acid. These degradation products were the same as those found to occur spontaneously in paper.

These studies of the fate of DBNPA together with the toxicological data supported the conclusion that the ingestion of small amounts of DBNPA leached from paper into food did not constitute a hazard. Furthermore, the demonstration that the excretory products of DBNPA were the same as those occurring spontaneously in paper negates the necessity of conducting separate toxicological studies on the degradation products.

The foregoing examples have been utilized to illustrate how pharmacokinetics and toxicology are associated inextricably in assessing the potential hazard of chemicals. The objective of toxicological studies is to elucidate qualitatively and quantitatively the untoward effects of chemicals. Since toxicity is manifest by the presence of the chemical at the tissue receptor site, pharmacokinetics is utilized to estimate the concentration of the chemical at the receptor site as a function of dose and those intra- and interspecies functions which influence delivery and clearance of the chemical from the receptor site. In essence, acquisition of pharmacokinetic data reduces the variables encountered when extrapolating manifestations of toxicity encountered with high doses to predict the potential toxicity of low doses or when using toxicity discerned in one individual or species to predict the likelihood of its occurrence in aother.

For the toxicologist, nonlinear or dose-dependent pharmacokinetics is of paramount importance because the toxicity of many chemicals is incurred only with doses which overwhelm detoxification processes. As a consequence, there may be a disproportionate increase in toxicity, including carcinogenesis, with increasing doses. Chemicals for which there exists evidence for dose-dependent pharmacokinetics include styrene, ethylene glycol, aniline, carbon disulfide, 2-naphthylamine, benzopyrene, bis-hydroxycoumarin, salicylamide, amphetamine, sulphobromophthalein, bromobenzene, acetomenophen, isonicotinic acid hydrazide, vinyl chloride (Gehring, 1976). It is likely that as more compounds are evaluated, dose-dependent pharmacokinetics will be found to be the rule rather than the exception. Only those compounds having very high toxicity are likely to be exceptions by virtue of causing death or severe manifestations of toxicity at doses below the threshold of saturation of detoxification mechanisms.

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OPEN FORUM

COL. CARTER (6570 Aerospace Medical Research Laboratory): I'd like to address this question to Dr. Young. What kind of success have you had in getting the regulatory agencies to accept pharmacokinetic data as part of their overall hazard evaluation of specific chemical compounds?

DR. YOUNG (Dow Chemical Company): I have talked to groups at the various regulatory agencies about these points. I don't believe that they are ready to accept these concepts at face value but they are listening. They are interested in the data and I believe that we have to gather more and more evidence of these situations to sort of overwhelm the resistance to using these studies for making hazard assessments. Let me use an example. One important way we could use pharmacokinetic studies is in establishing the dose levels to conduct chronic toxicological studies. We occasionally get into trouble because we start chronic studies at dose levels that are too high and don't know it until the study is all over with, and then it's too late to do anything. Chronic toxicity studies conducted on dioxane are probably a very good example of that. The studies were run several years ago. The dose levels that were used were way above what anyone would now consider a maximum tolerated dose and soon after the study started, half of the animals died. After looking at the pharmacokinetics of dioxane, it's quite obvious what was wrong with the dose levels selected for study. With the complete pharmacokinetic picture that we have now we could quite easily predict what a reasonable top dose level should be in a long-term study. I think that, at least at the present time, would be the most fruitful use of pharmacokinetics. Going back retroactively and trying to explain these situations is much more difficult.

LCDR ANDERSEN (Naval Medical Research Institute): I have two comments to make about your presentation, Dr. Young. You've discussed three compounds wherein the compound itself is toxic and elimination mechanisms change, the dose is changed. You avoided the issue of materials that are made toxic by metabolism activity. In the latter case, many of the considerations you discussed for 2,4-T and dioxane change appreciably. In those cases, saturation kinetics may produce a maximum amount of toxins at low concentrations of the materials and at very high doses, you have a completely different situation. I think the work that you've presented is elegant. You can't, however, generalize about materials like 2,4-T and dioxane and compare these types of materials with compounds like vinyl chloride which are enzyme activated. They require two distinctly different treatments and you only showed one of them. I think that a general treatment of dose related pharmacokinetics is required before you can make such wide generalizations. The other remark

I would make regards your response to Col. Carter's question about your success with the regulatory agencies. You suggested the use of pharmacokinetics to pick the proper dose level to use when we do experiments. I believe that the experiments should be done the way they have been done - at high concentrations. The experiments should be done to find out if and why high concentrations are different from low concentrations. And at some point our science has to make an impact on the regulatory agencies to explain why the low concentration exposures are different. You just can't fix the dose and make sure you don't have an effect.

DR. YOUNG: In response to your comment about the validity of using very high dose levels, I think I would have to question the validity of data obtained from a chronic study where you've got half-dead rats with necrotic livers. It's difficult for me to understand how rats in that state could be used for extrapolation down to those exposed to lower dose levels where the animals are in a healthy state. There are probably situations where necrotic livers lead to possibly some tumor formation. I disagree with you when you say that these very, very high dose levels are relevant to lower dose levels. They are a part of an acute toxicity study. I think that in the case of dioxane, if we had the approach of the National Cancer Institute, and selected a dose level such as the maximum tolerated dose the animals would have survived, and I think that the data would have been much more meaningful. Regarding your first statement, I think that these compounds can be handled in the same way as the way that we are looking at detoxification now. We have the same situation if we look at intoxification. I think that probably vinyl chloride is a good example of that. Just recently, analysis of the pharmacokinetic studies on vinyl chloride have helped explain some of the data obtained by Maltoni on vinyl chloride at very high dose levels that didn't fit into the straight line extrapolation. This problem was resolved by using Michaelis-Menten kinetics to generate the dose response line with which all of Maltoni's data could be used. We have a much better estimate of the dose response curve for vinyl chloride and better statistics because more data points were used using this. If I gave the impression that pharmacokinetics could only go in one direction, I didn't mean to do that. With time we probably will get examples of exactly what you're talking about. We need to look more carefully at mechanisms where activation may be the primary cause for toxic response such as reactive intermediates that react with DNA causing mutations. With pharmacokinetics, we can look at the dose which reacts with DNA in vivo as a function of dose level. We can use essentially the same models, the same type of mathematical expressions we have for detoxification to quantify intoxification. I certainly didn't mean to exclude that from the realm of possibility.

DR. NEBERT (National Institutes of Health): Except for TCD data, is there good evidence that metabolic activation is not important in p-dioxane or 2,4,5-T toxicity? I don't know of any.

DR. YOUNG: Once again, I believe in order to answer that question that the molecular mechanism of toxicity is going to have to be known.

LCDR ANDERSEN: Dr. Young, I believe that the work that you do is very necessary and very fine. I think maybe I make my comments a little more succinct. I think you stressed so much that what happens at high doses may be toxic and may be unusual, and it seemed to me that you implied that downward extrapolation is always incorrect. What I'm saying is for materials that are metabolized quite differently than 2,4,5-T, it may not be true that at high levels you have increased toxicity. It may be that what is happening at the low levels in a saturable process requires a much better understanding of the intoxication process. I realize this could be done for materials and should be, but when you present your data, it leaves me with the impression that you've tried to present only those materials for which some type of saturation mechanism occurs and that they are more toxic at high concentrations. This will not always be the case. That's really the point I was trying to make.

DR. YOUNG: We have, of course, studied compounds where we have not seen saturation kinetics. I think TCDD is one example. If I understand the point that you are making, it is that the very, very low dose levels where we are extrapolating is still unknown. That may be true but I think that we may make errors in extrapolation depending on the shape of the curve in the area where we are collecting our data. It can go either way. It could be too conservative or it could be the opposite. If you have a curve which is increasing and plateauing and you only look at points on the plateau, you may grossly overestimate in your extrapolation. If you look on the rapidly descending part of the curve which then plateaus at a lower dose, you may grossly underestimate your toxicity. I agree with you that the direction of the extrapolation done at very, very low levels is uncertain. I think that what we need to do is to take very high specific activity compounds and study their molecular interactions and see what these interactions do as functions of dose levels, to define the low dose part of the curve much better.

DR. DREW (Brookhaven National Laboratories): Both the first and the third papers this morning have, I think, some severe implications toward the NCI bioassay program for carcinogenesis. It's my recollection that the NCI bioassay program calls for the use of the C3/BL6 mouse in most of their studies and the Fisher 344 rat. They also call for the use

of an MTD, and they define the MTD or Maximally Tolerated Dose as that dose which does not change the life span of the animal nor result in any pathology of some 30 organs examined nor does it change any weight loss or any weight of the test animals other than by greater than 10% after 6 weeks exposure. In Dr. Young's paper, with regard to the dioxane study, the doses used would not meet the criteria of an MTD in that they caused severe toxicity. Would he care to comment, however, on the use of the MTD because that in many cases is a much higher dose than would be normally encountered? Would you, Dr. Nebert, care to comment on the use of that particular mouse and whether that particular mouse responds in the way that you described your system and also on how rats respond in this system?

DR. YOUNG: Dr. Drew, the use of the maximum tolerated dose makes a lot more sense than the way some studies have been conducted in the past for reasons I've already commented on. I think that the way a study of the pharmacokinetics of a compound can help define that maximum tolerated dose is by determining whether the kinetics at this maximum tolerated dose level is going to lead to accumulation of a body burden of the compound which would not be seen at lower dose levels. Pharmacokinetic studies could add additional perspective to guidelines for identifying the maximum tolerated dose.

DR. NEBERT: The $C_3H/BL/6$ F_1 which the NCI has selected is interesting in that both strains are responsive. This is in the progeny of a C_3H crossed with a $C_{57}BL/6$ mouse. The progeny are agouti colored, multiply like rabbits and are very healthy. I think this is one reason, perhaps, for choosing this hybrid strain. Both inbred strains are responsive as are the F_1 generation hybrid. It's of interest, however, if somebody kept these mice and bred them in a brother/sister mating to get the F_2 generation, one in 16 is nonresponsive. This is an expression of a double homozygous recessive trait and this is our best evidence to show that we have at least two regulatory loci. We have congenic inbred lines to further sort this out. Another point with respect to cancer studies is that this mouse would be heterozygous at the H_2 locus. It would have an H_2K and an H_2B allele which is important for immunologic surveillance; one being extremely susceptible to spontaneous tumors, the C_3H allele, and the other being extremely resistant. This could be an advantage or a disadvantage. Again, if these were bred further in the lab, there would be a breakdown of homozygous H_2 loci in both directions. With respect to rats, our laboratory has looked at more than 20 inbred strains of rats, and we

find all strains inducible to various degrees. We have also found numerous wild strains of mice trapped from various locations in several countries to be inducible. My impression is that the wild type animal, and as far as I can tell, all rats are responsive. We've looked at more than 70 inbred strains of mice and they are roughly 50/50 distributed between responsive and nonresponsive strains. So there seems to be some evolutionary advantage for the nonresponsive mouse who is the mutant to survive in these colonies. We find differences in rabbits. Some are responsive; other strains are nonresponsive.

DR. HODGE (University of California, San Francisco): First, Dr. Kun, I'd like to express my admiration of this study giving us for the first time some real notion of how the mitochondrion gets its essential source of energy. These questions are the ones I'd like to raise. Does fluorocitrate produce its toxic effect by combining with the esterase closest to the outer portion of the inner membrane or with the hydrolase on the inner most part? Secondly, you indicated that certain cells apparently have mitochondria with pores that would carry citrate in or carry citrate out which involves a different arrangement of esterase/hydrolase geographically in the membrane. How do you know that arrangement is the specific one on the inner side of the mitochondrion for the cardiac and that it's different from the arrangement in the type of mitochondrion where you have both ingress and egress?

DR. KUN (University of California, San Francisco): To our knowledge, the thioester synthetase and hydrolase both react with fluorocitrate to form a macromolecular thioester. I have no evidence to show that one or the other would be preferentially reactive. The chances are that both of them are reactive because the way that we isolated these two enzymes first was by affinity labeling. The second question, "How do we know the vectorial aspect of this reaction?" If you take an isolated heart mitochondrion, this will show no inward oriented citrate flux. If we couple the intramitochondrial citrate synthesis to extramitochondrial fatty acid synthesis, this will show an outward oriented vectorial movement. As far as the topography is concerned, if you sonicate a mitochondrial membrane, the inner membrane turns inside out and what was inside is outside and in that case, of course, you can show that the thioester synthetase reaction does occur on this inverted inner mitochondrial membrane. That would be the approximate topography of these two enzymes.

MR. VERNOT (University of California, Irvine): Dr. Young, assuming that the increase in nasal cancer which was seen at the high levels of administration of dioxane was significant, have you developed any hypothesis for the genesis of the cancer? If the nasal cancer was related to the unchanged dioxane itself, does this have implications for inhalation exposures to dioxane?

DR. YOUNG: I don't think I should speculate on the carcinogenic mechanism for dioxane. I think that one might have some ideas that would stimulate some experiments. I mentioned dioxane induced tumors and the fact that a large fraction of inhaled dioxane was excreted in the breath only as an interesting point. I could speculate that possibly it's the dioxane itself which may be causing the nasal cancer but it would only be sheer speculation.

MR. VERNOT: The possibility exists, however, that the feeding studies themselves might not elucidate either the hazards which might accrue from inhalation exposure of the material because during an inhalation exposure, the nasal mucosa would be continuously exposed to the dioxane itself.

DR. YOUNG: There was a two-year inhalation study conducted on dioxane at 111 ppm and there were no adverse effects noted in that study. I think your line of reasoning there was very good but at least at the concentration tested, it didn't seem to be the case.

DR. WINSTEAD: I would like to ask Dr. Nebert if he would care to make any comments on the scientific basis, not other issues involved, for the use of genetic screening for worker placement in areas where they are going to be exposed to various chemicals?

DR. NEBERT: That day is probably coming but not at the present time. It's too early because the tests aren't sensitive enough to determine genetic differences. I think it should be possible to determine a person's capacity for enzyme induction or metabolism by measuring several forms of P-450 in blood or the urine metabolites of several challenge drugs, but not in 1977 or the immediate future.

SESSION IV

INHALATION TOXICOLOGY

Chairman

Henry J. Trochimowicz, Sc.D.
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PAPER NO. 13

COMPARATIVE TOXICOLOGY OF TETRANITROMETHANE AND NITROGEN DIOXIDE

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Acute toxicity studies were conducted on several atmospheric pollutants resulting from the manufacture of munitions, to evaluate the community and environmental health hazards associated with their emission. These studies were undertaken at the request of the U. S. Army to establish criteria for setting environmental or emission standards.

One of the compounds, tetranitromethane (TNM), has been a hazard associated with the manufacture of trinitrotoluene (TNT). Since this byproduct has the capability of escaping into the atmosphere and affecting areas surrounding munition plants, the U. S. Army has a strong interest in determining its toxicity characteristics.

The results of acute studies done in our laboratory are shown in Table 1. The rat 4-hour LC_{50} was determined to be 17.5 ppm while the mouse LD_{50} was 54.4 ppm. Responses of the animals, within each species, were consistently dose-related and followed a general pattern of lethargy and inactivity with some nose and eye irritation at the toxic levels. All animals remained inactive during exposure with noticeable decrease in rate and depth of respiratory movements. Deaths which occurred following exposure generally occurred within 12 hours. If the animals survived this time period, they usually lived through the 14-day observation period.

TABLE 1. ACUTE TOXICITY OF TNM TO RATS AND MICE

	RATS	MICE
Oral LD_{50} (95% C.L.)	130 (83-205) mg/kg	375 (262-511) mg/kg
Intravenous LD_{50} (95% C.L.)	12.6 (10.0-15.9) mg/kg	63.1 (45.0-88.7) mg/kg
4-Hour Inhalation LC_{50} (95% C.L.)	17.5 (16.4-18.7) ppm	54.4 (48.0-61.7) ppm

Intravenous administration of TNM to rats and mice resulted in LD_{50}'s of 12.6 and 63.1, respectively. As was seen in the results of the inhalation exposures, rats are much more susceptible to the compound than mice.

Intragastric administration of undiluted TNM to fasted male rats produced an LD_{50} value of 130 mg/kg. Similar administration of TNM to male mice produced an LD_{50} of 375 mg/kg. Most deaths occurred during the 12-hour period immediately following dosing.

The difference between LD_{50} values determined for the oral and intravenous routes suggested different mechanisms of toxicity dependent upon the route of administration. Exploring this difference, groups of two rats were each given single peroral doses of TNM and blood samples were taken after 90 minutes. The blood samples were analyzed for methemoglobin content and a dose dependent response was measured as shown in Figure 1. In the rats dosed at 62.5 and 125 mg/kg, a second set of methemoglobin samples was collected three hours after dosing and the measured values had only decreased slightly below those found at 90 minutes.

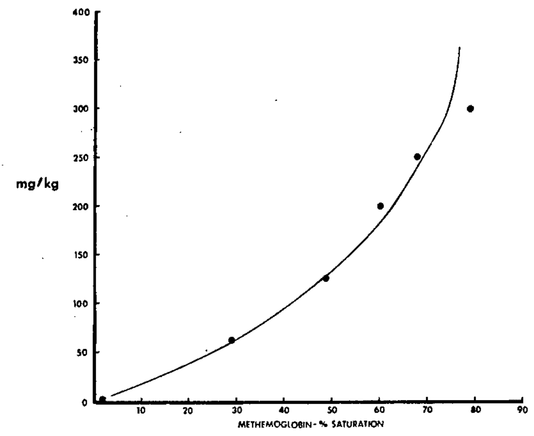

Figure 1. Methemoglobin response in rats given a single oral dose of tetranitromethane.

The toxic signs observed in the rats and mice after oral administration of TNM and the measured methemoglobin values are consistent with acute methemoglobinemia which is believed to be the toxic mechanism involved in lethality following this route of entry.

In order for TNM to form methemoglobin, the compound must be metabolized to nitrite ion. If NO_2 were liberated from the molecule it could be converted to nitrate and nitrate ions. The nitrate ion would then be converted to nitrite ion by bacterial action in the intestine. Since the mechanism of toxicity of ingested TNM appeared to be nitrite induced methemoglobinemia, the rat oral LD_{50} was compared with the rat oral LD_{50} reported for sodium nitrate by Smyth et al. (1969) of 180 mg/kg. Calculation of the NO_2 content of each compound gives an adjusted LD_{50} for NO_2 of 120 mg/kg TNM and 122 mg/kg for sodium nitrite.

The signs of toxicity observed in the inhalation and intravenous exposures of rats and mice were consistent with acute pulmonary irritation and respiratory deaths. At necropsy, animals exposed by these routes had congested and hemorrhagic lungs. Two rats were given an IV injection dose of 15 mg/kg TNM, slightly above the LD_{50} dose, and sampled for methemoglobinemia at 90 minutes and had the same methemoglobin values as untreated controls (0-3% saturation).

Animals exposed to TNM by the inhalation and intravenous routes reacted as if they had been exposed to nitrogen dioxide gas (NO_2). If one assumed that NO_2 was liberated from TNM in the lung on an equimolar basis (4 volumes of NO_2 for each volume of TNM) the measured TNM 4-hour LC_{50} value of 17.5 ppm would yield 70 ppm NO_2 which is comparable with the 4-hour LC_{50} for NO_2 of 88 ppm reported by Gray et al. (1954). Furthermore, a calculation of the dose of TNM inhaled by a 200 gram rat at the 4-hour LC_{50} concentration of 17.5 ppm assuming 100% absorption and a minute volume of 75 ml/min yields 12.6 mg/kg, a value identical to the IV LD_{50}.

Since the lethality of NO_2 is also due to its lung irritant properties leading to lung edema and hemorrhage, it seemed reasonable to hypothesize the toxicity of TNM was due to the action of the NO_2 moieties in the molecule leading to an LC_{50} approximately 1/4 that of NO_2. In order to determine whether the relationship between the toxicities of NO_2 and TNM held constant at subacute levels, an additional study was done with the rationale that a constant ratio at acute and subacute levels would imply a like relationship for chronic exposure. Exposure levels, both industrial and public, could then be set for TNM on the basis of presently accepted values for NO_2.

Simultaneous, two-week continuous exposures were conducted on equivalent concentrations of TNM and NO_2 assuming the NO_2 groups in TNM to be acting independently. NO_2 concentrations were 4 times those of TNM except for the last exposure which was over 5 times the TNM concentration. Exposure groups consisted of 100 male rats, housed 10 per cage. Similar numbers of control rats were exposed to air alone. The concentrations used in the experiment are shown in Table 2.

TABLE 2.

Experiment No.	TNM Conc., ppm	NO_2 Conc., ppm
1	7.5	30
2	5.0	20
3	3.5	14
4	7.5	40

Several problems occurred during the third and fourth day of the first exposure to 7.5 ppm TNM which resulted in concentration excursions. Two excursions, although for short duration, exceeded the 4-hour LC_{50} of 17 ppm. The following day 4 TNM rats died. Although the problems were eliminated and concentration control was satisfactory thereafter, the second exposure to 7.5 ppm TNM (Experiment No. 4) was planned because of the uncertainty caused by the excursions. Since the 30 ppm NO_2 had proceeded satisfactorily, an exposure to 40 ppm NO_2 was included in Experiment No. 4.

All rats were examined daily for general appearance, behavior, signs of toxic stress and lethality with body weights recorded immediately prior to the start of exposure and at the conclusion 14 days later.

Twenty rats per group including controls were reserved for methemoglobin determinations (MacEwen and Vernot, 1970) at the conclusion of the 14 day exposure period. In addition, groups of rats (consisting of 10 per group from the first experiment and 20 per group for all other experiments) had lungs precisely removed for wet weight determinations (Vernot and Kinkead, 1975). The wet lung weights of each group were statistically analyzed for determinations of edematous effects. The remaining animals were sacrificed and livers and kidneys weighed during necropsy.

Gross and histopathologic examinations were made on all animals that died during exposure or were sacrificed at the conclusion of the 14-day study. Organ weights of lung, liver and kidneys were recorded for all animals. Statistical comparisons (Student's t test) were performed on the mean organ weights.

The contaminant concentrations were continuously monitored using a colorimetric method in which a modified Saltzman reagent was allowed to mix and react with the sampled air in a glass delay coil. The resultant color developed was then related to the sample concentration and read using a Technicon AutoAnalyzer system.

Rats exposed to the highest levels of both contaminants showed lethargy, dyspnea, kyphosis and general poor health, but TNM exposed animals exhibited these symptoms of toxic stress to a greater degree than those exposed to comparable NO_2 concentrations. In addition, TNM caused a noticeable yellowing of the fur. Although the toxic signs decreased with decrease in concentration, they were still visible at the lowest concentrations tested.

The methemoglobin concentrations found in the blood of rats after 2-weeks of exposure to various concentrations of TNM and NO_2 are listed in Table 3. It is obvious that inhalation of either toxicant does not cause significant methemoglobinemia.

TABLE 3. METHEMOGLOBIN IN BLOOD OF RATS EXPOSED TO TNM OR NO_2 FOR TWO WEEKS

Contaminant	% Methemoglobin[1]	Contaminant	% Methemoglobin
Control	0.76 ± 0.11[2]	Control	1.01 ± 0.13
3.5 ppm TNM	0.92 ± 0.15	7.5 ppm TNM[3]	1.23 ± 0.31
14 ppm NO_2	0.99 ± 0.16	30 ppm NO_2	1.25 ± 0.27
Control	0.95 ± 0.12	Control	1.26 ± 0.22
5.0 ppm TNM	1.00 ± 0.16	7.5 ppm TNM	1.08 ± 0.13
20 ppm NO_2	1.21 ± 0.16	40 ppm NO_2	1.30 ± 0.26

[1] % of total hemoglobin.
[2] 95% confidence limits.
[3] Exposure which experienced concentration excursions.

The effect of the concentration excursions on the third and fourth day of the first exposure at 7.5 TNM, which twice exceeded 17 ppm for short periods, was an increase in mortality during the period from 5 to 9 days. Thereafter, the mortality in the initial and repeat 7.5 ppm studies were very similar. Figure 2 shows separate comparisons made between the mortality curves resulting from the second TNM exposure and those from 30 and 40 ppm NO_2. There appear to be no similarities between either of the NO_2 curves and that of TNM. In both figures, the 7.5 TNM curve intersects with the NO_2 curves. Mortality in the 40 ppm exposure increased rapidly until the third day, then leveled off until the tenth day when an increase began again.

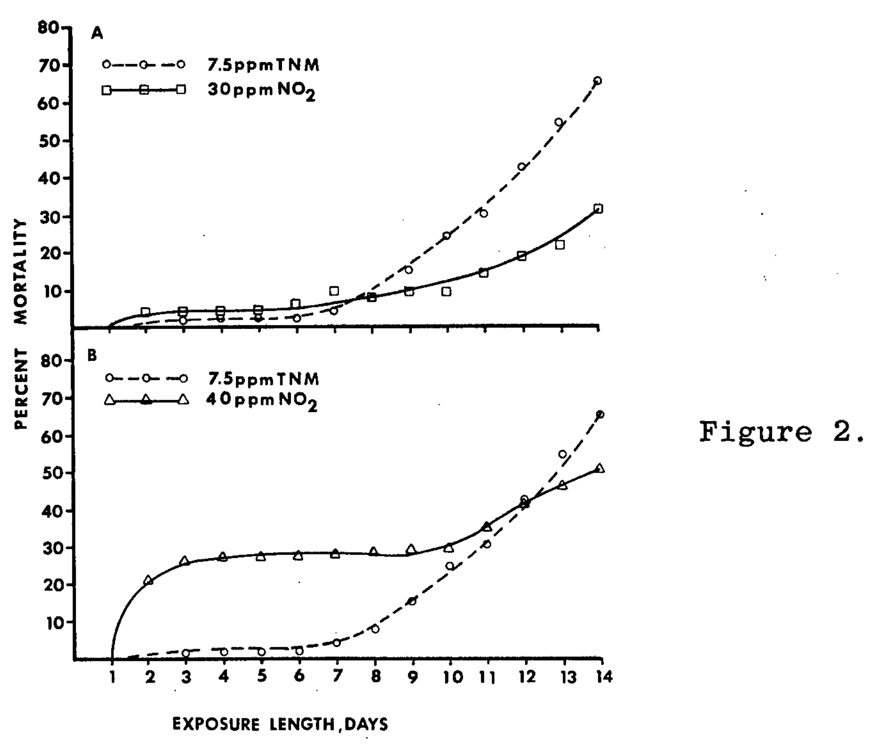

Figure 2.

Figure 3 shows the dose related effects of TNM and NO_2 on mean body weight obtained at termination of exposure. It is obvious that there are dose related decreases in body weight.

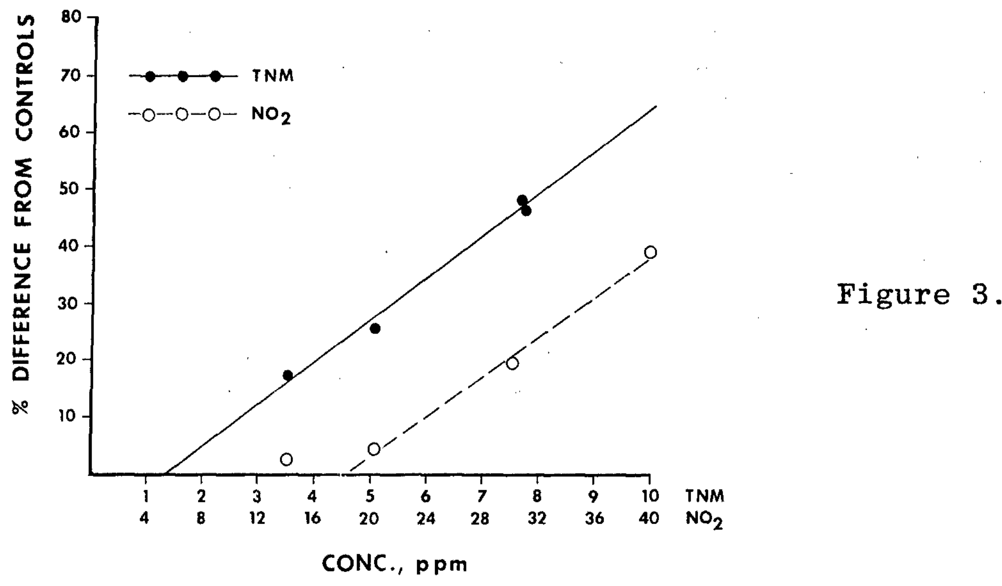

Figure 3.

Figure 4 shows the dose-related effects of exposure on the wet lung/body weight ratios. Here again, the effects are obvious with a dose related effect shown for each animal. Consistency in the control values and reproducibility of the 7.5 ppm TNM values show the validity of this test parameter.

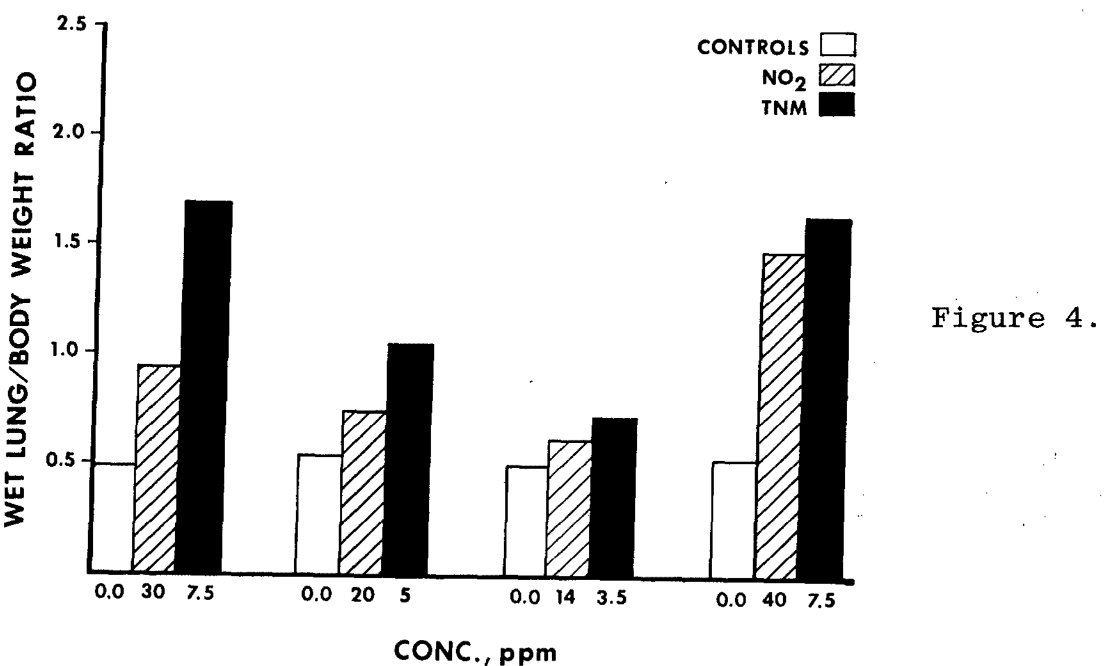

Figure 4.

Table 4 shows the incidence of respiratory disease in rats exposed to the two compounds. Histopathologic lesions seen were of three types: (1) Changes in the respiratory system that were related to exposure, in most cases, in a dose-related way. These changes were attributable to irritation of epithelium in the tracheobronchial tree and the alveoli. The primary changes due to irritation are largely exudative, but some proliferative lesions were seen. Lung edema was deemed the most severe primary lesion seen and its incidence was closely correlated with mortality. Secondary lesions were predominantly pneumonia and structural changes in lung architecture. (2) Lesions seen in other organs (liver, heart, and kidneys) were associated with death of the animal or altered hemodynamics due to advanced disease in the lungs. These lesions were predominantly distention of blood filled vasculature. (3) Incidental findings occurring sporadically within all systems examined, but not related to exposure of the contaminants.

TABLE 4. INCIDENCE OF RESPIRATORY DISEASE IN RATS EXPOSED TO TNM AND NO_2

	C	3.5 ppm TNM	14 ppm NO_2	C	5 ppm TNM	20 ppm NO_2	C	7.5 ppm TNM	30 ppm NO_2	C	7.5 ppm TNM	40 ppm NO_2
Pneumonitis	13	97	83	10	77	85	39	69	76	43	76	73
Catarrhal Bronchitis and/or Bronchiolitis	5	90	78	0	60	68	1	66	60	12	82	71
Catarrhal Tracheitis	0	10	3	0	20	22	4	33	30	2	36	24
Lung Edema	0	7	0	0	14	0	1	68	26	0	83	84
Bronchopneumonia	2	22	2	0	34	0	0	45	18	0	29	3
Histiocytic Pneumonia	2	28	3	3	18	23	3	9	18	7	13	23
Number of Animals	60	60	60	60	65	60	69	85	82	60	72	70

Lung edema, characterized by exudation of a protein-rich fluid into the alveolar spaces, was the most severe lesion seen directly attributable to the inhaled TNM. This lesion was noted only once in all control animals. Lung disease seen as secondary changes to the primary catarrhal lesions and lung edema were observed in a high percentage of rats exposed to TNM.

Lung edema was not seen in rats exposed to 14 and 20 ppm NO_2. One-fourth of rats exposed to 30 ppm NO_2 had lung edema, while the highest dose group (40 ppm) had this lesion in 84% of the animals - clearly a concentration dependent response once the effective threshold of injury is achieved. Secondary pulmonary disease was seen sporadically in rats exposed to NO_2.

Comparison of the mortality curves suggests that there is a difference in the mode of action of NO_2 and TNM. Although at 14 days the mortality resulting from exposure to 7.5 ppm TNM is much higher than that from 30 ppm NO_2, there is an indication of higher mortality early during exposure to NO_2. This is seen much more clearly in the 40 ppm NO_2 exposure where the course of deaths is biphasic with 21 deaths after 2 days and a phase between the 4th and 9th days where no deaths occurred. In contrast, the mortality curve resulting from TNM exposure rises smoothly.

Although the effects of the toxicants on rat body weight appear to be similar in that the dose response lines are parallel, this does not provide a constant potency ratio, and the potency of TNM is always greater than 4 times that of NO_2. No simple comparison can be made for lung weight effects. Above 3.5 ppm, TNM lung effects are relatively greater than 4 to 1 compared to NO_2, and below 3.5 ppm they are relatively less.

Using the parameters of mortality, lung weight increase and body weight rate decrease as indices of comparison, it is seen that the initial hypothesis of 1 to 4 molar equivalency of toxicity of TNM to NO_2 is not confirmed by the experimental facts. Quantitatively, the ratio is different from 1:4 in all parameters tested, lung weight, body weight and mortality. There appear to be qualitative differences as well as shown by the biphasic character of the 40 ppm NO_2 mortality curve and the appearance of the lung weight plot.

Examination of the histopathologic lesions induced by exposure to various concentrations of TNM and NO_2 indicates that the incidence of lung edema is the best gauge of the toxic action of TNM and NO_2. No edemagenic effect occurs upon exposure to 14 and 20 ppm NO_2 for two weeks while there is a concentration related incidence of edema in all exposures to TNM. Edema formation is the same in TNM and NO_2 exposed rats only at the 7.5 ppm TNM and 40 ppm NO_2 levels, a concentration ratio of 5.3 NO_2/1 TNM.

The actions of TNM and NO_2 during 2-week inhalation exposures are similar in the sense that the only effects appear to be those related to lung irritation and edemagenesis with no primary lesions in any other organ.

Results of this series of 2-week exposures demonstrate that the two compounds are not toxicologically equivalent on a 4 mole NO_2 to 1 mole TNM basis as postulated from acute toxicity test results.

Data developed in this study can be used to establish emergency exposure limits for TNM since concentrations having minimal effects after two weeks exposure would not represent hazards for short term exposure.

Because this study has demonstrated the toxicologic nonequivalency of TNM and NO_2, it cannot be used for the recommendation of chronic exposure limits (TLV's) for TNM. Only long-term animal studies using this contaminant would be satisfactory for such recommendation.

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PAPER NO. 14

A SEMIAUTOMATIC SMALL ANIMAL INHALATION FACILITY

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GENERAL DESCRIPTION

A "smart" data acquisition system coupled to a system for feedback control of gas concentration allows exposures in inhalation chambers to be controlled and documented easily and with reduced technical error. The capabilities of such a system far exceed those possible with manual operation, both in terms of accuracy of measurements and the complexity of exposures which may be performed.

Standard automatic data acquisition, whether from the periodic operation of a discrete sampling device such as a gas chromatograph, or from a continuous sampling device such as an infrared gas analyzer, saves time and labor by allowing the monitoring of chambers in the relative absence of human participation. In addition, such a system takes data uniformly, thus eliminating some errors likely with manual operation. However, without extensive hand processing, the resulting data represent only a point by point indication that the gas concentration was approximately what it should have been.

The addition of computing capability to the standard data acquisition system eliminates this and some other less obvious problems. A calibration is performed at several different concentrations in the general range of the desired exposure. From this an equation is derived which converts the measurement of gas concentration, generally a voltage or integral of a voltage, to the numerical value of the concentration being measured. This value is stored along with the time of the observation so that it may be presented in a format conducive to the meaningful evaluation of the exposure as a whole and the degree to which it matches the desired profile. The standard documentation of concentrations and times may also be presented as needed.

The system's computing capability may also be used to evaluate the daily calibration by comparing it with previous standards and by measuring how well the calibration data fit the form (generally linear) of the conversion equation. By establishing acceptance criteria, not only will human errors in calibration procedures be detected but so will most machine problems. By accumulating daily calibration data over a long period, a very good estimate can be made of the overall accuracy of the measurement system, and various equipment artifacts such as long term drift can be identified.

The final step in minimization of human participation is to provide a means by which the acquisition system controller can regulate the concentration within the chamber. Thus once the system has been set up, the operator need only run through a calibration and load the chambers, then return at the end of the exposure and unload the chambers. Since the control system constantly checks and adjusts the chamber concentration, the actual exposure will follow the desired profile more consistently than would be expected with human operators. In addition, it now becomes quite easy to change the concentration in virtually any preprogrammed manner. Multiple compound exposures are treated as independent runs in the same chamber, using the same or different analyzing instruments depending on specific circumstances.

SYSTEM DESIGN STRATEGY

Several decisions had to be made during the design of this system. The most basic of these was what portion of the system would be analog and what portion digital. Since the digital equivalents of analog components were generally more versatile, although also more expensive, a reasonable strategy was to make as much of the system digital as possible.

Next were decisions on the general system organization, for example, how many chambers were to be monitored, how much provision for multiple gas exposures was to be allowed, what types of analyzing instruments were to be used, how were the flows of exposure gases to be controlled, and what type of computing machine was necessary.

In our case the last question was answered first. Since we were not able to obtain a mini-computer, we purchased the best programmable calculator we could find. Concealed in that choice was the decision that all control would rest with the central processing element. An alternative, particularly

attractive for facilities with large numbers of chambers, is to handle each chamber with a dedicated local control element, such as a microprocessor linked to a central controller for data handling and overall control functions.

We chose to provide nine channels of operation for six chambers, thus allowing one-half of them to be run with two exposure compounds at any given time. Each could operate with either a gas chromatograph, infrared analyzer, or $NO-NO_2$ analyzer as required, although the infrared analyzers were used preferentially, since their response was faster and more easily monitored.

In order to control flows from various gas-generating systems an electrically operated metering valve was needed. Servo valves are available commercially; however, they require a small analog control system of their own and an analog reference signal. As an alternative to this, we were able to have metering valves mounted via clutch assemblies onto stepping motors (motors which rotate 1/400 revolution for each pulse received). A solenoid valve was added in series with each metering valve to provide on-off function. The resulting assembly (Figure 1) has proven quite adequate as long as the pressure of the gas supply is regulated within an appropriate range.

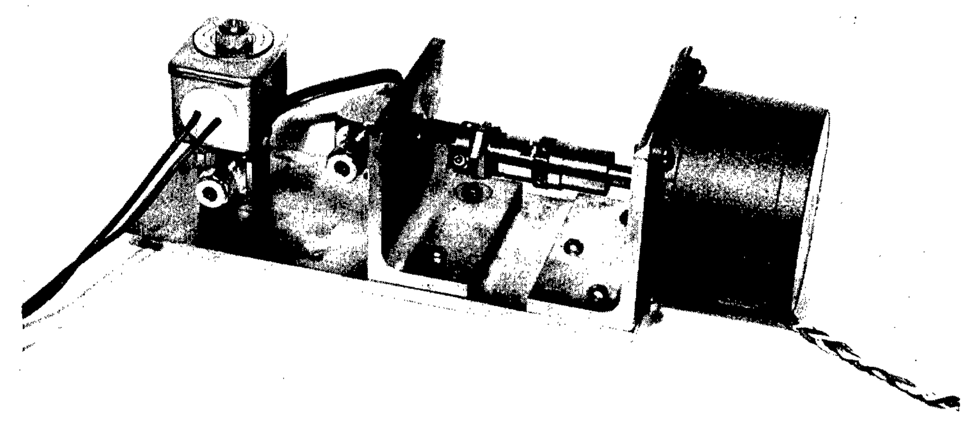

Figure 1.

HARDWARE DESCRIPTION

Chamber gas concentrations are measured by Miran Model 1-A, infrared gas analyzers, Varian Series 1400 gas chromatographs equipped with auto sampling valves, and a Beckman Model 952 NO-NO_2 analyzer (Figure 2). The outputs of these instruments are converted to 12-bit parallel digital format by one of the two Terrasyn Model 220 analog-to-digital converters operating at a fixed sampling rate of ten hertz. Each converter is equipped with a 16-channel multiplexer allowing the conversion of up to thirty-two independent analog signals. Since only nine channels are used for the analyzers, the remaining inputs are available to monitor additional variables such as temperature or humidity.

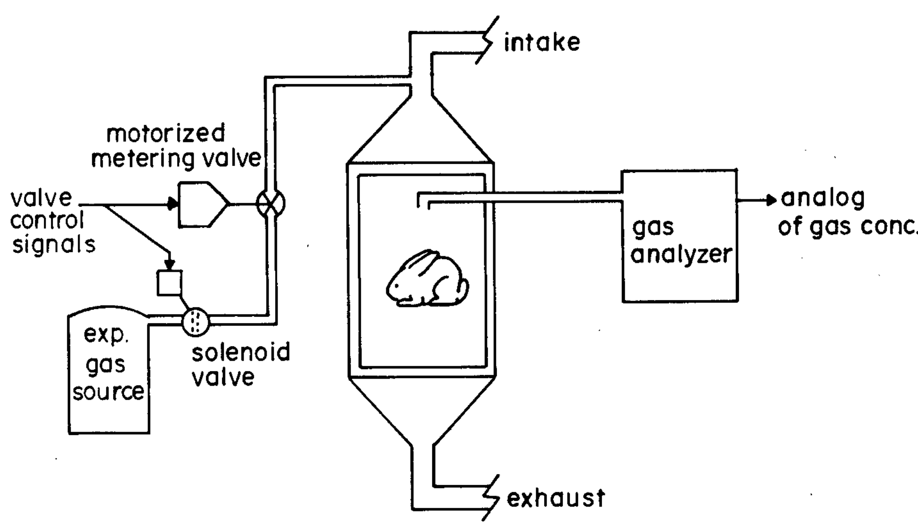

Figure 2. Machine controlled chamber.

Chamber control valves are driven by a single translator connected through a demultiplexer to up to twelve stepping motors. The operator interface is provided by a status board displaying calibration and status information for each chamber on four lights and one hexadecimal digit. Operator commands are entered using two digital switches, one identifying a channel and the other the desired command. The converters, stepping motor translator and status board, along with a clock and interval timer are interfaced to a Hewlett-Packard 9825 A programmable calculator, equipped with a magnetic tape, flexible disk memory and printer and plotter (Figure 3).

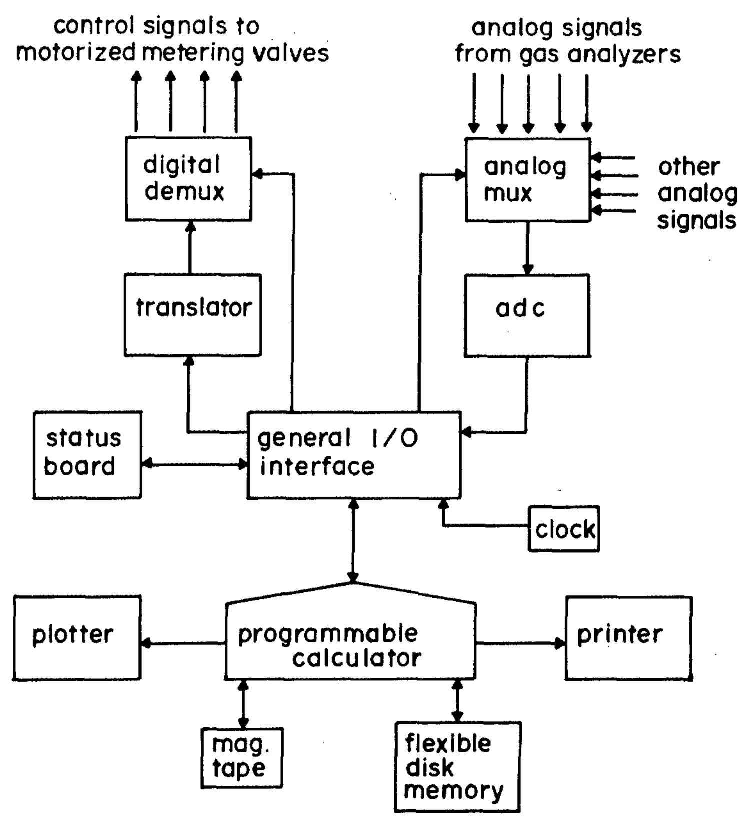

Figure 3. Functional diagram of control hardware.

SOFTWARE DESCRIPTION

The organization of system software is based on three primary functions of system operation: calibration, data collection, and chamber control.

Calibration consists of two distinct procedures. Immediately after assembling the apparatus necessary for a particular exposure, a characterization of system parameters is performed. Information concerning exposure conditions is entered at the calculator keyboard, then ten successive calibrations of three concentrations each are performed and analyzed. Bad calibrations, those significantly different from the group, are deleted and replaced by additional ones. From these initial data, measures of the distribution of slopes and adjusted ordinate means are calculated for use in evaluation of daily calibrations. The chamber is then run up to an average concentration, and various dynamic properties such as time constants and gains are measured.

Each day a single calibration consisting of three different concentrations is performed, tested against previous calibrations, and repeated if necessary. The slope and intercept of the regression line computed from these points are then used as constants for the conversion equation for that day.

During the daily exposure as each data point is taken, the voltage measurement is converted to the corresponding value of concentration and stored in integer format along with the elapsed time in a data file of the flexible disk memory. At the same time, this information is also printed on a whole system record, along with other channels and system control information as a "last resort" file.

At the end of a day's run an output routine prints a consolidated copy of the day's run. The integral of the concentration and of the concentration error, and other evaluations of performance are presented as consolidated measures of the exposure, along with calibration data and a point by point listing of concentrations and elapsed times.

On the first day of a series of exposures, a concentration vs. time profile is entered into a file on disk. This file is then used as the reference for chamber concentration throughout the exposure. Actual control is somewhat tricky since there is considerable delay between any adjustment and the resulting steady state concentration. This alone is of little concern; however, since many chambers are handled by the same system, corrections on any one channel occur only about every one-half time constant. Thus the correction scheme must be relatively accurate to insure good stability and reasonable response. To this end the steady state concentration is predicted from current and previous data and used to compute the necessary correction. In addition the gain of the control equation is modified to conform with current measurements.

CONCLUSION

An example of the use of a programmed concentration profile is shown in Figure 4. The solid line represents the results of monitoring morpholine concentration in ambient air during a simulated industrial situation. An exposure profile derived from these data was then programmed and run in an inhalation chamber. The resulting exposure, depicted by the dotted line, although not identical to the original, is a much closer approximation than a typical constant value exposure.

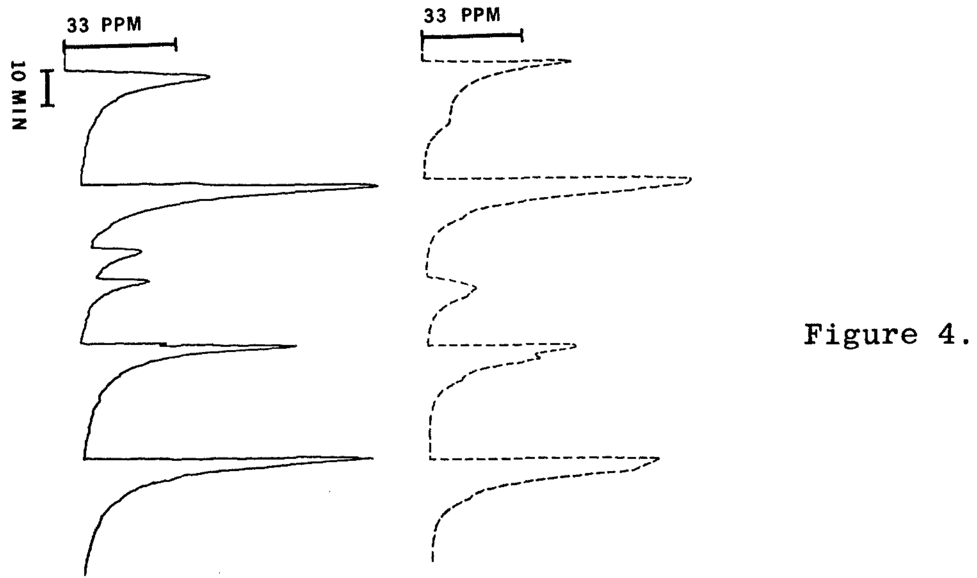

Figure 4.

Several additional features enhance the operation of this facility. To allow automatic restarts after power failures, the system is designed to load the control program at power up. Data and necessary control constants are stored on disk, which is unaffected by power loss, and the clock is connected to an auxiliary power source.

A second feature is a set of diagnostic programs which verify the operation of each component or group of related components. Another special function program plots an infrared frequency scan of air, air and exposure compound, and their difference, to aid in monitor frequency selection. To aid gas chromatograph setup, the same program can plot the equivalent of a strip chart recording for any window of time after the sample is taken.

Machine management of inhalation chambers offers many improvements in facility operation, not the least of which is the need for less operator attention, thus permitting the execution of more complex exposures. The magnitude of, and probability of committing technical errors are reduced as are the time and effort necessary to evaluate the quality of a day's run or the accuracy of an entire exposure regimen.

PAPER NO. 15

PULMONARY DEFENSE MECHANISMS AND INHALATION TOXICITY

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INTRODUCTION

Alveolar clearance of inhaled particles involves the action and interaction of several pulmonary defense mechanisms. Such mechanisms have been discussed in recent reviews (Lauweryns and Baert, 1977; and Green et al., 1977). Particle bombardment of the bronchus initiates changes that alter the aerodynamic patterns within the lung in order to eliminate or minimize particle deposition. These events are clearly seen in guinea pigs exposed to subacute concentrations of sulfuric acid mist where the resulting lesion is manifested as a distinct pattern of affected and nonaffected regions scattered throughout all lobes of the lung (Cavender et al., 1977a). Bloodstream defenses include the dissolution of particles, the killing of invading microorganisms, and/or the phagocytosis of debris in the parenchyma of the lung. These mechanisms are readily manifested in tuberculosis or in exposure to particulates, e.g. coal dust, fibers or aluminum chlorhydrate. These aerodynamic changes and blood defenses must mesh with the production of surfactant and mucous, the lymphatic circulation, and the mucociliary escalator without jeopardizing the gas exchange functions of the lung.

The studies presented herein have resulted in pulmonary lesions that are not the result of direct toxic effects of the inhaled compounds but these lesions have been produced as a result of "overstimulation" of specific pulmonary defense mechanisms.

Except where noted, all exposures were conducted in 1.3 m^3 stainless steel chambers (Hinners et al., 1968). Animals were exposed 6 hours/day, 5 days/week with appropriate chamber control animals exposed to clean filtered air. Exposure details have been published for sulfuric acid mist (Cavender et al., 1977a) and for aluminum chlorhydrate (Steinhagen et al., 1977).

Recruitment of Granulocytes to the Lung

An inflammatory response in the lung is mediated through thymic dependent (T) lymphocytes. T-lymphocytes are initially of bone marrow origin and play a central role in initiating and mediating delayed immune functions. They attain immunologic maturity under the direction or humoral influence of the thymus (Miller and Osoba, 1967). Mature peripheral T-lymphocytes are able to recognize foreign antigens. Specifically, the interaction of T-cells with a foreign antigen results in the synthesis of lymphokines. The association of a foreign antigen with specifically sensitized T-lymphocytes results in the activation of those cells. Within minutes after interaction with an antigen, alterations in calcium metabolism and membrane morphology occur in T-cells and within hours these cells synthesize new proteins (Allwood et al., 1971; Rosenberg and Levy, 1972). Lymphokines are among these newly synthesized proteins (Altman et al., 1973). Lymphokines are instrumental in the recruitment to and activation of macrophages and polymorphonuclear leukocytes (PMN) in the lung. Lymphocyte-derived chemotactic factor (LDCF) attracts macrophages and PMNs to a local tissue site, migration inhibitory factor (MIF) may immobilize these cells at the local site (David et al., 1964) and these cells undergo a process of activation which enhances both their phagocytic and bactericidal activities (Shima et al., 1972). Activation is probably initiated via the release of C_5 fragments of the complement system. The time course for granulocyte recruitment to airways exposed to endotoxin aerosols shows a peak number of PMNs at 4 hours in guinea pigs and at 6 hours in hamsters (Hudson et al., 1977). Recent studies have shown the increased bactericidal activity to correspond to PMN production of the deadly superoxide radical, O_2^- (Salin and McCord, 1974).

In summary, the normal defense mechanisms for microbial invasion or the deposition of other antigenic substances in the lung is as follows:

1. The three cell types of interest in this sequence are T-lymphocytes, macrophages, and polymorphonuclear neutrophils as shown in Figure 1. Normally, only macrophages and an occasional lymphocyte are present in the lung as illustrated in Figure 2.

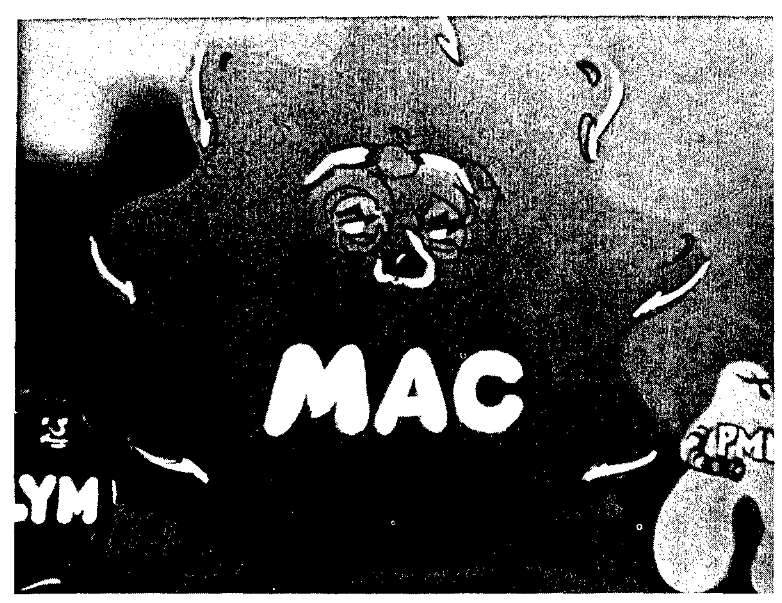

Figure 1. The three free-cell types of importance in pulmonary maintenance: lymphocyte (LYM), macrophage (MAC), and polymorphonuclear neutrophil (PMN).

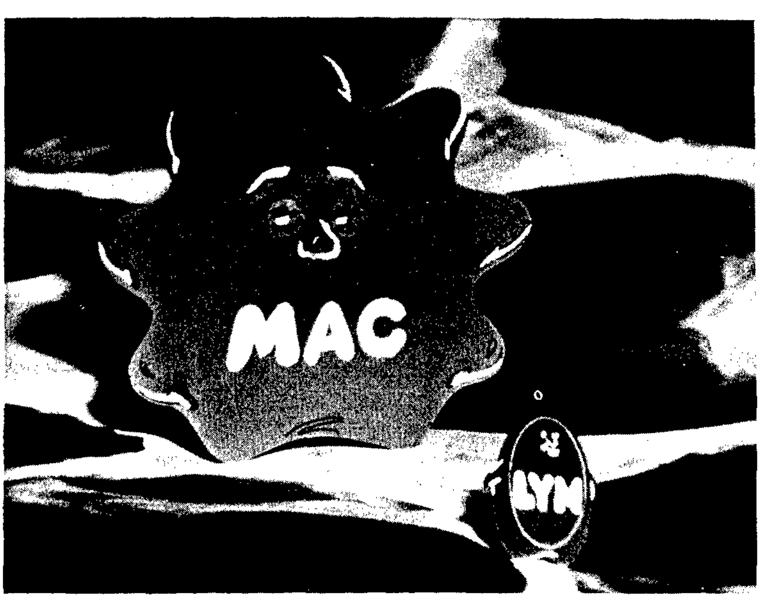

Figure 2. Macrophage and lymphocyte on lung background.

2. Upon antigenic deposition in the lung, the T-lymphocytes release chemotactic substances which attract macrophages and PMNs to the lung as shown in Figure 3.

Figure 3. Lymphocyte and macrophage reacting to bacterial invasion. Macrophages phagocytize invading bacteria while lymphocytes release chemotatic stimuli for granulocyte recruitment.

3. Activated macrophages and PMNs attack the invading antigen. PMNs kill the bacteria with superoxide and both macrophages and PMNs phagocytize and digest the bacteria as shown in Figure 4.

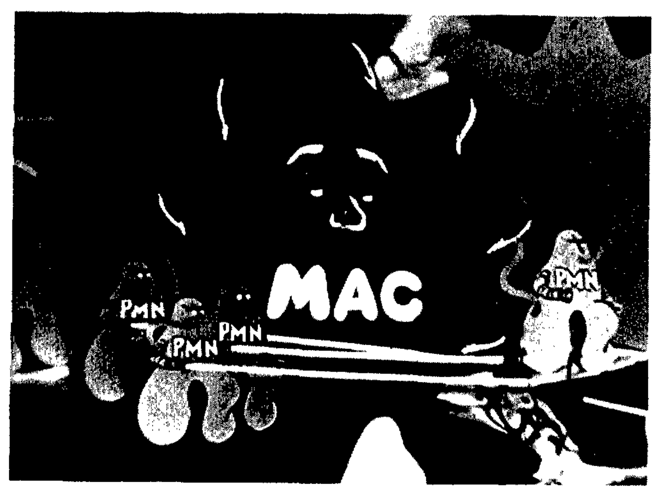

Figure 4. Polymorphonuclear neutrophils and macrophages combating invading bacteria. Polymorphonuclear neutrophils bombard the bacteria with the lethal superoxide radical, O_2^-. Both macrophages and polymorphs phagocytize the bacteria.

4. Lysosomal action in digesting the bacteria is shown in Figure 5.

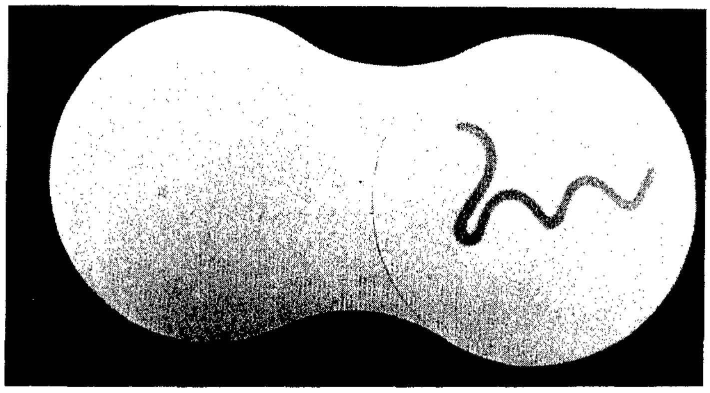

Figure 5. Lysosomal digestion of phagocytized bacteria.

5. This process continues until the lung is free of the invading species. The clearance of these phagocytic cells is illustrated in the schematic shown in Figure 6. The cells migrate to the mucociliary escalator or are removed via the lymphatic circulation.

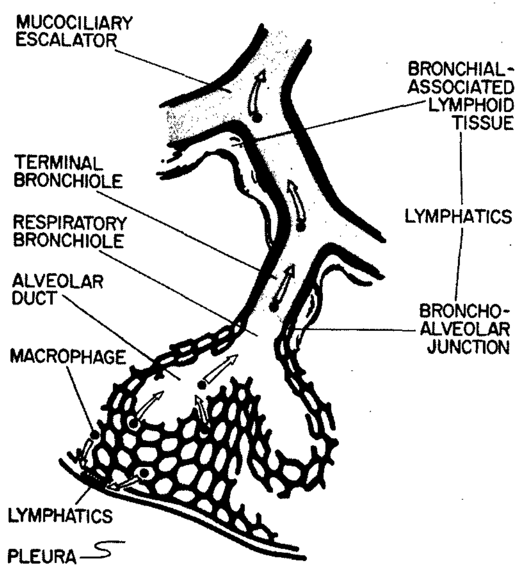

Figure 6. Schematic view of macrophage movement from alveoli.

Under certain conditions, the defense mechanism fails to clear the lung properly and the inflammatory response leads to granuloma formation. The classical example of such failure is in tuberculosis where, even in remission, there are pockets of bacteria "holed-up" within the formed tubercule or granuloma. These tubercules are "smoldering" pockets of the bacteria ready to erupt under appropriate conditions.

Granuloma formation can also occur in the lung upon short term exposure to seemingly nontoxic materials, e.g., talc. Another compound that has not shown any toxic response when applied to skin, even upon abrasion, is aluminum chlorhydrate (ACH). However, inhalation exposure of rats and guinea pigs to 25 mg/m^3 ACH produces granulomas within 5 days. Since skin tests have shown ACH not to be antigenic in and of itself, how do these granulomas form?

The normal function of pulmonary alveolar macrophages (PAMs) is to phagocytize particulates and cellular debris that may accumulate in the lung. When presented with latex beads in _in vitro_ studies, PAMs will engorge themselves as they phagocytize the beads. In the lung, they must phagocytize the antigenic substance and migrate to the mucociliary escalator or to the pulmonary lymphatic system in order to clear the lung (Figure 6). It is perhaps at this stage that PAMs create pulmonary troubles when exposed to nonantigenic particles such as ACH. The sequence of granuloma formation may occur as follows:

1. A few PAMs react to the deposition of ACH particles in the lung and begin phagocytosis as shown in Figure 7.

Figure 7. Macrophage phagocytizing aluminum chlorhydrate particles.

2. At least a few of these PAMs "overengorge" themselves and cannot clear the lung. This results in their death in the alveolar spaces as illustrated in Figure 8.

Figure 8. More macrophages and more ACH. As additional ACH deposits in the lung at least some macrophages "overengorge" themselves with ACH and cannot clear the lung. Their death in the lung releases protein which initiates the recruitment of polymorphonuclear neutrophils.

3. The release of cellular debris in the presence of ACH, some of which is protein bound, initiates an antigenic response in the lung as shown in Figure 9.

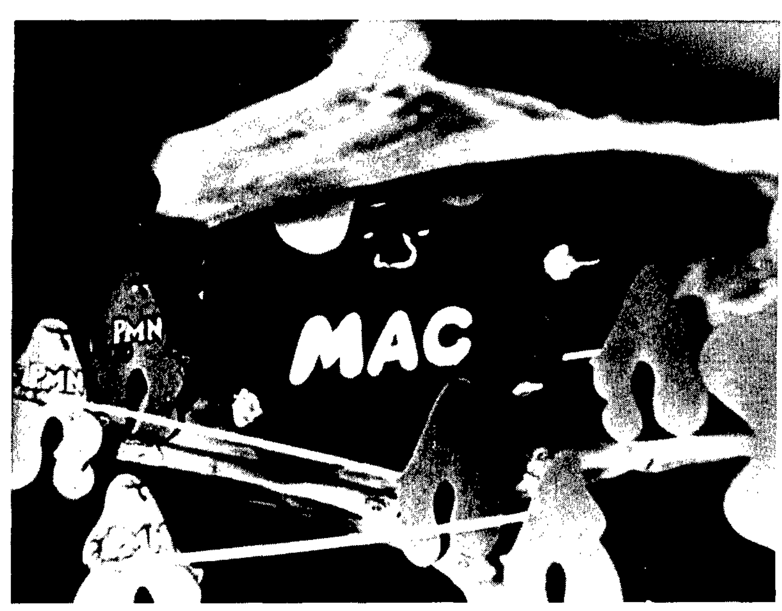

Figure 9. Polymorphonuclear neutrophils arrive and bombard each other with superoxide.

4. This process continues with more PAMs and PMNs arriving until a pile-up of dying cells (granuloma) occurs as is illustrated in Figures 10 and 11.

Figure 10. More macrophages and more polymorphonuclear neutrophils join in the melee.

Figure 11. The pile up of dying cells forms necrotic regions or granulomas.

An actual granuloma from ACH exposure is shown in Figure 12. Necrosis involves seven or eight layers of alveoli adjacent to the central region of the granuloma. Many PAMs and PMNs do clear the lung during this process as evidenced by the formation of microgranulomas in the bronchus-associated lymphoid tissue which is illustrated in Figure 13. These cells actually burden the lymphatic circulation as shown in Figure 14. In summary, the activation of PAMs to phagocytosis and subsequent overengorgement of ACH particles results in an inflammatory response in the lung that ends in granuloma formation. Once the granuloma is formed, removal of the stimulus (ACH) does not lead to regression of the lesion.

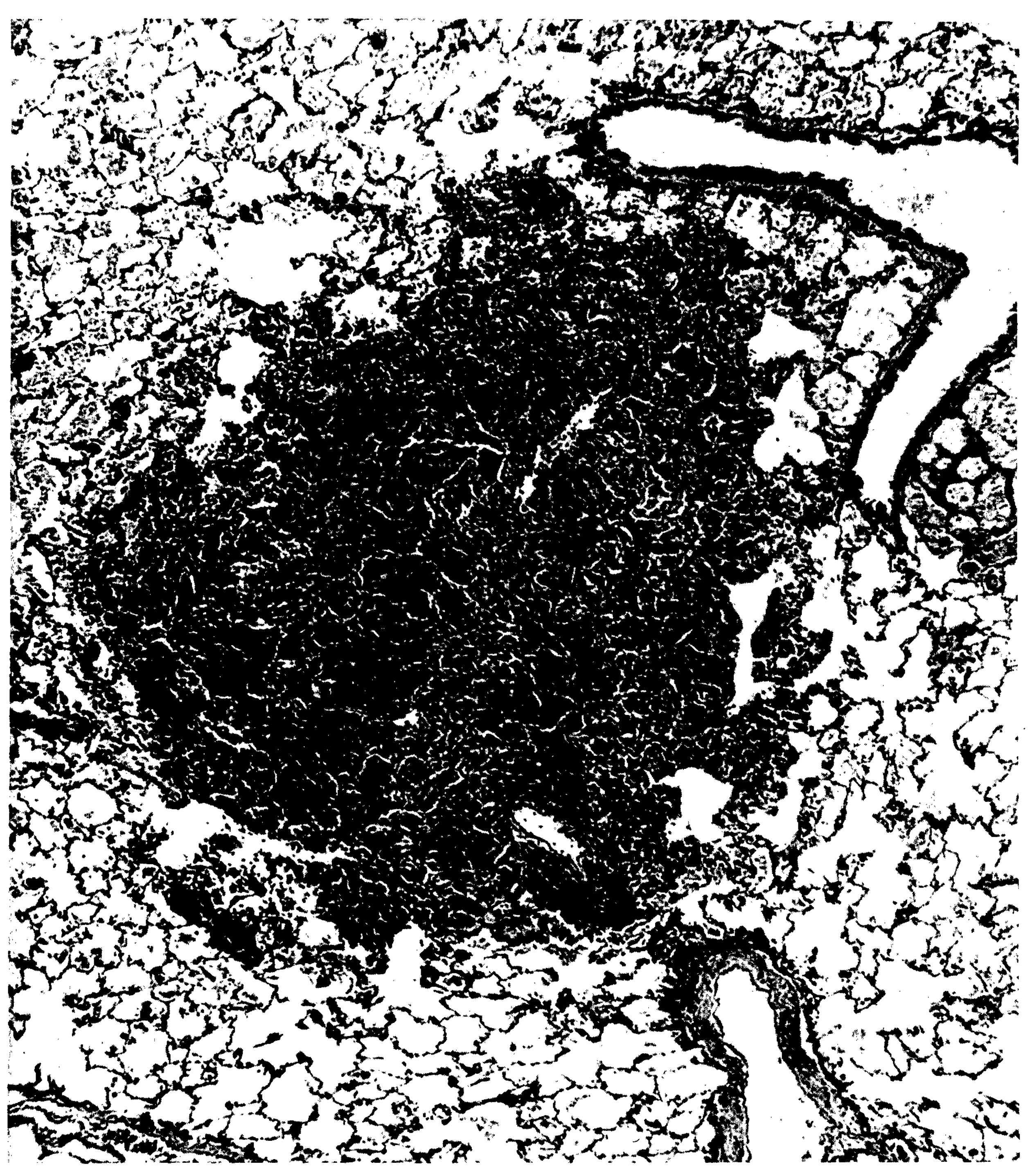

Figure 12. Focal granulomatous reaction in the lung of a rat exposed to 25 mg/m^3 of ACH. The reaction is characterized by accumulations of mononuclear cells, lymphocytes and large macrophages. The alveolar lumina surrounding these areas of heavy cellular infiltrations contain large macrophages with foamy cytoplasm. (H & E Stain Paraffin Section, X250).

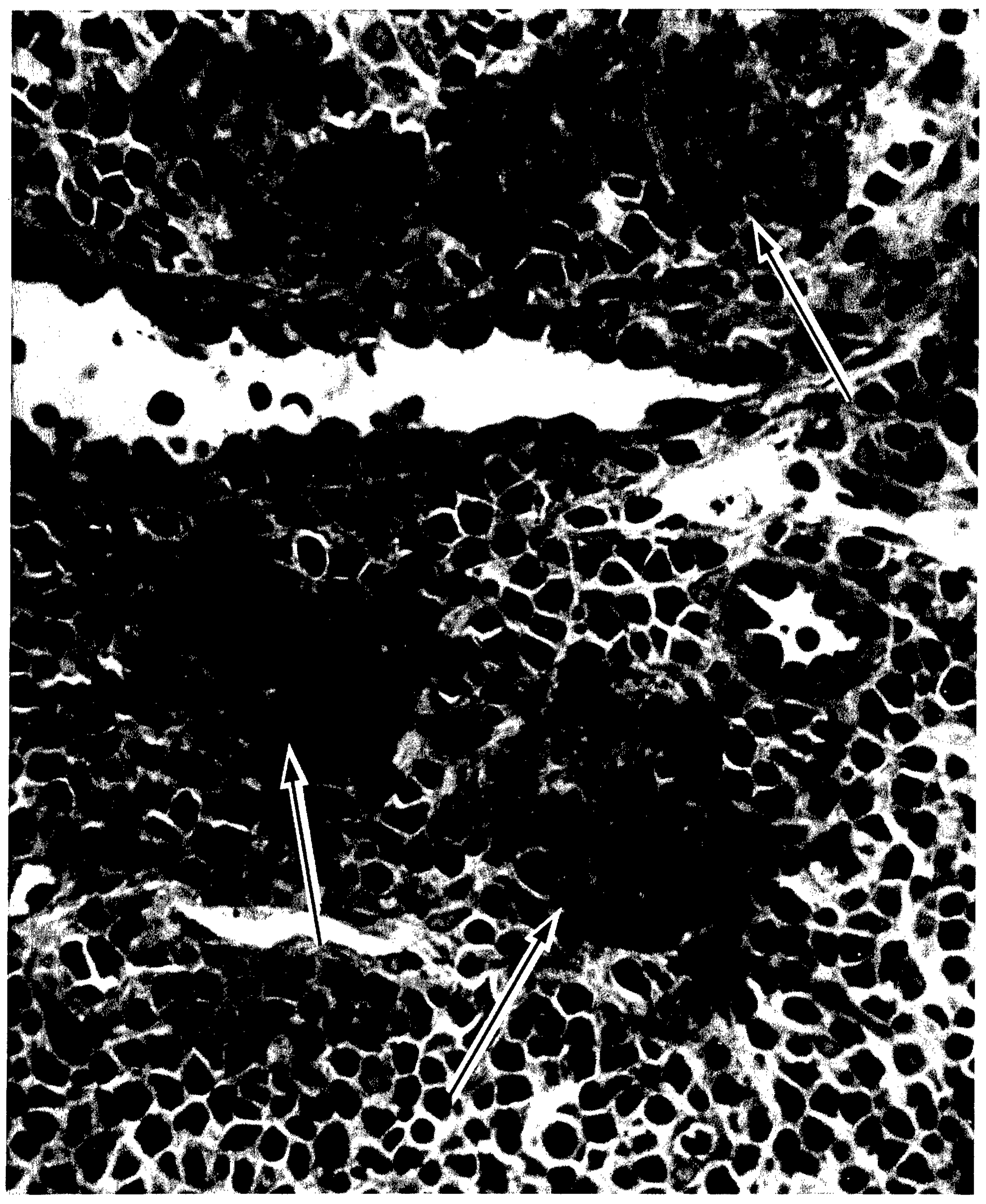

Figure 13. A peribronchial lymph node from a rat exposed to 25 mg/m^3 ACH illustrates multiple foci of particle-laden macrophages. (Epon Embedded Toluidine Blue Stain, X1,000).

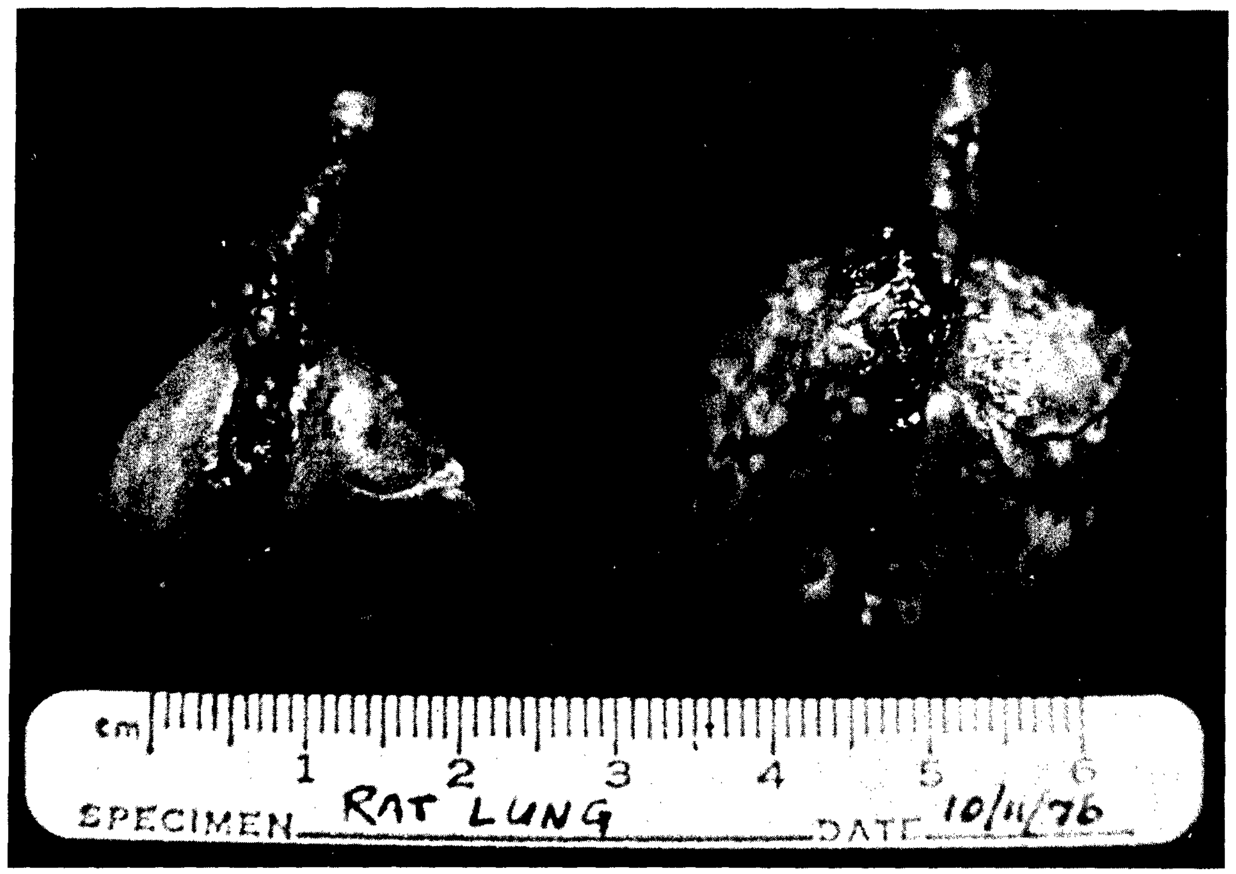

Figure 14. Gross appearance of a control lung (left) and a lung from a rat exposed to 25 mg/m^3 ACH.

Aerodynamic Changes Due to Particle Deposition in the Lung

The methodology for the measurement of changes in pulmonary resistance and compliance in unanesthetized guinea pigs exposed to environmental pollutants was developed by Amdur and coworkers (Amdur and Mead, 1955). These continuing studies have been employed for toxicologic evaluation of deep lung irritants. In summary, a guinea pig is placed in a plethysmograph and its resting values of resistance and compliance are measured over a thirty minute span. Thus, each animal serves as its own control. The animal is then exposed for up to six hours to an environmental pollutant and the changes in resistance and compliance are measured. In this way changes in resistance and compliance and their absolute values are recorded. It has been assumed that changes in pulmonary resistance and compliance represent subthreshold levels of direct toxic effects which can be measured and recorded long before histopathologic lesions appear. These studies have been used to rank compounds as to relative toxicity and many of the environmental standards concerning oxides of sulfur have been based on these and related studies.

Since their initial use in the mid-1950s, these elegant studies have filled important gaps in our basic knowledge of respiratory physiology and inhalation toxicity. However, the data obtained for guinea pigs have not been consistent with toxicologic data obtained for other animal species. Perhaps this divergence in irritant response is greatest for sulfuric acid mist where the LC_{50} for guinea pigs is 25-30 mg/m^3 (Cavender et al., 1977a) while for mice, rabbits, and rats, the LC_{50} is greater than 718 mg/m^3 (Treon et al., 1950). In acute and subacute exposures of guinea pigs to sulfuric acid mist, exposure related microscopic alterations in the lungs were characterized by diffuse regional, nonsuppurative alveolitis. However, in chronic exposures of rats and guinea pigs to 10 mg/m^3 sulfuric acid mist, 6 hr/day, 5 days/week, no significant histopathologic lesions were found in the respiratory tracts of either species. The severity of lesions in guinea pigs in subacute studies contrasted with the lack of histopathologic lesions in these chronic studies led to an investigation of the pathogenesis of the acid mist induced lesions.

The work of Amdur and coworkers has provided the following facts:

1. Irritant gases can be divided into at least two distinct types. One type, which includes sulfur dioxide and acetic acid, exhibits a biphasic response in the lung. At low concentration most of these vapors are adsorbed in the upper airways and the increase in resistance is due to this upper airway stimulation leading to a reflex bronchial constriction. At higher concentrations, at least some of the vapor reaches the bronchial tree and the increase in resistance is proportionally higher. It is this type of gaseous irritant that can be potentiated by simultaneous administration of sodium chloride aerosols. These irritants continue to increase pulmonary resistance over several hours of exposure; however, when the exposure is terminated, the resistance returns to control levels within three hours postexposure. The second type of irritant gas, which includes formaldehyde and formic acid, is more potent and exhibits a unimodal response of increasing resistance with increasing concentration. These irritants are not potentiated by sodium chloride aerosols and usually reach maximum effects within one hour of exposure. When the exposure is terminated, the resistance decreases markedly but has not returned to control levels within three hours postexposure. A summary of these experiments has been published (Amdur, 1959; Amdur, 1966).

2. Irritant gases like sulfur dioxide can be potentiated by inert aerosols of sodium chloride. Sodium chloride aerosols take up water upon entering the high humidity of the respiratory tract and serve to dissolve sulfur dioxide. Sulfur dioxide now penetrates to the lower respiratory tract dissolved in the aerosol, even at very low concentrations. This sulfur dioxide droplet aerosol behaves like the more potent type irritant gases such as formaldehyde. There is a possibility that some sulfur dioxide is further oxidized to sulfuric acid. All aerosols tested that can dissolve sulfur dioxide upon entering the respiratory tract potentiate the response, while dry aerosols such as ferric oxide do not. The more soluble sulfur dioxide or another pollutant is in the droplet aerosol, the greater the potentiation. Apparently, the process by which these aerosols are rendered more potent also converts them into aerosols that produce a response requiring more than three hours postexposure for the resistance levels to decrease to control values (Amdur and Underhill, 1968; McJilton et al., 1976).

3. Insoluble aerosols do not produce alterations in pulmonary flow resistance in concentrations up to 10 mg/m^3. Thus, they act strictly as nuisance dusts. Some soluble aerosols, when given alone, e.g., sodium chloride, do not produce alterations in pulmonary resistance; however, when given with a gaseous irritant, these soluble aerosols potentiate the response of the irritant gas (Amdur and Underhill, 1968).

4. Soluble aerosols containing sulfates or amines act as irritant aerosols and cause alterations in pulmonary flow resistance. Particle size is of extreme importance in eliciting a change in pulmonary resistance. These aerosols must penetrate the upper airways in order to effect an increase in resistance (Amdur, 1969; Amdur, 1970; Amdur and Corn, 1963).

One question that has never been resolved in these studies is: Are these changes due to direct toxic effects, or is it possible that bronchoconstriction is a pulmonary defense mechanism that precedes and attempts to prevent direct toxic effects? If this were the case, certain stimuli may cause severe bronchoconstriction without causing direct toxic effects. Such stimuli may act through specific receptors, e.g., sulfate, or through physical impaction on the bronchus. Under selective conditions, bronchoconstriction may be so severe as to result in anoxia in guinea pigs. These extreme conditions are seen in histamine exposures (Nadel et al., 1965; Amdur, 1966). Guinea pigs exhibit increased resistance accompanied with a marked decrease in compliance. As exposures continue, labored

breathing ensues followed by death via bronchoconstriction and laryngeal spasm. This sequence is also seen in acute exposures of sulfuric acid mist (Treon et al., 1950; Amdur et al., 1952; Pattle et al., 1956; and Amdur, 1958). When this is related to particle size data (Amdur and Corn, 1963) in terms of numbers of particles impacting on the bronchus, the prediction would indicate a greater stimulation for submicron particles (it takes one thousand 0.1 micrometer unit density particles to equal by weight one 1.0 micrometer unit density particle). Thus, stimulation, via physical impaction or special receptors located on the bronchus, leads to bronchoconstriction in a fashion similar to the nonirritant aerosol histamine. Bronchoconstriction probably involves the release of histamine or other vasopressor agents and includes the degranulation of Mast cells (Charles et al., 1977). The process can be visualized in Figures 15, 16, and 17.

Figure 15. Cross-sectional view of a respiratory bronchiole in a control guinea pig lung.

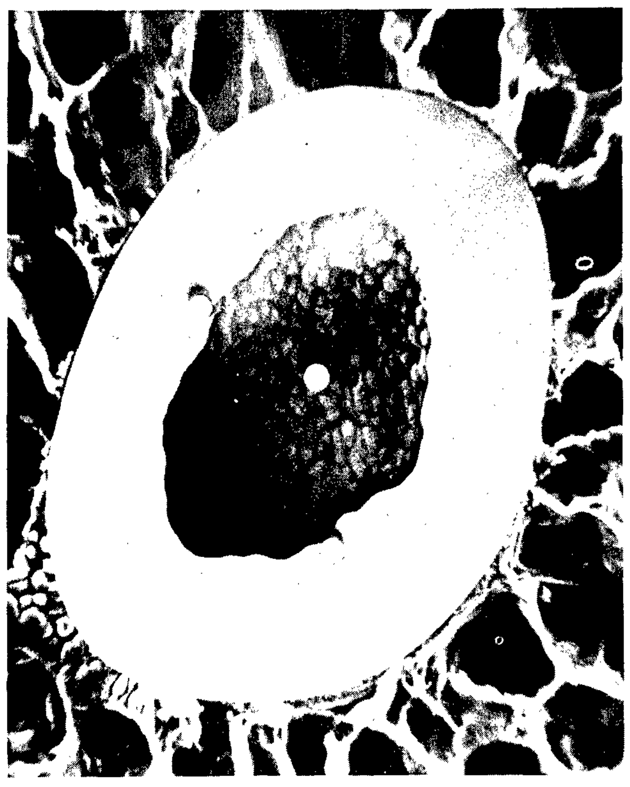

Figure 16. Constriction of the terminal bronchiole is shown and the activation of mast cells is portrayed by the three circles.

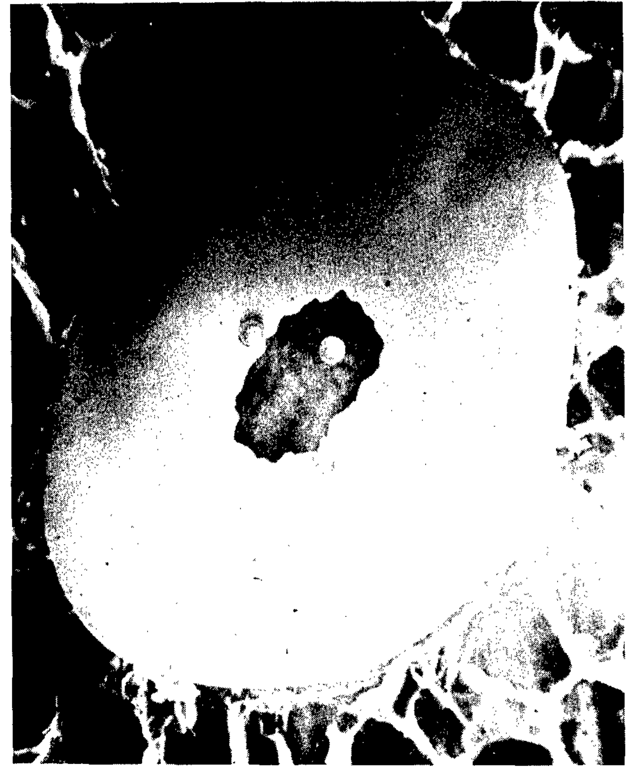

Figure 17. Further constriction of the bronchiole and further activation of the mast cells.

Is there further evidence that the results in guinea pigs may be due, at least in part, to nontoxic stimulation causing bronchoconstriction at low levels of these pollutants? The evidence is two fold: (1) In chronic studies at 10 mg/m^3 sulfuric acid mist, no exposure related histopathologic effects attributed to sulfuric acid were found during the two year study; and (2) Testing of sulfuric acid mist in numerous other species reveals no acute or subacute toxicity at concentrations thirty to forty-fold higher than the LC_{50} for guinea pigs (Treon et al., 1950; Pattle et al., 1956; and Schwartz et al., 1976). Similar acute and subacute data have been reported for sulfur dioxide, indicating a relatively nontoxic status for the oxides of sulfur (Rajagopalan and Johnson, 1976).

In contrast, two other studies should be mentioned. In chronic studies involving sulfuric acid mist (Alarie et al., 1973, 1975), guinea pigs after 52 weeks exposure exhibited no deleterious effects while minimal but definite effects on pulmonary structures and deterioration in pulmonary function were seen in cynomolgus monkeys following 78 weeks exposure. In a recent industrial exposure (Smith et al., 1977), distinct loss of 1-sec forced expiratory volume was found in workers exposed to sulfur dioxide, respirable particulates, sulfates and copper. The effects were attributed to sulfur dioxide although the authors failed to acknowledge the possible interactions of the other exposure species in terms of the high relative humidity of the respiratory tract. Changes in solubility and humidification occur within fractions of a second and could, in this instance, play an important role in the interpretation of results (Cavender et al., 1977b).

From these data, it is reasonable to interpret small changes in pulmonary resistance and compliance in the guinea pig as pulmonary defense mechanisms. When these changes are recorded as direct toxic effects, a very different interpretation is reached using guinea pigs when compared with data using other species. For the oxides of sulfur, toxic concentrations are forty-fold different for guinea pigs than for other rodents and laboratory animals. This fact once again emphasizes the importance of using more than one species in any toxicologic evaluation. This is especially true when establishing environmental and/or industrial regulations.

SUMMARY

Pulmonary defense mechanisms must protect the lung from infectious agents, chemical toxins, mineral dusts and immunologic particles. Each day a surface as large as a tennis court is exposed to a volume of air containing these foreign materials that would fill a swimming pool. Respiratory

diseases represent relative failures of pulmonary defense mechanisms. Certain pulmonary lesions result from an overstimulation of specific defense mechanisms. The recruitment of granulocytes in rodents in response to aluminum chlorhydrate inhalation and bronchoconstriction in guinea pigs in response to sulfuric acid mist impaction on the bronchus appear to be two pulmonary defense settings that can be stimulated to the extent of causing pulmonary lesions. The measurement of pulmonary resistance and compliance in guinea pigs, which represents the extent of bronchoconstriction, has been utilized to rank the relative toxicities of various environmental contaminants and in establishing industrial and environmental regulations without supporting data in other species. Since the data for guinea pigs may be a measure of a pulmonary defense mechanism, i.e., bronchoconstriction, and does not represent direct toxic effects, these regulations are not based on sound toxicologic evaluation. The requirement for use of two or more species in the toxicologic evaluation of environmental pollutants must be strongly emphasized.

ACKNOWLEDGEMENT

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PAPER NO. 16

A LONG-TERM INHALATION TOXICITY AND CARCINOGENICITY STUDY OF VINYL BENZYL CHLORIDE IN RATS AND MICE*

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INTRODUCTION

Vinyl benzyl chloride (VBC) monomer is an alkylating intermediate in the production of polymers. Alkylating chemicals have been found to have carcinogenic activity (Shimkin et al., 1966; Walpole, 1958; Haddow, 1959; Ross, 1962; Wheeler, 1962). Included among carcinogenic alkylating agents are some of the alpha-haloethers such as bis(chloromethyl)ether (Van Duuren et al., 1968; Gargus et al., 1969; Leong et al., 1971; Laskin et al., 1971; Drew et al., 1975; Laskin et al., 1975; Kuschner et al., 1975). Since VBC is an alkylating agent, it has been studied in rats and mice exposed by inhalation to elucidate its potential to induce chronic toxicity with special emphasis on carcinogenicity.

In a preliminary study, repeated 6.5 hour exposures of dogs, rabbits, guinea pigs and rats to 8 ppm of VBC resulted in eye and nasal irritation, pneumonia, and death of some animals on study. At the termination of the 6-month exposure period, the animals were killed for histopathologic examination. The only apparent adverse effect was respiratory irritation. Other organs such as the liver and kidneys were not injured (Torkelson, 1959). In another study on the odor and sensory irritation threshold of VBC on human subjects, VBC could be detected at a nominal concentration of 0.07 parts per million (ppm). At 0.5 ppm,

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the vapors caused considerable irritation to eyes, nose, and respiratory tract. At 1.9 ppm, the irritation was intolerable to human subjects within minutes. However, the chronic effects of VBC at concentrations at or below the sensory irritation threshold have not been studied, especially with respect to the potential carcinogenicity of the compound.

This paper presents the findings of a study in which Sprague-Dawley rats and Swiss-Webster mice were exposed to 1 and 0.1 ppm of VBC in air daily for 6 months, followed by a lifetime observation period.

MATERIALS AND METHODS

Sample

The sample (supplied by The Dow Chemical Company, Midland, Michigan) contained 67.91% meta- and 30.58% para-isomers of VBC. The sample was 98.49% pure with 0.14% of vinyl toluene, 0.20% of alpha chlorinated vinyl toluene, 0.47% of beta chlorinated vinyl toluene, 0.23% of dichloroethyl toluene, and 0.24% of unknown impurities.

Vapor Generation

Exposures were conducted in 3.7 m^3 stainless steel chambers under dynamic airflow conditions. The exposure atmosphere in each chamber was generated by metering liquid VBC into a heated glass vaporizer at a calculated rate. The vapors emerging from the vaporizer were diluted with room air at a rate to provide the desired VBC concentration. The nominal concentration of VBC in the chamber atmosphere was the ratio of the rate of VBC delivery to the rate of total chamber airflow (the volume of air ejected from the vaporizer plus the volume of the make-up air).

Chamber Atmosphere Analysis

The concentration of VBC in the chamber was determined by analysis. For analysis, 20 liters of air from the chamber was drawn at the rate of 1 liter/minute through 25 ml of cold methanol in a fritted bottom bubbler. The VBC dissolved in methanol was extracted with 10 ml of carbon disulfide (CS_2). The quantity of VBC in a 2 ml sample of VBC-CS_2 solution was analyzed by means of a gas chromatographic technique. The gas chromatographic conditions were as follows:

- Instrument, Varian Aerograph Hy-Fi Model 500 equipped with a tritium electron capture detector

- Column, 5 ft. x 1/8 inch diameter, stainless steel, packed with 20% SE-54 on Chromosorb W (80-100 mesh)

- Inlet temperature, 165 C

- Column temperature, 160 C

- Detector temperature, 150-170 C

- Carrier gas, helium at 20-30 ml/minute

- Sample size, 2 µl

The concentration of VBC in each sample was found by interpolation from a curve prepared with standard VBC solutions in CS_2.

Experimental Design

Three groups of animals each consisting of 120 male Sprague-Dawley rats (Spartan Research Animals, Haslett, Michigan) were used. One group was exposed to 1 ppm (6.2 mg/m^3) of VBC and a second group to 0.1 ppm (0.62 mg/m^3). The third group served as controls and was maintained outside of the chambers under ambient conditions. The duration of exposure was 6 hours per day, 5 days per week for a total of 131 exposures in 6 months. Food and water were removed from all animals during the exposure period. Even though controls were not placed in chambers, food and water were removed from them during the exposure period. After the 6 month exposure period, the rats and mice were kept under ambient conditions and observed for the duration of their lifespan (maximum of 30 months for rats, 25 months for mice). Animals were observed throughout the study for changes in physical condition or demeanor. Body weights were recorded once a week for the first 3 months of the study and monthly thereafter.

Hematology

During week 12 of the exposure period blood samples were obtained from the tail veins of 10 rats from each of the control and the 1.0 ppm exposure groups. These blood samples were used in determination of the packed cell volume (PCV), red blood cell count (RBC), hemoglobin (HGB), white blood cell count (WBC) and differential. From the rats killed on day 1 and day 5 of the postexposure period (week

27 of the study), blood samples were collected into heparinized test tubes from the cervical blood vessels at time of decapitation. The same hematologic determinations were carried out again during weeks 52 and 104 of the study on blood samples obtained from five rats/exposure level.

Pulmonary Exfoliative Cytology

Four rats from each group were subjected to pulmonary exfoliative cytologic examination on day 1 of the postexposure period.

Following deprivation of food overnight, each of these rats was anesthetized with methoxyflurane; the trachea was exposed prior to clamping with a hemostat; and the trachea and lungs were removed as a unit following decapitation. Using an adaptation of the method of Gross et al. (1969), a saline wash was used to collect cells from the tracheobronchial system.

Cells in the saline washings of the tracheobronchial system were sedimented by centrifugation and then resuspended in 0.5 ml of the supernatant. Two drops of the cell suspension was spread on glass slides and fixed by application of an aerosol hair spray. After drying, a fixed smear from each rat was stained with Giemsa's stain and another was stained by the Papanicolaou method. The prepared slides were examined microscopically using the oil immersion objective.

The cell population on slides from each rat was evaluated for atypical or neoplastic cells and for altered cellular distribution resulting from inflammatory or other disease processes. In addition, a representative number (100 cells) of cells on the Papanicolaou stained smear was classified according to morphological type. These animals were also used for pathological examination as described below.

Cytogenetic Evaluation of Bone Marrow Cells

On day 5 of the postexposure period, 4 rats from each group were submitted for cytogenetic evaluation of bone marrow cells. These rats had been deprived of food overnight. Four hours prior to sacrifice, each rat was given 4 mg/kg colchicine via intraperitoneal injection. Each rat was rendered unconscious by placement in a CO_2 chamber. The trachea was then exposed and clamped with a hemostat prior to decapitation. Both femurs were removed immediately after death and the enclosed bone marrow was aspirated into Hank's Balanced Salt Solution for preparation of slides for chromosomal cytogenetic examination.

Pathologic Examination of Rats

Rats killed for pulmonary exfoliative cytologic evaluation and for cytogenetic evaluation of bone marrow cells were also used for pathologic evaluation.

Each rat was subjected to a complete gross examination. The heart, brain, liver, kidneys, and testes were removed from each rat and weighed. Organ to body weight ratios were calculated. The trachea and lungs were removed as a unit and fixed by intratracheal infusion of 10% formalin. Fixation with formalin occurred after washing of the tracheobronchial tree with saline in animals used for pulmonary exfoliative cytologic examination. Representative portions of thoracic lymph nodes, thymus, trachea, thyroid, parathyroid, heart, liver, kidney, testes, adrenal, accessory sex glands, spleen, pancreas, spinal cord, brain, eye, stomach, small intestine, large intestine, olfactory epithelium, urinary bladder, external nasal mucosa, pituitary gland, and all gross nodules were routinely preserved in 10% formalin. Following fixation, standard histologic procedures (including decalcification of nasal turbinates) were used to prepare hematoxylin and eosin stained sections from all rats for histologic examination.

Pathologic Examination of Mice

All mice were held for observation until they died or were killed in a moribund condition. Each mouse was subjected to a thorough necropsy examination, with specific emphasis on the presence or absence of tumorous lesions. All major organs and tissues were preserved in 10% formalin. Microscopic examination was conducted on all lungs and livers in which gross necropsy examination had revealed the presence of nodular lesions suggestive of a proliferative pathologic process, such as tumor formation.

Data Analysis

Statistical analysis of hematologic parameters, body weights, and organ weights were carried out using an analysis of variance and Dunnett's Test. The mortality data and tumor incidence were analyzed with Fisher's Exact Probability Test. The level of significance in all statistical analysis was $p < 0.05$.

RESULTS

Chamber Atmosphere Concentrations

The analytical concentrations (mean ± S.D.) for the two chambers were 0.11 ± 0.05 and 0.93 ± 0.29 ppm for the low and high exposure levels, respectively. Although there were day-to-day variations in chamber VBC concentration, 80-90% of the total exposure days were within 50% of the designated levels. There was no overlapping of concentration between the high and low range throughout the exposure period.

Clinical Observations

During the exposures, the rats and mice exposed to 1 ppm VBC exhibited slight transient eye and nasal irritation. The signs of irritation disappeared soon after termination of exposure to VBC each day. The mean body weights of rats and mice are summarized in Figures 1 and 2, respectively. The mean body weights of VBC-exposed animals were not significantly different from those of the control animals for either species.

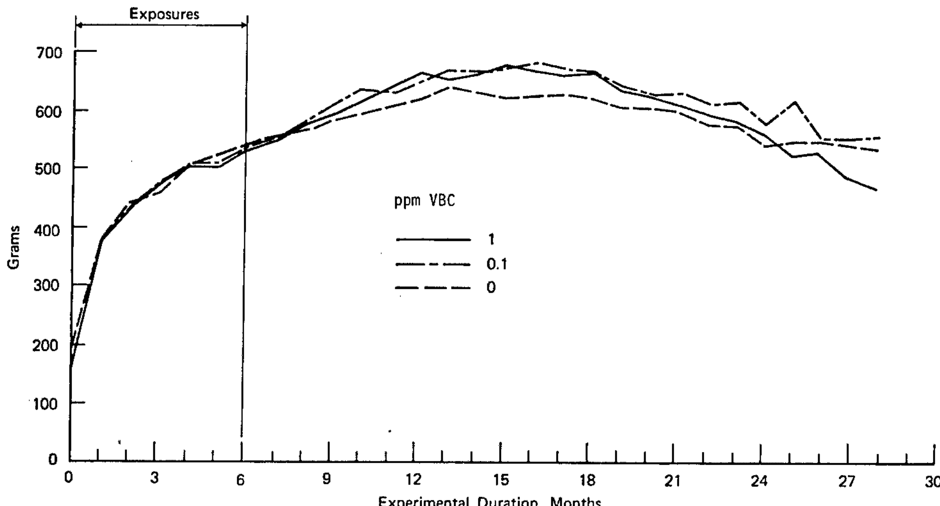

Figure 1. Mean body weights of male rats exposed to vinyl benzyl chloride vapor 6 hours/day, 5 days/week for 6 months and observed for 24 subsequent months.

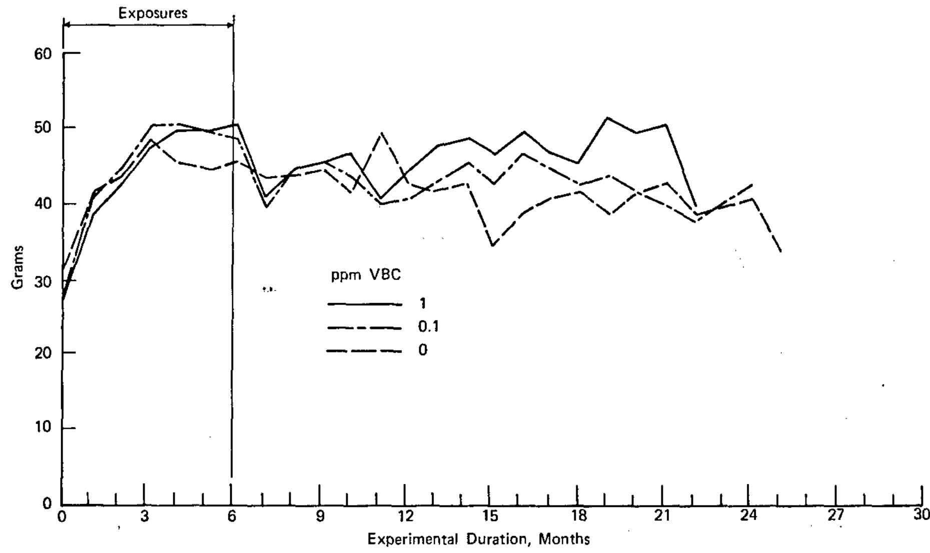

Figure 2. Mean body weights of male mice exposed to vinyl benzyl chloride vapor 6 hours/day, 5 days/week for 6 months and observed for 19 subsequent months.

Mortality

The cumulative percent mortality of rats and mice is summarized in Figures 3 and 4. The graph reveals that only a few deaths occurred among rats during the 6-month exposure period. For the study overall, the incidence of mortality as a function of time was not significantly different in rats exposed to VBC vapors compared with control rats.

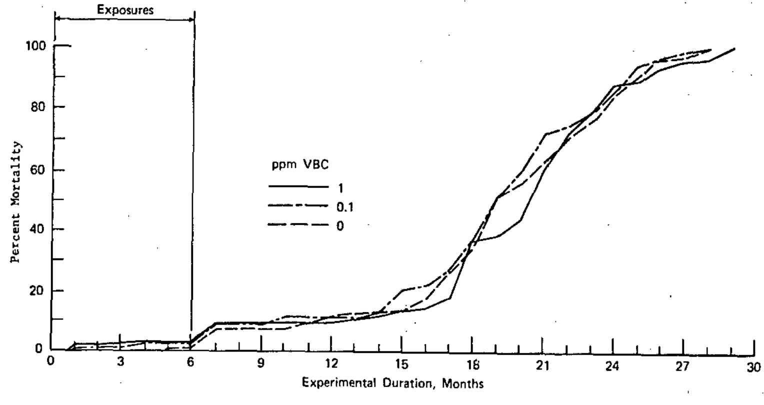

Figure 3. Cumulative percent mortality for male rats exposed to vinyl benzyl chloride vapor 6 hours/day, 5 days/ week for 6 months and observed for 24 subsequent months.

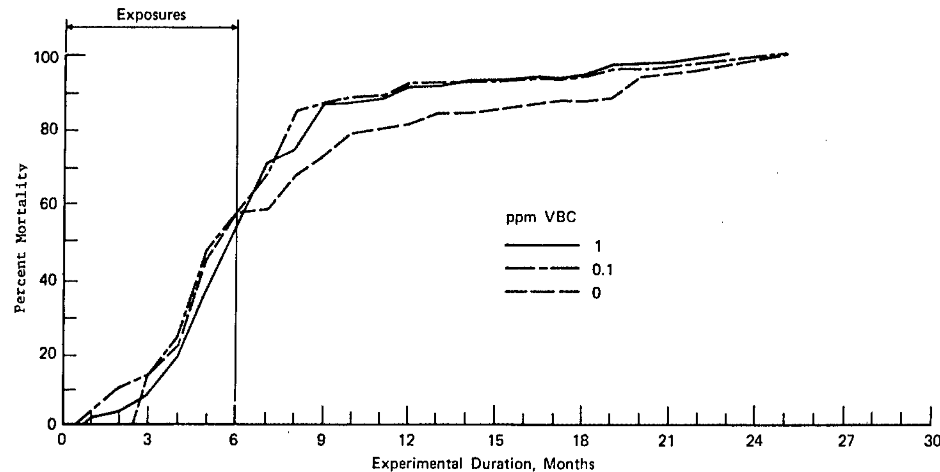

Figure 4. Cumulative percent mortality for male mice exposed to vinyl benzyl chloride vapor 6 hours/day, 5 days/week for 6 months and observed for 19 subsequent months.

Deaths of mice exposed to VBC began by the fifth exposure week and continued until the second or third month of the postexposure period. There were no deaths among the control mice until the third month of the exposure period, at which time a similar mortality pattern began to occur. Necropsy examination revealed an ascending urinary tract infection in a majority of the exposed and control mice. Necropsy examination revealed a necrotic purulent balanoposthitis, purulent urethritis, urinary retention in the urinary bladder and urecystitis. Microbiologic cultures (Veterinary Medical Diagnostic Laboratory, Michigan State University, East Lansing, Michigan) from these cases revealed the presence of various bacterial organisms, including species of Salmonella, Proteus, Pseudomonas, E. coli, and Staphylococcus. Some of the mice had traumatic lesions indicative of cannibalism.

Hematology

Mean hematologic values during week 12 of exposure to 1.0 ppm VBC were comparable to controls (Table 1). Hematologic values for rats on day 1 postexposure and day 5 postexposure revealed no alterations considered related to VBC exposure. In determinations done on postexposure day 1, the RBC counts of rats in both treatment groups were significantly higher than the control values but were well within the expected range for rats of this age, sex and strain. The WBC counts of all rats appeared to be lower on both postexposure day 1 and day 5 due to the collection of the blood sample into a test tube containing excess heparin at time of decapitation. The reversed neutrophilic-lymphocytic ratio noted

TABLE 1. HEMATOLOGIC VALUES (MEAN $\pm$ S.D.) OF RATS EXPOSED TO VINYL BENZYL CHLORIDE VAPOR 6 HOURS/DAY, 5 DAYS/WEEK FOR 6 MONTHS AND OBSERVED FOR 24 SUBSEQUENT MONTHS

Exposure Level (ppm)	Number of Animals	PCV Percent	RBC $\times 10^6/mm^3$	Hgb g/100 ml	WBC $\times 10^3/mm^3$	WBC Differential Count (%)				
						Neut	Lymph	Mono	Eosin	Baso
Week 12 Exposure										
0	10	52.2±3.7	8.3±0.8	17.2±0.9	20.8±3.2	14.2	84.7	0	1	0
1	10	53.6±3.6	7.8±0.8	17.6±0.9	23.3±5.2	19.3	78.2	0	2	0
Day 1 Post 6-Month Exposure (27 weeks)										
0	4	48.5±1.3	7.8±0.1	15.0±0.2	5.2±1.2	32.3	65.3	0	2	0
1	4	49.8±1.3	8.7±0.3*	16.0±0.6	5.5±0.6	19.0	79.8	0	1	0
0.1	4	49.5±0.6	8.3±0.1*	15.6±0.3	5.5±0.7	19.8	79.3	0	1	0
Day 5 Post 6-Month Exposure (27 weeks animals used in cytogenetic study)										
0	4	51.5±2.1	9.0±0.4	16.0±0.4	7.0±0.4	63.0	35.5	0	2	0
1	4	50.7±2.8	8.6±0.2	16.3±1.0	6.9±1.8	75.0	23.5	0	1	0
0.1	4	51.3±1.7	8.8±0.5	16.4±0.7	7.6±1.9	70.3	28.3	0	2	0
6 Months Post 6-Month Exposure (52 weeks)										
0	5	53.0±1.4	8.3±0.4	16.6±1.0	13.6±1.6	11.6	87.4	0	1	0
1	5	53.4±2.6	8.8±0.7	16.4±0.9	14.6±3.6	17.0	80.0	0	2	0
0.1	5	50.6±2.9	8.7±0.5	16.1±0.6	15.3±3.8	20.8	78.0	0	1	0
18 Months Post 6-Month Exposure (104 weeks)										
0	5	48.6±6.8	7.5±1.1	16.1±1.9	16.5±6.6	32.0	63.0	3	2	0
1	5	43.2±7.4	7.1±0.6	14.3±2.5	18.3±3.9	48.0	48.5	3	0	0
0.1	5	42.8±5.8	6.6±1.3	13.9±2.3	16.5±8.2	39.0	58.0	2	1	0

*Significantly different from contemporary controls by Analysis of Variance and Dunnett's Test, $p<0.05$.

in the WBC differential on postexposure day 5 was due to the administration of colchicine (required for cytogenetic evaluation) which caused mitotic arrest of the lymphocytic elements of the body. Hematologic examination of VBC-exposed rats at 52 and 104 weeks of the study revealed no differences from the controls.

Pulmonary Exfoliative Cytology

Examination of prepared smears revealed no atypical or neoplastic cells. There was some variation in the distribution between respiratory epithelial cells and macrophages (Table 2); this was attributed to variation in the degree of force used in flushing the tracheobronchial tree.

TABLE 2. RESULTS OF PULMONARY EXFOLIATIVE CYTOLOGIC EXAMINATION FOR INDIVIDUAL RATS EXPOSED TO VBC VAPOR 6 HOURS/DAY, 5 DAYS/WEEK FOR 6 MONTHS

	Distribution of Cell Types Based* on Evaluation of 100 Cells			
	Number of Cells Classified as:			
Conc. of VBC Vapor	Macrophages	Polymorphonuclear Leukocytes	Respiratory Epithelial Cells	Unidentifiable Cells
0 ppm Control	91	2	7	0
	88	1	10	1
	85	3	11	1
	83	1	16	0
1.0 ppm	34	0	64	2
	64	1	32	3
	86	0	14	0
	73	2	23	2
0.1 ppm	28	0	72	0
	82	0	18	0
	73	1	26	0
	64	0	36	0

*No abnormal cells noted on examination of smears for atypical or neoplastic cells in any sample.

Cytogenetic Evaluation of Bone Marrow Cells*

There were no indications of alterations related to exposure to either level of VBC. These results are tabulated in Table 3.

TABLE 3. RESULTS OF CYTOGENETIC EVALUATION OF CHROMOSOMES OF BONE MARROW CELLS COLLECTED FROM RATS EXPOSED TO VINYL BENZYL CHLORIDE VAPOR 6 HOURS/DAY, 5 DAYS/WEEK FOR 6 MONTHS

Group	No. Rats Examined	Mitoses Scored	Gaps	Breaks	Reunions	>10 Aberrations
0 ppm Control	4	150	1	0	1 (dicentric)	0
1.0 ppm	4	166	3	0	0	0
0.1 ppm	4	169	4	1	0	0

Organ Weights

Mean body and organ weights (both absolute and relative) of rats killed upon completion of the 6-month period are tabulated in Table 4. There were no alterations in the body weights or the absolute or relative weights of the liver, kidneys, brain, heart, or testes of VBC-exposed rats relative to control rats.

Gross and Microscopic Pathology of Rats (Week 1 Postexposure)**

Gross examination of rats killed on day 1 or day 5 postexposure revealed no lesions considered related to VBC exposure. Microscopic examination of H&E stained sections disclosed increased numbers of inflammatory cells in the mucosa and submucosa of the nasal turbinates from 2 of 4 rats exposed to 1.0 ppm VBC and killed on postexposure day 1, which suggested an irritation effect related to treatment. However, as similar lesions were not observed in rats exposed to 1.0 ppm VBC and killed on postexposure day 5, the inflammatory changes were reversible. Both control and treated rats used

*Evaluated by M. C. Benge and D. J. Kilian, Medical Department, Texas Division, The Dow Chemical Company, Freeport, Texas.

**Detailed compilations of the detailed pathologic observations are available from the authors on request.

TABLE 4. MEAN (± S.D.) BODY AND ORGAN WEIGHT AND ORGAN TO BODY WEIGHT RATIOS OF RATS EXPOSED TO VBC VAPOR 6 HOURS/DAY, 5 DAYS/WEEK FOR 6 MONTHS

Group	Body Weight (g)	Brain		Heart		Liver		Kidneys		Testes	
		g	g/100g	g	g/100g	g	g/100g	g	g/100g	g	g/100g
Exfoliative Cytology[a]											
0 ppm	510.5 ±37.3	1.98 ±0.03	0.39 ±0.02	1.50 ±0.12	0.29 ±0.02	13.50 ±1.16	2.64 ±0.04	3.51 ±0.24	0.64 ±0.09	4.02 ±0.34	0.79 ±0.06
1.0 ppm	520.0 ±38.9	1.89 ±0.08	0.37 ±0.03	1.50 ±0.14	0.29 ±0.01	13.46 ±1.65	2.58 ±0.16	3.40 ±0.34	0.65 ±0.03	4.07 ±0.32	0.79 ±0.09
0.1 ppm	491.5 ±37.2	1.93 ±0.05	0.39 ±0.03	1.37 ±0.10	0.28 ±0.01	12.45 ±1.57	2.53 ±0.23	3.18 ±0.33	0.65 ±0.05	4.13 ±2.37	0.84 ±0.05
Cytogenetic Evaluation[b]											
0 ppm	538.0 ±22.6	1.89 ±0.10	0.35 ±0.01	1.64 ±0.12	0.30 ±0.02	15.52 ±1.14	2.88 ±0.19	3.79 ±0.64	0.70 ±0.10	4.19 ±0.42	0.78 ±0.07
1.0 ppm	555.3 ±64.2	2.00 ±0.03	0.36 ±0.04	1.58 ±0.12	0.29 ±0.02	15.88 ±3.61	2.84 ±0.32	3.74 ±0.61	0.67 ±0.06	4.18 ±0.45	0.76 ±0.09
0.1 ppm	570.5 ±53.4	1.99 ±0.06	0.35 ±0.03	1.58 ±0.13	0.28 ±0.01	15.98 ±2.47	2.80 ±0.26	3.87 ±0.33	0.68 ±0.03	4.13 ±0.46	0.73 ±0.08

No significant differences between treated and control groups by analysis of variance and Dunnett's test, $P<0.05$.

[a]These groups (n=4 per group) were killed on day 1 following 6-months of exposure.

[b]These groups (n=4 per group) were killed on day 5 following 6-months of exposure.

in the cytogenetic study had microscopic alterations associated with mitotic arrest subsequent to administration of colchicine; these were not considered related to VBC exposure. A majority of the rats used in the cytogenetic study had focal reddened areas in the lungs, sometimes accompanied by diffuse congestion and atelectasis; these changes were attributed to the method of euthanasia (using a CO_2 chamber) specified in the cytogenetic protocol. There were other findings common to the strain of rats used which were considered unrelated to the exposure to VBC.

During the latter stages of the 6-month period of exposure, pathologic examination of animals found dead or moribund revealed lesions indicative of a respiratory infection in those groups of rats exposed to 1.0 or 0.1 ppm of VBC. This was grossly evident as variable degrees of pulmonary congestion and consolidation, and sometimes pleural adhesions, hydrothorax, and nasal exudate. Microscopically, these affected rats had a bronchopneumonia, typically caseopurulent to necrotic in type, and sometimes accompanied by fibronecrotic pleuritis, inflammation of the thoracic lymph nodes, or purulent pericarditis/myocarditis. The bacterium *Corynebacterium kutscheri* was cultured (Veterinary Medical Diagnostic Laboratory, Michigan State University, East Lansing, Michigan) repeatedly from the lungs of affected rats. During the initial 6-month period, this infection was noted in 2 rats from each of the groups being exposed to 1.0 or 0.1 ppm VBC. The control rats, which were in an adjacent holding room and not placed in exposure chambers, did not have any deaths due to this bacterial respiratory infection during this initial 6-month time period. During months 6-12 of the study (initial 6 months of postexposure observation period) there was only one additional death attributed to bacterial pneumonia in a rat of the 1.0 ppm group. During months 13-18 (second 6-month postexposure period), the bacterial pneumonia was noted in 6 rats of the 1.0 ppm group, 7 rats of the 0.1 ppm group, and 3 rats of the control group. During this time period, there were a few cases in which the bacterial infection had disseminated to other organs, such as liver and kidney, which had inflammatory reactions similar to those in the lungs.

During months 19-24 (third 6-month time period of postexposure observation) 22, 10, and 7 rats died with lesions of a bacterial pneumonia in rats exposed to 1.0, 0.1, and 0 ppm of VBC, respectively. It is assumed that the higher incidence of bacterial pneumonia in the groups exposed to VBC vapors may have been associated with the physical stress of the exposure regimen or with the effect of VBC upon pulmonary tissues.

In addition to the respiratory lesions associated with the bacterial pneumonia, a wide range of inflammatory and/or degenerative changes of a wide spectrum of organs and tissues occurred in a scattered pattern among the control and exposed rats; none of these changes appeared to be related to VBC exposure since they were typical of those seen in other studies of this strain of rat in our laboratory.

Tumor Incidence in Rats

Table 5 is a collation of all tumors noted during the entire study. All types of tumors and their incidence were as expected in a long-term study using rats of this strain. Tumors of endocrine organs were the most frequently occurring, involving the pancreas, thyroid, adrenal, and pituitary glands. Statistical evaluation of the total tumor incidence compiled for the entire study revealed no differences between the exposed groups and the control group in regard to the total number of rats with tumors or in the incidence of any of the 49 individual tumor types and classifications diagnosed in these rats.

Statistical analysis of the tumor data compiled for sequential 6-month time intervals of the study also revealed no differences between exposed and control groups, except for one isolated case. This case was a statistically increased incidence of pancreatic islet cell adenomas occurring in the lower exposure group of rats that died during months 19-24 of the study. This observation was considered not related to exposure to VBC due to (1) no difference in the overall incidence of this type of tumor occurring during the entire study; (2) no difference in the incidence of this type of tumor occurring during the other sequential 6-month intervals of the study; and (3) lack of any suggestion of a dose-response relationship.

TABLE 5. TUMOR INCIDENCE IN RATS EXPOSED TO VINYL BENZYL CHLORIDE VAPOR 6 HOURS/DAY, 5 DAYS/WEEK FOR 6 MONTHS AND OBSERVED FOR 24 SUBSEQUENT MONTHS

Number of Rats With Tumors According to Location and Classification	Vapor Concentration		
	Control (0 ppm)	1 ppm	0.1 ppm
Total Number of Rats Examined	112	108	112
Total Number of Rats with Tumors	56	50	59
Number of Tumors per Group[a]	71	67	85
Mean Number of Tumors/Rats with Tumors[a]	1.27	1.34	1.44
Subcutaneous Tissue			
Subcutaneous fibroadenoma (mammary)	5	6	2
Subcutaneous fibroma	2	1	6
Subcutaneous neurofibroma	2	2	4
Subcutaneous adenoma	0	0	3
Subcutaneous myxoma	1	1	0
Subcutaneous fibrosarcoma with metastasis to lung	0	0	1
Fibrosarcoma with metastasis	0	1	1
Subcutaneous lipoma	0	1	0
Subcutaneous malignant schwannoma	1	0	0
Adenoma of preputial gland	1	0	0
Integument			
Squamous cell carcinoma of integument	0	1	0
Cutaneous papilloma	0	1	0
Sebaceous gland adenoma	0	1	0
Basal cell tumor of integument	1	0	0
Squamous papilloma of integument	1	0	0
Gastrointestinal			
Gastric adenomatous polyp	1	0	1
Adenocarcinoma of small intestine	1	0	2
Undifferentiated sarcoma from wall of stomach with metastasis to regional lymph node	1	0	0
Cornified polyp of gastric mucosa	0	2	0
Mucocystoadenocarcinoma of small intestine with scirrhous areas	0	1	0
Lymphoreticular			
Lymphosarcoma (generalized)	4	5	2
Lymphosarcoma of thoracic lymph node	0	0	1

No significant differences from control values when analyzed by Fisher's Exact Probability test $p<0.05$.

[a]Entries not statistically analyzed.

Data listed as number of rats in each group in which the observation was noted. Total number excludes those killed for interim evaluation, lost or cannibalized.

TABLE 5 (continued)

Number of Rats With Tumors According to Location and Classification	Vapor Concentration		
	Control (0 ppm)	1 ppm	0.1 ppm
Central Nervous System			
Reticulosarcoma of brain stem and cerebrum	0	1	0
Meningioma of brain	0	1	0
Astrocytoma of brain	2	1	0
Adrenal			
Adrenal pheochromocytoma	14	15	14
Thyroid			
Thyroid adenoma	4	5	6
Thyroid adenocarcinoma	1	0	1
Ear Canal			
Sebaceous gland carcinoma of external auditory canal	2	0	1
Squamous cell carcinoma of external auditory canal with metastasis to lung	1	0	0
Miscellaneous			
Intraabdominal malignant schwannoma	0	2	1
Mediastinal malignant schwannoma	0	0	1
Mesenteric unclassified sarcoma	0	0	1
Paravertebral neurofibrosarcoma	0	0	1
Hemangioma of kidney	1	0	0
Lung and Trachea			
Pulmonary adenoma	0	1	0
Submucosal adenoma in trachea	0	0	1
Polyp formation in trachea	0	0	1

No significant differences from control mean when analyzed by Fisher's Exact Probability test $p<0.05$.

Data listed as number of rats in each group in which the observation was noted. Total number excludes those killed for interim evaluation, lost or cannibalized.

TABLE 5 (continued)

Number of Rats With Tumors According to Location and Classification	Vapor Concentration		
	Control(0 ppm)	1 ppm	0.1 ppm
Liver			
Unclassified autolyzed malignant tumor within liver	0	1	0
Hepatocellular carcinoma	0	0	1
Pancreas			
Pancreatic islet cell adenocarcinoma	1	0	0
Pancreatic islet cell adenoma	2	4	6
Pancreatic acinar adenoma	5	2	6
Pancreatic acinar adenocarcinoma with metastasis to lung and lymph node	1	0	0
Pancreatic acinar adenocarcinoma	1	0	0
Reproductive			
Interstitial cell tumor of testicle	2	0	2
Parathyroid			
Parathyroid adenoma	0	1	0
Musculoskeletal System			
Osteoma of thoracic rib	0	0	1
Pituitary Gland			
Pituitary adenoma	14	10	17

No significant differences from control mean when analyzed by Fisher's Exact Probability test $p<0.05$.
Data listed as number of rats in each group in which the observation was noted. Total number excludes those killed for interim evaluation, lost or cannibalized.

Lung and Liver Tumor Incidence in Mice

Table 6 lists the results of gross and microscopic examination of lungs of mice used in this study. Pulmonary adenomas and adenocarcinomas, both of probable alveolar origin, were noted in both control and exposed groups of mice. The incidence was highest in the control group of mice, with lower incidences in both groups of exposed mice.

TABLE 6. RESULTS OF GROSS AND MICROSCOPIC EXAMINATION OF LUNGS OF MICE EXPOSED TO VINYL BENZYL CHLORIDE VAPOR 6 HOURS/DAY, 5 DAYS/WEEK FOR 6 MONTHS AND OBSERVED FOR 19 SUBSEQUENT MONTHS

Vapor Conc. (ppm)	No. of Mice with Grossly Visible Nodule(s) in Lung/No. Mice Examined	No. of Mice with Microscopic Evidence of Lung Tumor		No. of Mice with other Salient Microscopic Observations on Lungs
		Pulmonary Adenoma	Pulmonary Adenocarcinoma	
0	9/157	6/9	3/9	
1.0	9/144	3/9	3/9	1/9 Focal interstitial inflammation
				2/9 Focal alveolar histiocytosis
0.1	4/141	0/4	2/4	1/4 Focus of mononuclear cells in lung
				1/4 Focal alveolar histiocytosis

The pulmonary tumors observed in the mice of this study were of the type commonly seen as spontaneous occurrences in mice of this strain.

Table 7 lists the results of gross and microscopic examination of livers of mice used in this study. Some mice from all groups, control and exposed, had gross and microscopic evidence of tumor development originating within the liver. The incidence was highest in the control group. Morphologically, tumors of hepatocellular origin included 1 benign hepatic tumor in a mouse exposed to 1.0 ppm VBC and

4 malignant hepatocellular carcinomas, of which 3/4 occurred in the control group and 1/4 occurred in the low exposure (0.1 ppm) group. One control mouse had a tumorous mass within the liver that was classified as a hemangiosarcoma. No tumors of the latter type were noted in the 2 groups of mice exposed to vapors of VBC.

TABLE 7. RESULTS OF GROSS AND MICROSCOPIC EXAMINATION OF LIVERS OF MICE EXPOSED TO VINYL BENZYL CHLORIDE VAPOR 6 HOURS/DAY, 5 DAYS/WEEK FOR 6 MONTHS AND OBSERVED FOR 19 SUBSEQUENT MONTHS

Vapor Conc. (ppm)	No. of Mice with Grossly Visible Nodule(s) in Liver/No. of Mice Examined	No. of Mice with Microscopic Evidence of Tumor With Liver			No. Mice with Other Salient Microscopic Observations on Livers
		Benign Hepatic Tumor	Hepatocellular Carcinoma	Angiosarcoma in Liver	
0	5/157	0/5	3/5	1/5	1/5 Hepatic necrosis and inflammation secondary to urinary infection
1.0	4/144	1/4	0/4	0/4	1/4 Pyogranulomatous peritonitis and hepatitis
					1/4 Disseminated amyloidosis of liver and other organs
					1/4 Centrilobular hepatic degeneration
0.1	1/141	0/1	1/1	0/1	

DISCUSSION

Clinical observation indicated transient eye and nasal irritation in both mice and rats during exposure to 1.0 ppm but not 0.1 ppm VBC vapor. The presence of nasal irritation was confirmed microscopically in 2 of 4 rats examined on day 1 of the postexposure period. By day 5 postexposure no inflammation was observed in the nasal turbinates.

Other noteworthy effects in the study were the ascending urinary tract infections in mice and the *Corynebacterium kutscheri* respiratory infections in rats. Both infectious processes occurred in the exposed and control groups, but occurred earlier in the groups exposed to VBC. Factors other than exposure to VBC were involved. Each exposure group was housed in one animal rack during the exposure period, resulting in close contact of animals within the group and thus greater opportunity for transmission of the infectious agent among members of the group. Also, only the animals exposed to VBC were placed in chambers; the control groups were held

in an animal holding room nearby. The added stress of being housed in chambers and exposed to VBC could have enhanced susceptibility to infection for both mice and rats. The greater occurrence of the respiratory infection in rats exposed to VBC also suggests that the demonstrated pulmonary irritation effects of VBC could have increased susceptibility of rats to the respiratory infection.

With respect to carcinogenesis, no increase in any type of tumor was found in either mice or rats at either concentration of VBC.

In summary, the results of this study are interpreted to indicate that lifetime observation of rats and mice after inhalation of 0.1 or 1.0 ppm VBC for 6 hours/day, 5 days/week for 6 months revealed no ill effects, including carcinogenesis, except for reversible eye and nasal irritation in animals during exposure to 1.0 ppm VBC.

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PAPER NO. 17

REPETITIVE HUMAN EXPOSURES TO DIMETHYLFORMAMIDE VAPOR

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INTRODUCTION

Dimethylformamide (DMF) is a widely used industrial solvent which is known to be hepatotoxic (Massman, 1956; Clayton et al., 1963). To protect the worker, the American Conference of Governmental Industrial Hygienists (ACGIH) has set 10 parts per million (ppm) DMF, by volume, in air as the threshold limit value (TLV) (American Conference Governmental Industrial Hygienists, 1975). Since DMF is readily absorbed through skin as well as lungs, air concentration measurements may not accurately define the total exposure experience. Measurement of total individual exposure under these circumstances is often best accomplished with a biological monitoring system.

Previous studies (Barnes and Ranta, 1972; Maxfield et al., 1975) in this laboratory have shown that excretion of monomethylformamide (MMF), the major urinary metabolite of DMF, can be used as an indicator of total DMF exposure. In a human study (Maxfield, 1975), single 6-hour exposures to 10 ppm DMF vapor and single dermal applications of liquid DMF showed noticeable variation in MMF urinary excretion patterns among subjects.

Kimmerle and Eben (1975) found similar results in man at exposure concentrations of 26 and 87 ppm DMF. Repeated exposure to these DMF concentrations did not increase MMF concentrations in blood or the amount excreted in the urine.

This human study was designed to provide detailed information on individual MMF excretion patterns during and after repeated exposure to an airborne concentration of 10 ppm DMF and to assess the usefulness of urinary MMF concentrations as a biological monitor of total DMF exposure.

MATERIALS AND METHODS

Technical grade DMF was obtained from the Industrial Chemicals Department, E. I. du Pont de Nemours & Company. Analysis of the sample showed less than 29 ppm impurities.

Eight healthy males, 20 to 47 years old, 70 to 84 kg in weight, and 173 to 183 cm in height, were subjects. The experiment was conducted according to the specifications of informed consent and safety recommended by the human experimentation code of ethics of the World Medical Association (Declaration of Helsinki) (Ladimer, 1970).

The subjects, dressed in short-sleeved shirts and work trousers, were exposed to a nominal 10 ppm (v/v in air), 30 $\mu g/m^3$, six hours daily on five consecutive days. The exposures were split into two three-hour exposures separated by an hour break. Due to chamber size limitations, exposures were done during two consecutive weeks, with subjects 1 through 4 exposed during the first week and 5 through 8 during the second week.

To simulate working conditions, the subjects stepped up and down a single 7-3/4" high step at a rate of 23 cycles per minute for five minutes per hour during exposure. Between exercise periods, the subjects were seated. Drinking water was permitted but not smoking or eating in the chamber.

The Mylar® exposure chamber has been described previously (Reinhardt et al., 1971), and was located in a large controlled temperature room. Chamber temperature was maintained at 22-28 C. Communication between observers and subjects was provided by both an intercom and window.

Liquid DMF was vaporized by injecting it through a needle into a stream of house air. A hassock-type fan distributed the vapor throughout the chamber.

Vapor concentration entering the chamber was controlled by adjusting either infusion pump speed or air flow rate. An exhaust fan maintained a slight negative pressure in the chamber.

The DMF concentration in the subjects' breathing zone during exposures was monitored continuously by drawing chamber air into an infrared analyzer (Miran-I Portable Gas Analyzer Model 5653, Wilks Scientific Co.). The air sample was exhausted from the instrument back into the exposure chamber. Response was recorded continuously and the instrument was calibrated daily. Interference with the infrared absorption of DMF by organic substances exhaled by the subjects was corrected for by conducting an identical exposure without DMF.

All urine voided by the subjects was collected from the beginning of the first exposure to 24 hours past the end of the last exposure. These specimens were collected immediately before and after each exposure and approximately bihourly during nonexposure working hours. Additional urine samples were collected four days before exposure and just prior to entering the chamber on the first exposure day. A final overnight sample was collected 48-56 hours after the last exposure. Volume and time of collection for each urine sample were recorded and samples were refrigerated until analyzed. The MMF concentration in urine extracts was determined by gas chromatography (Barnes and Henry, 1974) with a detection limit of 0.1 mg MMF/liter.

The subjects were given a complete physical examination, including clinical biochemistry 7 days before and 3 days after exposure. Blood samples were taken after the subjects had fasted overnight. Twenty-four indices were determined: red and white cell counts, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobn concentration (MCHC), total protein, albumin, globulin, albumin/globulin ratio, calcium, phosphorus, cholesterol, total lipids, bilirubin, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total lactic dehydrogenase (LDH), alkaline phosphatase, urea nitrogen, uric acid, glucose, and creatinine.

The subjects' oral temperatures were measured at the start and end of the first three exercise periods on exposure days one and five. Respiration rates were determined immediately after the subjects entered the exposure chamber as well as before and after the first exercise period on days one and five.

After exposure measurements of total respiratory volume, respiration rate and expiratory volume were measured with a pneumotachometer and dry gas meter.

Histograms and standard tests of normality indicated that the urinary metabolite data were log-normally distributed. Therefore, to better satisfy the assumptions behind the analysis of variance (i.e., normally distributed data with variances independent of means), the data (X) were transformed to log (X + 1). Log (X + 1) rather than log (X) was used since some samples did not contain detectable amounts of MMF. When the analysis of variance (AOV) showed no important differences between the first and second week data, the weeks were combined for a better estimate of variation. Split-plot AOV's were used to test for differences in average daily MMF (μg/ml), total daily MMF (μg), 7-hour MMF (μg/ml), and 7-hour total MMF (μg). Significance was judged at the 0.05 probability level.

RESULTS

The DMF air concentration during exposures, corrected for baseline drift, relative humidity changes and exhaled organics, averaged 8.8 ± 0.7 ppm (Table 1) and ranged from 7.9-12.9 ppm.

TABLE 1. RESULTS OF DAILY AIR ANALYSIS FOR DMF

Exposure Day	Average Concentration, ppm	
	Week I	Week II
1	8.4 ± 0.7*	9.1 ± 0.4
2	9.1 ± 0.8	8.3 ± 0.6
3	9.0 ± 0.8	8.7 ± 0.5
4	8.7 ± 0.8	9.1 ± 0.6
5	8.4 ± 0.7	9.1 ± 0.6
Average	8.7 ± 0.8	8.9 ± 0.6

Combined (Week I and II) 8.8 ± 0.7

*Mean ± standard deviation, 26 samples (15 minute intervals).

Relative humidity ranged from 26% to 67%, averaged about 35% at the start and increased to about 56% by the end of an exposure. Organic substances exhaled by the subjects interfered to the extent of about 10% with the Miran's infrared absorption frequency used for DMF.

There were no differences between results of pre- and postexposure physical examinations. Body temperature and heart rate were not affected. Results of pulmonary function measurements ranged from an average of 9.8 ± 2.5 liters/minute during rest to 22.8 ± 2.7 liters/minute during exercise. Based on these data, time weighted average of total respiratory volume during a 6-hour exposure was 4.06 ± 0.81 M^3.

MMF was not detected in urine samples taken prior to the first exposure. Measurable amounts of MMF were found in the urine of all subjects during and after each 6-hour exposure. No MMF was detected in the urine of any subject two days after the last exposure.

Based on total daily MMF excreted and daily MMF concentrations there was no evidence that the amount of MMF in the urine increased during the 5-day exposure (Figures 1 and 2). A variance analysis performed on both concentration and total excretion data from 7 and 24-hour samples showed no statistically significant ($p < 0.05$) day-to-day increase of MMF during the five days of exposure.

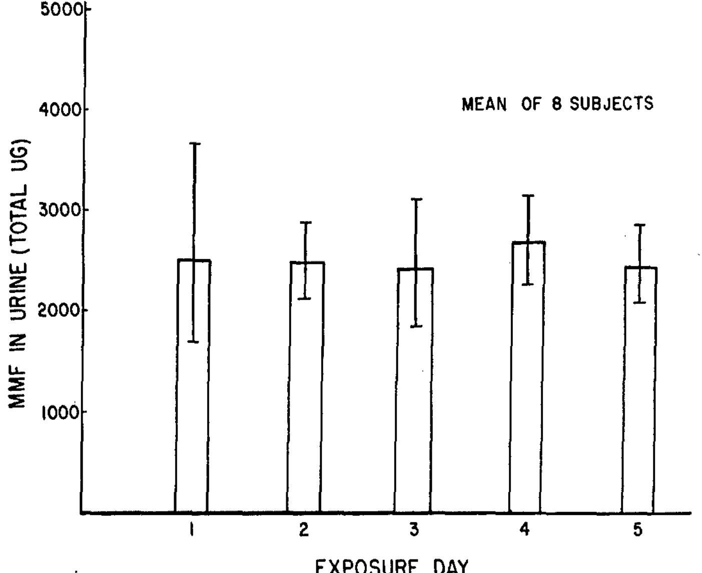

Figure 1. Daily total MMF (µg) in urine after repeated exposure to DMF vapors. (Mean ± 1 S.D.)

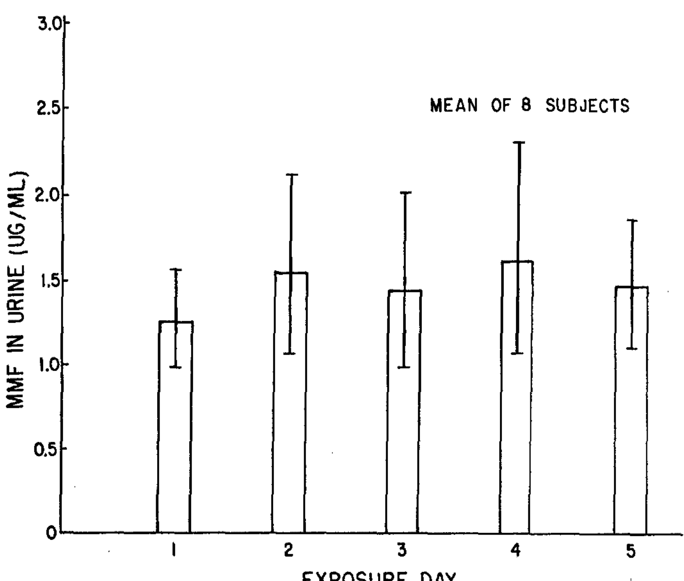

Figure 2. Daily MMF (µg/ml) in urine after repeated exposure to DMF vapors. (Mean ± 1 S.D.)

The excretion rate (µg/hr) of urinary MMF increased rapidly during each exposure, peaked within 3 hours after end of exposure, and was nearly zero by the beginning of the next exposure 24 hours later (Figure 3).

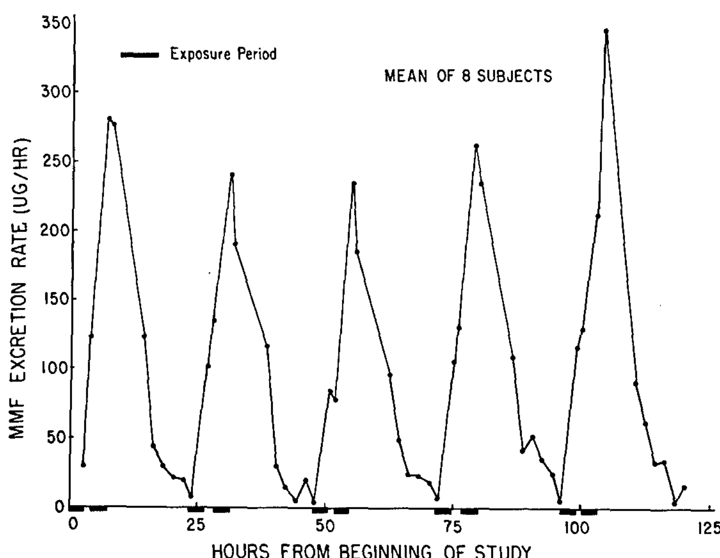

Figure 3. MMF excretion profile after repeated exposure to DFM vapors.

Seven hour end-of-exposure and 24-hour start-of-exposure samples were analyzed (Figures 4 and 5). The 7-hour mean daily concentration was 4.74 μg/ml and ranged from 3.81 to 5.90 μg/ml; the mean daily total was 736.8 μg and ranged from 665 to 873 μg. The 24-hour daily concentration was 0.28 μg/ml and ranged from 0.23 to 0.36 μg/ml; the mean daily total was 11.6 μg and ranged from 5.8 to 26.9 μg.

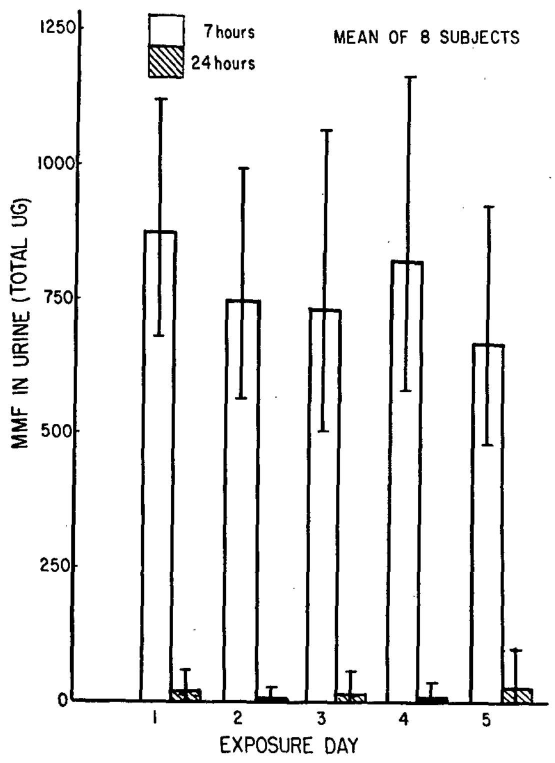

Figure 4. Urinary MMF levels (μg) 7 and 24 hours after repeated exposure to DMF vapors. (Mean ± 1 S.D.)

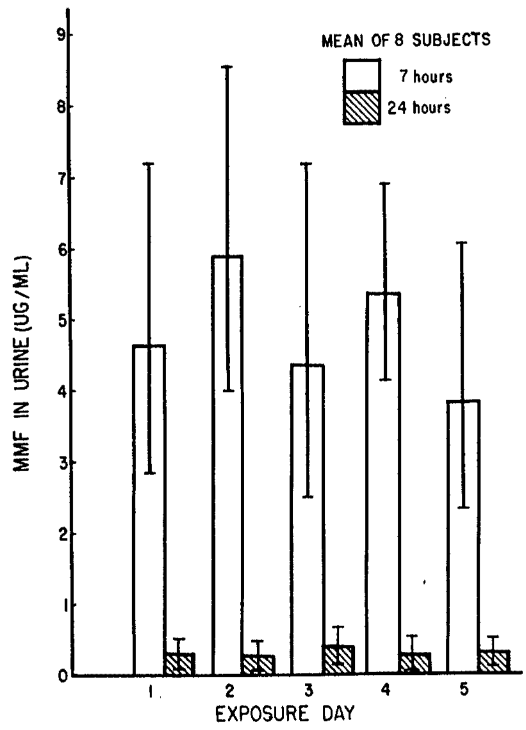

Figure 5. Urinary MMF levels (μg/ml) 7 and 24 hours after repeated exposure to DMF vapors. (Mean ± 1 S.D.)

DISCUSSION

The results of this human study showed that DMF vapor is absorbed by the body and can be correlated with urinary MMF after exposure.

Urinary levels of MMF rose soon after exposure began and returned to nearly nondetectable levels by 14 hours after end of exposure. There was no increased excretion of MMF in the urine following repetitive exposure.

The best index of DMF exposure was the total amount of urinary MMF excreted in 24 hours. The MMF in a single urine sample taken at a specific time after exposure was also useful. Individual measurements of total μg were more uniform than individual measurements of concentrations.

On combining results from this study, a previous Haskell study (Maxfield et al., 1975), and studies by Kimmerle et al. (1975), a linear relationship was found between total exposure (concentration X time) and urinary MMF (Figure 6).

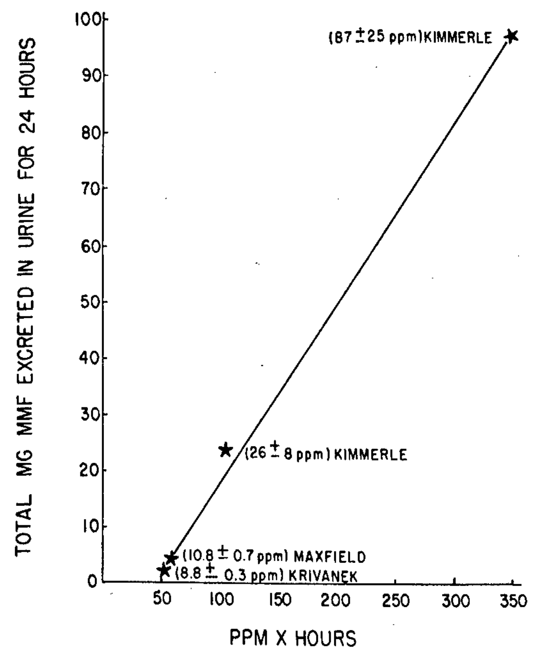

Figure 6. Relation of urinary MMF levels to exposure level and duration-compilation of 4 human DMF vapor exposure studies.

The amount of MMF in a subject's urine was related to duration and level of exposure, physical activity (total respiratory volume during exposure, subject mean = 4.06 M^3).

A worker's physical activity and thus respiratory volume may be two or three times that recorded for this study's subjects. Therefore, one could expect that workers exposed to the same DMF air concentration as subjects in this study would have higher urinary MMF outputs than reported here.

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OPEN FORUM

DR. TROCHIMOWICZ (E. I. du Pont Nemours and Company, Inc.): I'd like to begin this Open Forum with a question to Dr. Rampy. In his 2-year study, he exposed animals to vinyl benzyl chloride for 6 months followed by an 18-month postexposure holding period. Certainly in long-term animal testing, the question of how long to expose and what's an adequate exposure to simulate the human situation is a controversial one. I'd just like to hear some of your reasoning, Dr. Rampy, for selecting 6 months as the exposure period rather than continuing the exposure for 1 or 2 years.

DR. RAMPY (Dow Chemical Company): If someone were cynical, they might say I planted that question because I hoped it would be asked. There are a number of factors to be considered here. One is that the animal exposures in this study were obviously carried out some time ago since the study ran for nearly three years and the time required for obtaining the pathology results is another delay. The animal exposures were actually begun about 4-1/2 years ago. At that time, there wasn't much controversy over the length of a chronic toxicity study. We were particularly interested in comparing this compound with bis-chloromethyl ether. A study of bis-chloromethyl ether done at the same time in the same strain of rats at 100 ppb and 1/10 of a ppb produced nasal tumors in 90% of the rats at the higher level, the 100 ppb, for the same treatment period. So I think there would be some comfort to the worker who had to choose between vinyl benzyl ether and bis-chloromethyl ether. Added to that is the knowledge that vinyl benzyl ether is much less volatile than bis-chloromethyl ether. That's one factor. Another factor is that 6 months is a very significant part of a rat's life, and I think you could make an argument for conducting exposures for that period on that basis. We are told by many people that once you induce cancer, it's not a reversible process so if you expose them long enough to get the tumor started and if you observe them long enough, it should show up anyway. I'm not sure that I concur with that argument. I think that if we were to do that study today, we would undoubtedly expose the animals for a longer period of time. But I would like to point out that a study of this type has some use in cases where you can't afford to spend $500,000 to $700,000 and you need some animal exposure data to base a conclusion on regarding carcinogenesis.

MR. WANDS (National Academy of Sciences): I'd like to pursue that question just one step further. Did you have any means of plotting time to development of a tumor and was it necessary to carry them the full 30 months? Could you have gotten the same information in one year instead of two?

DR. RAMPY: Since we didn't find tumors other than spontaneous ones, we don't have any way to plot time to tumors. I think that our conclusion now based on several other studies of this type is that observing the animals throughout their lifetime is not productive. The reason is that the incidence of geriatric lesions becomes so enormous in those stragglers that live out beyond 2 years on study that it's difficult to make a histologic diagnosis in many cases. Although it is not consistent with good laboratory practice, I think that the interest in the animals and the attention they get the longer the study goes becomes less and the incidence of autolysis may become a little greater. For this particular strain of animals where mortality approaches 75-80% at 24 months, we have concluded that the study should be terminated at 24 months, not necessarily 24 months of exposure, but at 24 months after the beginning of the study. I don't think it is necessary or wise to carry them out to their ultimate lifetime.

MR. VERNOT (University of California, Irvine): I'd like to ask Mr. Moorman to discuss his calibration methodology.

MR. MOORMAN (National Institute of Environmental Health Sciences): That depends somewhat on the instrument and the type of compound. So far, we've been concentrating on organic solvents using the infrared analyzers. We construct a closed loop of known volume and inject into that an appropriate volume of what we're calibrating against and use that. Is that what you wanted to know?

MR. VERNOT: Is that the technique that you've been using up to this time exclusively?

MR. MOORMAN: That is correct.

DR. TROCHIMOWICZ: Dr. Rampy, you did do some microscopic examination of the nasal turbinate areas of rats exposed to vinyl benzyl chloride and reported some evidence of reversible inflammatory changes. Were there any other changes such as metaplasia in that area?

DR. RAMPY: No changes of that type were seen.

DR. TROCHIMOWICZ: The reason I ask is that I'm aware that there are at least three compounds that can produce squamous cell carcinoma in the nasal turbinate area, hexamethyl phosphoramide, epichlorhydrine and dimethyl carbamyl chloride. The changes that precede those squamous cell carcinomas are certainly inflammatory changes in the nasal turbinate area. The next thing that we see is a flattening of the cells, a squamous metaplasia, and then the actual tumor development. It seemed similar that we see some of the early changes here and I was wondering whether you think if you continued those exposures any longer, you might have exacerbated further changes?

DR. RAMPY: I don't know what would have happened if we had gone longer. I think that the kinds of inflammatory changes that we observed are the kind you would see with most any irritant. It's a rather nonspecific response to an irritant. Whether they would have gone on to produce those kinds of changes seen in those other studies, I don't know. Can you tell us how long they took to produce metaplasia with the compounds you described? Were they seen only at the end of the study or were they seen early?

DR. TROCHIMOWICZ: With hexamethyl phosphoramide, exposure to approximately 500 ppb for 6 months produced the actual tumor in rats. Some of those cellular changes in the nasal turbinate area had occurred in animals examined at a three month sacrifice. Some squamous metaplasia was very apparent in all the rats at 3 months, and by 6 months, actual gross evidence of tumors was seen. At lower levels like 50 ppb which was one of the levels tested, those early changes were not seen until about 6 months of exposure in rats. The actual tumor development at 50 ppb showed up after approximately 13 months of exposure.

DR. RAMPY: Then I'd have to say that in comparison to that study, vinyl benzyl chloride doesn't look like a carcinogen or at least not a very potent one.

MR. WANDS: Dr. Rampy, do you have any data on the metabolic pathways or the pharmacokinetics of vinyl benzyl chloride in the rat and any information on how that might compare to the way the human handles this material?

DR. RAMPY: No, unfortunately, we don't have pharmacokinetic information on vinyl benzyl chloride. The only comparative data that we have for humans is the observation that it is an irritant at levels similar to those seen in the animal studies. In regard to the form that it's metabolized or excreted, we don't have any direct information.

MR. WANDS: Mr. Kinkead, I'd like to ask you one question. It appears that the decomposition of tetranitromethane to nitrate/nitrite probably takes place in the lung. If so, then one would look for some kind of metabolic activity there, mitochondrial involvement of some sort. Did you see any signs at all of adaptation or increased sensitivity in the animals as time went on? I realize it was a fairly short time.

MR. KINKEAD (University of California, Irvine): That was one of the problems. It was a short exposure time and we really didn't investigate that area. We were simply trying to compare the effect of the two compounds. If we conduct more in depth studies with tetranitromethane, we'll probably look at the mitochondria and determine whether there are any effects.

DR. RAMPY: Dr. Krivanek, I was pleased to learn of your human exposure studies with dimethylformamide. I'd like to ask you about these studies. It seems to me that one of the main values of conducting human exposures is to correlate the response in man to those seen in animals. I'm not that familiar with DMF toxicity information but I assume that a variety of comprehensive toxicity studies had first been done in animals. How do those studies compare? What did you learn from this study that tells you about the applicability of the animal studies for predicting hazards for man?

DR. KRIVANEK (E. I. du Pont de Nemours and Company, Inc.): Experiments were first conducted with animals. In feeding studies with DMF in rats, we were able to isolate out the MMF. Kimmerle ran some DMF in dogs and also in rats and isolated MMF and formamide as metabolites. He obtained approximately the same type of time distribution curves on the amount of material that was excreted by the animals as we found in man. In that respect, the metabolism of DMF is very similar in man as well as the other species examined. Another compound may act entirely differently. We did go from the animal studies to the human. We don't start with human studies.

MR. VERNOT: I have a question for Dr. Krivanek. How much of the inhaled dimethylformamide did you recover in the urine of your subject?

DR. KRIVANEK: I haven't done that calculation. Most of the DMF is metabolized. I can't give you a precise number but it's in a ball park range of 70-80%.

DR. MC CREESH (U.S. Army Environmental Hygiene Agency): I have a question for Dr. Krivanek also. You've talked about inhaled DMF. DMF also has a skin notation in the TLV lists of ACGIH. Did you feel there was any percutaneous penetration in these volunteers? Either it penetrates very rapidly and is metabolized or very, very slowly. And since DMF has an odor, did they shower and shampoo at the end of these tests or between exposures?

DR. KRIVANEK: On the second part of your question, at the levels we were exposing individuals, there was no indication of a noticeable DMF odor in the chamber. In regard to skin absorption properties of DMF, previous studies, published in the open literature, showed that skin absorption can contribute approximately 30% of the total DMF uptake. Skin absorption can be a significant factor in inhalation exposure to DMF.

DR. CROCKER (University of California, Irvine): Dr. Krivanek, you stated that the exposure was perhaps different in the person who is exercising actively as compared to the person who is not exercising at all when the air concentration was uniform. I would like to imagine that the actual exposure depended upon the total amount absorbed which your urinary excretion finding would match up with. It would also be interesting to know how much of that which was inhaled was retained. I'm trying to get away from the air concentration as a measure of exposure and get to the very measure you used which is excretion and to another one which is the amount retained per breath at the beginning and at various times during the exposure in the expectation that there may be some progressive change in the capacity of the subject to retain the inhaled material. In other words, absorption may not be uniform at all times. The way I would measure absorption would be to measure the concentration of DMF in the inhaled air and the exhaled air. Did you have any experience doing that?

DR. KRIVANEK: We haven't done any work along that line.

DR. CAVENDER (Becton, Dickinson and Company): I'd like to ask Dr. Rampy one question. I noticed in the study you reported that you did not use any chamber controls. Perhaps some of the pneumonia seen could have occurred because the control animals were held during the first 6 months in another room and then later they were housed in the same room with the exposed animals, as I understand the experiment, and the infections came during this later time. Is this a normal procedure that you would recommend for others? I'd like to know your reasoning for your answer.

DR. RAMPY: You're quite right that the difference of the time in incidence was related to the way they were housed and where they were housed. I wouldn't recommend doing out of chamber controls routinely. At the time, we were faced with a cramped facility where we were trying to do as many studies as possible and we made the choice of maintaining controls in animal holding rooms. In a large number of studies done with controls out of chamber, we only rarely saw differences of this sort. But I think certainly if you have a chamber, you should put the control animals in it.

MR. WANDS: Another question for Dr. Krivanek. As I remember his first slide, metabolism goes beyond monomethylformamide to the straight formamide. Is there any possibility of measuring such things as formic acid, or formates, or whatever else might be an end product of that process? Does that process continue to CO_2?

DR. KRIVANEK: Formamide is found in the urine. However, if you are going to select metabolites, the MMF is about 30 times more concentrated than the formamide. Kimmerle's work went into the question. Their recommendation was that if you are going to examine the metabolites, look at the MMF rather than the formamide. There's more of it and it is excreted sooner. If you are talking about practical work experience, you can get an end of shift sample that's a good indication of the exposure. With the formamide, you might have to wait another 8 to 10 hours for its excretion to come out.

MR. JOHNSON (University of California, Irvine); I would like to ask Mr. Moorman a question about his system. What language is it programmed in?

MR. MOORMAN: The system is set up on a Hewlett-Packard 9825 calculator which has Hewlett-Packard's own unique language. It's patterned after Fortran and is roughly that level of power.

MR. JOHNSON: The other question I wanted to ask you is about automatic systems. Do you have individual air control on your main air flow or is that affected by the systems coming up?

MR. MOORMAN: The main air flow is controlled by house blowers so all chambers go on and off together as the power is turned on and off. There is individual modification through manual valves which the desired flows are set at but they remain fixed at that level for the entire exposure.

MR. JOHNSON: Do you have an idea of the number of statements in the computer program? What are the memory requirements?

MR. MOORMAN: It's difficult to answer your question because I shuffle parts of the program in and out of it as they are needed. The initial characterization program is used once in six months for a particular run. It's fairly lengthy so I keep that separate from the rest of the program. The monitor program is about 150 lines and there are probably an average of 5 to 6 statements per line.

DR. CAMPBELL (Environmental Protection Agency): Dr. Cavender, you posed a question about a distinction between defense mechanisms being activated and outright or direct toxic effect in the lung. Would you care to talk about this distinction or speculate on an answer on the dividing line, that magic line between the need for calling forth of defense mechanisms and toxicity or where calling forth of defense mechanisms becomes toxic?

DR. CAVENDER: Ozone, an immunogenic gas, is equally toxic in every species tested, more or less. There are some differences but a 0.2 ppm concentration will have some effects in all animal species tested. The things we were looking at today really depend upon the animals ability to handle the type of pollutant or contaminant that is being exhibited. In the guinea pig which has severe bronchial constriction capability, one must correlate it with another species before one says this is a toxic response. I'm not saying a small change in pulmonary function is not a toxic response. If it correlates in other animal species at similar concentrations, it is. But if it requires 30 or 40 times the LC_{50} to produce the same type of reaction in another species, then perhaps it is not indeed a direct toxic effect but just a mere measurement of different pulmonary defense mechanisms. I'm sure that there is a dividing line where you start getting direct toxic effects. In the case of bronchial constriction in the guinea pig, you get death due to the mechanism before you get any direct toxic effects. Now, how you set standards in relationship to this is difficult to determine. EPA has a severe responsibility in addressing these questions. How you resolve the difference in species response is something I cannot answer today. It is something that needs to be addressed, and unfortunately, standards have been set using information obtained from a single species rather than looking at the broad spectrum of response in many species.

SESSION V

ENVIRONMENTAL STUDIES

Chairman

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PAPER NO. 18

IMPROVING INFORMATION FEEDBACK LOOPS ON ENVIRONMENTAL QUALITY

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INTRODUCTION

There are already a number of information feedback loops concerning environmental quality. When someone turns the wrong valve and large volumes of a toxic material inadvertently escape into an ocean or a lake, fish die by the thousands and the fishkill, if sufficiently large, is reported in the newspapers and on television. When the pines die in the hills near Los Angeles, they are telling something about a change in the quality of the air. The inability of the Peregrin falcon and other birds to lay eggs that remain intact during the nesting process is a signal that something new and unpleasant has permeated the environment. It has been said that "it's not nice to fool Mother Nature"--it's impossible! Death is her final signal that she has not been fooled. Even the final signal does not impress some members of society, especially when it occurs with another species or with other humans in other places. The same tired arguments, do you want fish or jobs, you can't stop progress, and there is no evidence that the fish or the pines were killed by the air or the spilled toxicant, are trotted out. However, there is fairly clear evidence that the general public has become rather cynical about these excuses.

An alternative approach to establishing feedback loops from natural systems which will govern our relationships with them is to provide the best treatment possible and assume Mother Nature will accept it. This is called "best practicable technology." In short, we will treat our wastes as best we can (naturally being practical in doing so) which means, of course, the least treatment society will permit. It is interesting that this approach is not permitted inside the plants because the result may not be adequate. Manufacturers know full well that the product must meet certain quality specifications, and if the present "practicable"

technology does not do this, they must make a better one so that it will. In short, they have established a feedback loop involving both the product and the process that produces the product, and if the present technology is not adequate, it must be improved. The practical aspect is that if they cannot make adequate improvement they go out of business because someone else undoubtedly will.

There is no question that if a large number of installations are using old, outmoded equipment that is certainly not "the best," then getting the best represents a substantial improvement. It would be an exercise in self-delusion if we were to assume that the best waste treatment presently available would always be enough. Certainly no one would knowingly take off in a spaceship unless there was confirmation that the best practicable technology was also sufficient to permit adequate protection.

Still another alternative to using nature's signals as a guideline for disposal of societal wastes is the use of national and state standards based on a few specific instances. Since environmental quality varies regionally and has a major impact on biological effects of waste discharges (e.g., Water Quality Criteria of 1972), this is not the best method for optimizing the nondegrading use of environmental assimilative capacity. This is probably the approach to which industry objects most strenuously because meeting arbitrary standards not based on site specific information can lead to additional treatment with no demonstrable biological or ecological benefits (e.g., overtreatment). It is also probable that there will be an equal amount of undertreatment. Thus, average standards are a poor means of utilizing the assimilative capacity of ecosystems which have striking regional differences.

The least-tried, most common-sense alternative is a site-specific fitting of discharges to the assimilative capacity of the local ecosystem. The case both for and against the assimilative capacity concept has been discussed in more detail elsewhere (Cairns, 1977). Since ecosystem assimilative capacity varies both regionally and seasonally, it is evident that the feedback loops providing biological information about the condition of the receiving system must furnish regular or continual information about the condition of the local system.

It is not surprising that engineers, policy makers, manufacturers, etc. have chosen the other routes mentioned because they are easier to administer, more economical on a short-term basis, and perhaps most important, biologists have not provided them with either appropriate parameters to use for the quality controls or methods to implement the

acquisition of this information. Biology as a profession has failed to produce any consensus opinion on the following very important questions:

1. What biological and ecological parameters are useful indicators of ecological quality?

2. What are the best methods for making these measurements?

Generally, when biologists are asked to evaluate a problem there is the usual squabble over whether this parameter is better than that one or, if agreement is reached on a parameter, which way to measure it. As a consequence, policy makers tend to take the attitude "a plague on all squabbling biologists--let's wait until they reach an agreement." The failure of biologists to produce standard methods for parameters generally agreed upon as important is one of the serious problems facing the implementation of environmental quality control through the establishment of feedback loops designed to produce a rapidly responding, quickly correcting quality control system.

It will take at least ten years to get a substantial number of additional standard methods into place in such volumes as the *Standard Methods for the Analysis of Water and Wastewater* and various standards books by the American Society for Testing and Materials. In the meantime, a sufficient number of standard methods is in place to institute wide-scale biological monitoring. For example, by using one of the bioassay methods either produced by the American Public Health Association or American Society for Testing and Materials, one could make a monthly, or perhaps ideally once weekly, assessment of the toxicity of a waste discharge. Although much research is needed, we are only using a fraction of the available, practical technology and methodology.

RATIONALE FOR BIOLOGICAL MONITORING

Practical systems for continuously monitoring water quality are of critical importance for the management of watersheds. There are two reasons why it will only be through such continuous feedback information systems that sensible and efficient controls can be designed. First, a watershed's ability to receive waste fluctuates on a daily or even hourly basis. Second, without constant monitoring industrial spills do much damage to aquatic

ecosystems before anyone is aware of the problem. It is significant that Section 308 of the Federal Water Pollution Control Act Amendments of 1972 states that the Administrator of the Environmental Protection Agency shall require the owner or operator of any point source to "install, use and maintain such monitoring equipment or methods (including where appropriate, biological monitoring methods) as the Administrator may require in fulfilling the objectives and requirements of the Act." Continuous monitoring systems using aquatic organisms as sensors may come to supplement the stream surveys and standard bioassays that are routinely used at present for biological monitoring.

The value of a regional monitoring program using chemical and physical measurements has already been well established by ORSANCO's (Ohio River Valley Water Sanitation Commission) successful operation of automated monitoring devices for nearly ten years on the Ohio River (Klein et al., 1968). These devices record such things as dissolved oxygen and pH. They have become indispensable in the management of the watershed and are stimulating similar developments in a number of countries. The major difficulty with this type of monitoring system, however, arises in the analysis of the data and in making evaluations of a complex ecosystem from the measurements of a few physical parameters. A possible solution to these problems involves the use of biological monitoring techniques.

All industries discharging wastes are using the environment as an extension of their waste-disposal system. If this were not true, each industry could recycle wastewater since its quality would be equal to that of the water entering the plant. Once this simple fact is recognized and accepted by the general public and industrial management groups, a new perspective on our relationship with environmental problems is possible. Conservationists may not like industrial and municipal use of the environment as a receiving system for wastes, but this situation is the rule rather than the exception in all industrialized societies and many developing societies as well. However, each group must compromise if we are to have a gradual transition from our present environmental situation to a more amenable one.

If we are willing to regard the environment as an extension of the plant waste-disposal system, then it seems quite reasonable to extend the quality-control practices which exist within the plant to the area outside the plant. The essence of quality control inside the plant is the continuous or regular collection of data regarding the process quality at different points in the operation and alteration of the management practices as necessary to maintain the desired

quality. The same principle can be applied in the natural environment, although the techniques would be somewhat different. For example, in Figure 1 there are industrial, agricultural, and wooded areas, as well as one small city. There are two reservoirs marked X and Y in the upstream areas and several small tributaries, one running through an agricultural area containing two fruit orchards and a hayfield, and another running adjacent to a hayfield next to Industry 3. Two types of biological or environmental monitoring systems (Cairns et al., 1970) could be used: (1) An in-plant system which uses a mixture of the plant waste and water from the receiving river to see if the waste will prove harmful to one or more representative aquatic organisms. The in-plant monitoring systems are designed to determine something about the biological quality of the wastes before they actually enter the receiving stream. (2) In-stream monitoring units which provide data from the receiving system itself because our ultimate objective is to protect it. In-stream monitoring units should utilize ecological data that are derived from the complex interlocking cause-effect pathways characteristic of natural systems. At present these are based on diversity indices or modifications of them (one infers that a high diversity of species indicates a large number of cause-effect pathways), although ideally they should be based on the function of the system as well as on the structure. The justification for maintaining the diversity characteristic of a particular locale has been discussed elsewhere (Cairns, 1967).

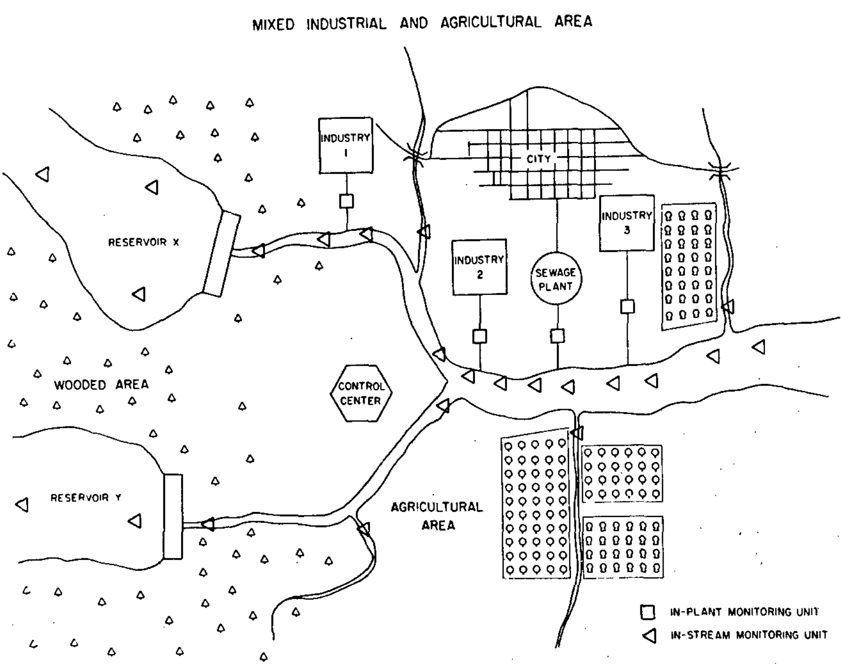

Figure 1. Mixed industrial and agriculture area.

Early Warning System

A number of papers discuss the use of aquatic organisms as "sensors" in an early warning system associated with an industrial waste discharge before it reaches the receiving system (e.g. Cairns et al., 1970, 1973b, Cairns and Dickson, 1974; Besch and Juhnke, 1971; Shirer et al., 1968). A simple schematic is given in Figure 2 for readers unfamiliar with this field.

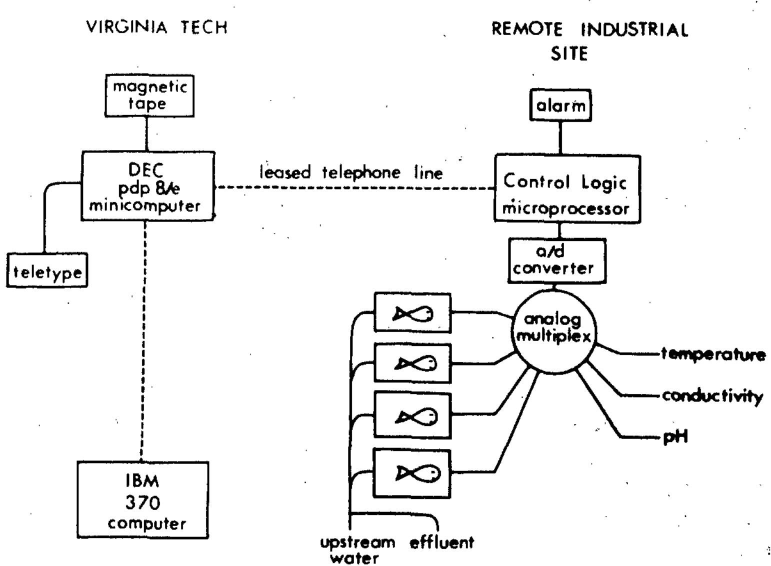

Figure 2. Schematic diagram of in-plant monitoring system.

Receiving System Monitoring

Aquatic microbial community structure has been used for years to assess the effects of pollution on aquatic ecosystems. Patrick et al. (1954) have obtained excellent results by fitting the structure of diatom communities to a truncated discrete lognormal model. By comparing the parameters of the model, evaluations can be made regarding the response of diatom communities to varying degrees of pollution. In addition, Kaesler and Cairns (1972) have shown that a portion of the microbial community (diatoms) is useful for estimating the response of the entire aquatic community. Generally the response of the microbial fraction of the entire aquatic community to adverse environmental conditions is more rapid than that of the macroinvertebrates and fish. Therefore, in terms of ecological quality-control measures, it is probably more efficient to look at this fast-response portion of the community, especially if we use changes in community structure as the basis for decision.

Unfortunately, using traditional taxonomic methods, the time required for proper identification of organisms is too great to permit a rapid response time which is the essence of an efficient quality control system (Cairns et al., 1970). Hohn (1961) reports that to fit a diatom community to the theoretical model would involve 25 to 30 hours from beginning to end of the analysis. This time lag is far too great for a rapid corrective response of the sort common to most effective quality-control systems. A rapid biological monitoring system with a short information feedback time should be coupled to the physical-chemical monitoring system now becoming commonplace. The interfacing of these systems would alleviate the dangers of attempting to predict the biological consequences of complex waste discharges based solely on a few chemical and physical parameters (Cairns et al., 1973b). A simple schematic using an optical processor for identifying diatoms (Figure 3) is provided for readers unfamiliar with this field.

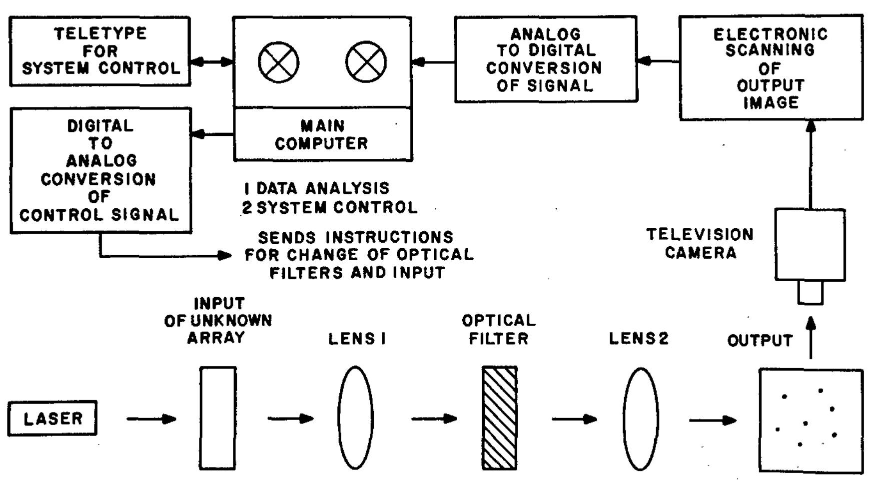

Figure 3. The fundamental arrangement of the prototype system (from Cairns et al., 1977).

QUESTIONS REGARDING IN-PLANT MONITORING

There are a number of questions regarding the conceptual and operational soundness of biological monitoring methods that must be answered more definitely before these methods are widely accepted by the academic community, potential industrial users, and regulatory agencies (Cairns, 1977). For an "in-plant" monitoring system, the most important questions are probably the ones listed here.

1. Will the system detect spills of lethal materials before they reach the receiving waters?

2. If only one organism is used as a sensor (for example, the bluegill sunfish), will this organism be so much more tolerant to the particular toxicant in question that it will pass undetected and harm other members of the aquatic community in the receiving system (for example, algae and invertebrates)?

3. Is it possible to monitor chemical-physical parameters and achieve the same results at lower cost and greater efficiency?

4. Since the biological response alone will not identify the particular toxicant causing the response but only indicate that some deleterious material is present, is it possible to couple a chemical-physical monitoring system with a biological monitoring system that will expedite the identification of the particular deleterious component causing the warning response?

5. Will a false signal cause an expensive shutdown of the plant or an undue expenditure of time and effort by the waste control personnel?

6. Should an organism indigenous to each receiving system be used, which would require a long site-specific developmental period for each new drainage basin, or can some "all-purpose" organism, such as the bluegill, be used for all types of systems (or perhaps one organism for a warm-water and one for a cold-water system)?

7. Is it possible to use in-plant biological monitoring systems to detect the presence of spills of materials having either acute lethality or long-term effects or only the former?

8. Are the in-plant monitoring systems only for very large industries with sizable waste control staffs, or is it possible to develop compact miniaturized reliable in-plant monitoring systems that can be used by persons inexperienced in monitoring without undue expenditure of time, etc.?

QUESTIONS REGARDING IN-STREAM SYSTEMS

The in-stream system has not been in development as long as the in-plant system (the in-plant monitoring program began in 1966; the in-stream in 1971) although the parameters based on community structure have been in use many years. Although

the basic conceptual problems in the laser unit have been satisfactorily resolved, research is in progress to improve the following areas: (1) signal-to-noise ratio, (2) problems of focus, and (3) effects of size and orientation.

Since the unit is still being developed, questions related to practical day-to-day use have not been addressed substantively.

WHEN SHOULD BIOLOGICAL MONITORING BE USED?

Clearly biological monitoring will not be used in every situation. Even when used properly it will not reduce risk to zero! It is also important to recognize that monitoring systems are designed to supplement more traditional assessments, not replace them! A few examples of situations where biological monitoring would be appropriate follow:

1. when potentially toxic chemicals are discharged or introduced into fragile or highly vulnerable ecosystems.

2. when one or more accidental spills of hazardous material have occurred and the operation continues. (This should be done until evidence of continuing effectiveness has been established even when additional engineering or operational safeguards have been installed.)

3. when hazardous materials are or may be discharged or introduced into an area containing rare or endangered species or species of particular commercial or recreational value.

4. in all situations where the expected environmental concentration is close to predicted "no biological effects" concentration or where the uncertainty about either or both of these concentrations is great. This process of hazard evaluation may be depicted graphically (Cairns, in press). In Figure 4a the "no adverse biological effect" level and the concentration that will result from introducing the chemical into the environment (e.g., see Mill et al., 1977) are well apart. In fact, only estimates of these concentrations are known and are indicated by dotted lines which envelope the solid concentration lines. This is because Tier I testing consists of comparatively crude short-term tests. Tier II testing is more sophisticated and expensive (e.g., continuous flow instead of batch) and Tier III even more so. Frequently Tier I testing will sufficiently improve the estimates so that one will, at decision point P, be able to determine that the concentrations are indeed different. Thus, there may be

justification for terminating testing in Tier I and concluding, at a certain risk level, that introduction of the chemical will not cause an environmental hazard. One should remember that risk cannot be reduced to zero. Both ecology and economics have externalities!

In Figure 4b the two concentrations are closer together. Now, testing must be carried through Tier III before the same statement can be made at a comparable risk level. Figure 4c depicts the case where testing up to the same point leads.

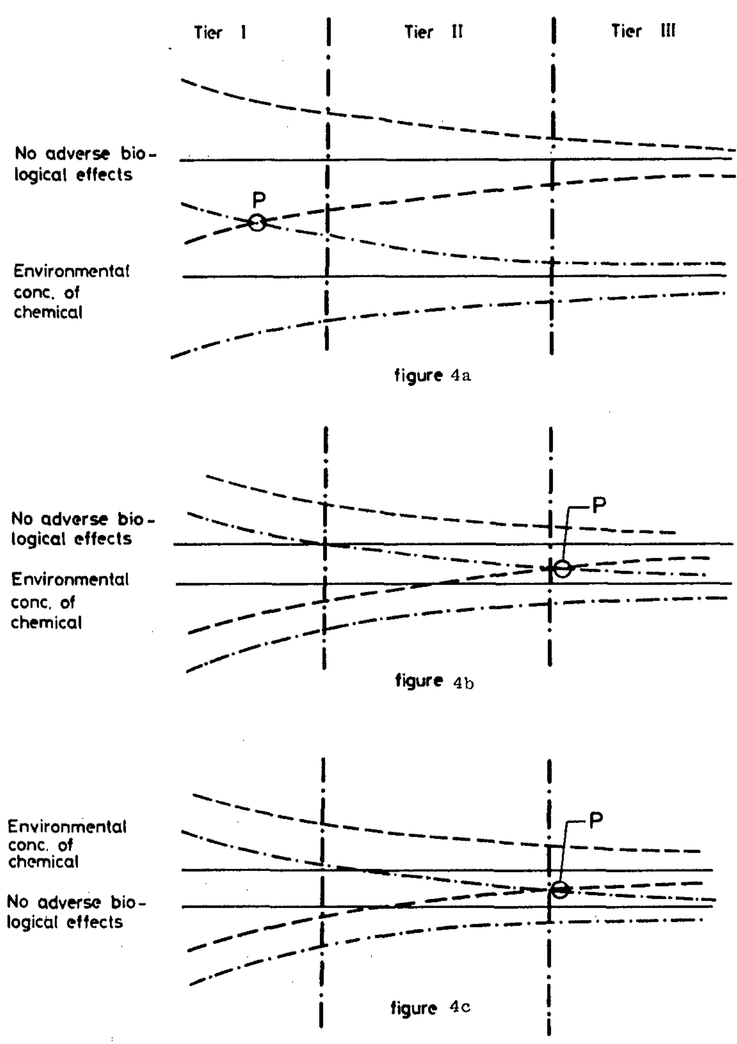

Figure 4. This figure depicts the relationship of a chemical concentration which produces no adverse biological effects with the actual environmental concentration of the chemical. Tier I bioassays or toxicity tests are preliminary or screening tests; Tier II are of intermediate complexity; and Tier III are the expensive, long-term sophisticated tests for sublethal responses. P indicates the point in the testing program where a decision is justified; see text for further discussion.

Biological monitoring using fish has also been used in Europe to assess the quality of water withdrawn from a river to be used in public drinking water systems.

CONCLUSION

Human society is now in a transitional period somewhat comparable to the agricultural revolution. At one time, the environment was a source of food but was not managed for that purpose. Then, continued population growth and concentration together with increased expectation provided the impetus for a managed food supply which we called the agricultural revolution. The industrial revolution has produced environmental stresses of a magnitude and intensity that at worst might seriously impair earth's life support system, and, at best, if unchecked, would degrade the quality of life. To optimize the advantages of natural and technological systems, management is needed. Effective management will require a continuous flow of information about the quality of the system being managed.

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PAPER NO. 19

A SYSTEM FOR CHRONIC TOXICITY STUDIES WITH MICROORGANISMS

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INTRODUCTION

Investigations of toxicity in lower bioforms are usually conducted for one or more of five reasons: (1) to determine the specific toxicity for the species of interest; (2) to screen compounds for potential toxicity as a precursor to studies in higher animals; (3) to obtain data for eventual extrapolation to man; (4) to evaluate the consequences attendant with the environmental intrusion of the compound of interest; and (5) to establish the value of specific organisms as indicators of environmental pollution. These bioforms, e.g. bacteria, various invertebrates, protozoa, algae, and plant and animal cell cultures are becoming increasingly attractive systems for toxicologic study as manpower, equipment, space, and time constraints become more costly and the volume of required research appears to increase logarithmically. Although data derived from vertebrate, and particularly mammalian, studies cannot be duplicated with these far simpler creatures or systems, they do function as laboratory tools that provide much useful information that is available on a reduced time basis and certainly at considerably lower costs. For those investigations that are concerned with environmental toxicity, the judicious choice of species representative of the target environments is essentially the only method by which the environmental impact of the potential pollutants can be ascertained (in conjunction with the appropriate studies of environmental interaction, i.e., stability, reactivity, and biological degradation and accumulation). Included in the selection of environmental representatives are those less complex life forms alluded to previously.

The specific species to be used in any of these researches, with the exception of those to which particular interest is directed, becomes a matter of subjective choice by the investigator, influenced to some degree by objective factors. These include availability of the test species (system), ease of maintenance and handling, freedom from interfering species (e.g., contamination of blue-green algal species by persistent bacteria), applicability to the experimental requirements/design, amenability to data collection procedures, peculiarities of the life cycle, and correlation of findings with other biological response data. Bioassays utilizing prokaryotic and simple eukaryotic forms are not of recent origin; the application of specific auxotrophic bacteria and fungi for quantitative analysis of vitamins and amino acids preceded the availability of contemporary physico-chemical and wet chemical techniques. However, the recognition of the sensitivity and in some cases selectivity of biological responses to toxic molecules has continued the interest in and utilization of simple biological entities for a variety of research and assay inquiries. Ewald et al. (1976) studied the effects of PCB's on several growth parameters of *Euglena gracilis*. Lichens, symbiotic relationships between particular fungi and algae, were used by Lundstrom and Hallgren (1973) to study pollution by oxides of sulfur. The toxicity of five heavy metal compounds was determined in the ciliated protozoan *Tetrahymena pyriformis* (Carter and Cameron, 1973). The common protozoan *Paramecium* was used by several early investigators as a bioassay system for various types of compounds including potential carcinogens (Epstein et al., 1963). Tissue cultures of various cell lines have been used to determine the effects/toxicity of a variety of toxic molecules and contaminants, e.g., nitrous oxide (Bruemmer, 1967), spacecraft contaminants (Hays, 1967), and potable water contaminants (Ehrlich, 1976). The serious consequences of water pollution by petroleum products have elicited innumerable studies on the resultant biological effects; target bioforms used in these studies range from bacterial and planktonic forms to various species of osteichthyes including such forms as barnacle larvae (Donahue et al., 1977) and mosquito larvae (Berry and Brammer, 1977). Unique characteristics of particular bacterial species have been the basis for specific bioassays. Williamson (1976) developed a method to determine the toxicity of wastewaters to be processed in sewage treatment plants, based upon nitrate removal by the bacterium *Nitrobacter*. Perhaps the most widely known bacterial bioassay for the screening of potential carcinogens is that of Ames (1975) which detects the mutation from auxotrophy or prototrophy in five strains of histidine-deficient *Salmonella typhimurium*.

To determine both a more accurate indication of and predictive capability for the environmental impact of a potential pollutant, model ecosystems have been studied that include a number of diverse species. These systems permit an investigation of the interrelationships among species inhabiting the affected ecosphere as well as the influencing physico-chemical factors (Metcalf, 1975). The greater majority of bioassay techniques, whatever the thrust of the investigation, are generally concerned with acute effects; however, many toxicological and environmental research problems must also include consideration of chronic exposures. Such studies are obviously more complex requiring specialized equipment and significantly increased logistic burdens. Continuous exposure systems have been developed for animal studies (Thomas, 1965), for fish with readily water soluble toxicants (Mount and Brungs, 1967), and for aquatic studies of highly volatile pollutants (Brenniman et al., 1976). All of these approaches are invaluable and indeed cannot be replaced by other, less complex systems. However, their complexity suggests that other systems for chronic studies be available that would obviate the cost and time constraints and provide a capability for screening and/or data acquisition preliminary or in addition to the more extensive methods. Such an approach may be found in the continuous culture systems of unicellular organisms, particularly with selected bacterial strains.

The growth of bacteria in the laboratory occurs predominantly under closed conditions, i.e., where nutrient sources and waste removal are fixed and become limiting, eventually resulting in the death of the culture. This type of growth when occurring in liquid culture is termed batch and gives rise to the familiar sigmoid growth curve which can be described in rather precise mathematical terms, provided all growth influencing parameters are known. In contrast is continuous culture as it occurs in the device termed "chemostat," first described by Monod (1950) and independently by Novick and Szilard (1950). The chemostat is an open system in which a limiting nutrient is supplied at a rate partly selected by the investigator and partly determined by the microbe, and waste metabolic byproducts and microbial cells are continuously removed. This type of continuous culture that is controlled by a rate limiting nutrient bears no relationship to continuous culture that is controlled intrinsically by the optical density (turbidity) of the growing culture. This approach is actually a "continuous batch" process in which a preestablished culture

turbidity, as monitored by suitable optical sensors, triggers the addition of fresh medium to the growth chamber to obtain a selected, lower cell density, volume being kept constant by an appropriate liquid withdrawal system. The growth kinetics of this "turbidostat" culture are quite different from those describing the extrinsically controlled activities in the chemostat. Many excellent treatises on the kinetics and application of this dynamic equilibrium approach to microbial growth have been presented (Ghosh and Pohland, 1971; Veldkamp and Jannasch, 1972; Powell, 1956; Herbert et al., 1956; Tempest, 1970; and Law et al., 1976). Several researchers have recognized the value of continuous culture as a unique tool for ecological investigations (Parker, 1966; Jannasch and Mateles, 1974).

The major difference, from an experimental viewpoint, between continuous microbial culture systems and all other methods/systems for the study of chronic toxicity is the unchanging nature of a continuous culture. Continuous, long-term exposure of any plant or animal system must consider time as a consequential factor, i.e., the system ages and hence changes biochemically and therefore in resistance/susceptibility. Certainly for some investigations this is a significant factor in the biological response of the experimental biosystem and must be considered in establishing both the toxicity and hazard potential of the substance of interest. However, if the research demands a system that remains physiologically constant during the period of exposure (especially if that period is extensive), the chemostat with its many possible modifications appears as the only means of accomplishing this requirement, albeit with bioforms at the lowest level of complexity.

The chemostat, then, establishes for as long as required the continuous addition of low concentrations of toxicant to the steady state culture permitting determination of bioaccumulation, biodegradation, bacteriocidal or bacteriostatic effects, differential toxicity in mixed cultures, mutation, and biochemical changes. Selection of strains marked by increased resistance or increased metabolic activity can be accomplished as dictated by experimental design. Environmental factors that contribute to qualitative metabolic stasis and growth kinetics, e.g., temperature, agitation, redox potential, medium composition, light flux (for photosynthesizing species), etc. can be readily altered to study chronic exposure effects over varying conditions.

Chemostat systems may be as simple as the original design of Novick and Szilard (1950) or as complicated as the experiment requires. The purpose of this paper is to describe a 5-unit chemostat system that was designed to study the effects of chronic exposure of selected bacterial strains to potential environmental pollutants. The general requirement that guided the approach was two-fold: (1) the system should be comprised of commercially available equipment of moderate price and flexible design; and (2) the system should require a minimum of attention and should permit monitoring of significant growth parameters.

CONTINUOUS CULTURE SYSTEMS

The initial system design was based on the range of experimental requirements demanded by the physico-chemical characteristics of the pollutants to be studied, the physiological needs of the anticipated test bacterial strains, and the type of data to be obtained. Therefore, the capability to vary and control temperature, agitation, aeration, anaerobiosis, nutrient feed rate, and pollutant feed rate was mandatory. In addition, continuous monitoring of selected physiological and environmental parameters was necessary as well as the ability to obtain culture samples aseptically. Since the first series of experiments was concerned with the effects of water soluble compounds (hydrazine, monomethylhydrazine, and unsymmetrical dimethylhydrazine) and an aerobic soil organism, the system was conceived as shown in Figure 1. It must be emphasized that this modular design can be readily altered to accommodate the specific requirements of a different research protocol.

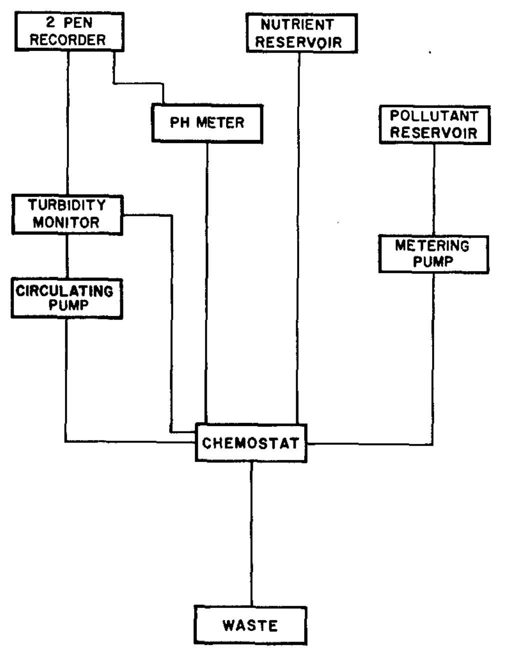


Figure 1. Block diagram of continuous culture system.

The nucleus of the system is the chemostat. Rather than fabricating this device from available component parts, the BioFlo Model C30 Bench Top Chemostat (New Brunswick Scientific Co., Inc., Edison, New Jersey) was purchased since this unit incorporated all of the control requirements in a compact design (see Figure 2). Figure 3 is a functional representation of the operating features and controls of the Bio-Flo. The growth chamber is a 1000 ml Pyrex vessel with a working volume of approximately 350 ml which is maintained by a simple overflow port. The chamber is fitted with a rubber stopper held in position by a threaded metal retaining ring. Penetrations through the stopper are provided for baffles, cold finger, heating element well, thermistor sensing probe well, medium inlet, sampler, thermometer, pH electrode, etc. Additional penetrations may be added to accommodate other sensor requirements. All materials in contact with the medium within the growth chamber are stainless steel (type 316), Pyrex glass, or Teflon. Agitation is accomplished with a magnetically coupled, stainless steel impeller that revolves on a glass nub fixed in the bottom of the chamber. Continuously variable agitation rates from 200 to 1000 rpm are available and held constant by a solid state controller. Temperature control is accomplished within the growth chamber by means of a heating probe and solid state thermistor regulator. The available temperature range is from ambient to 60 C with $\pm$ 0.2 C accuracy. Below ambient temperature control is possible with the cold finger. We operate at 25 C, maintaining this with the heating element and cold water circulating through the cold finger.

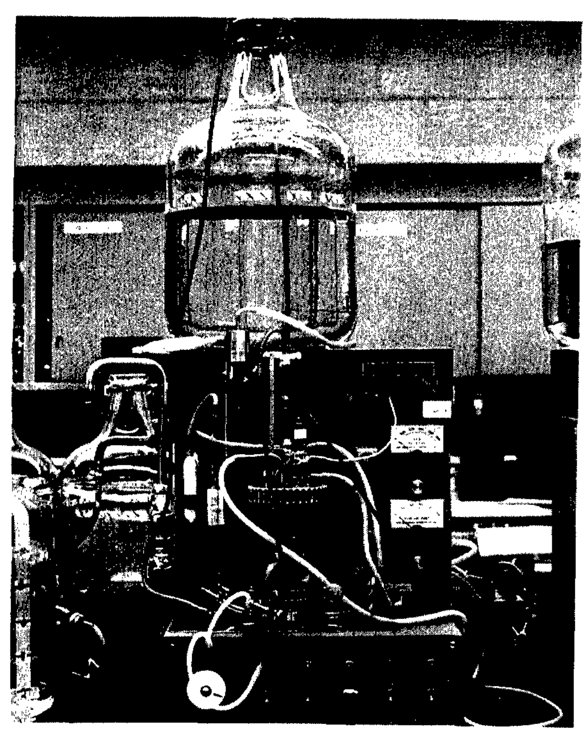


Figure 2. BioFlo with medium reservoir in operation.

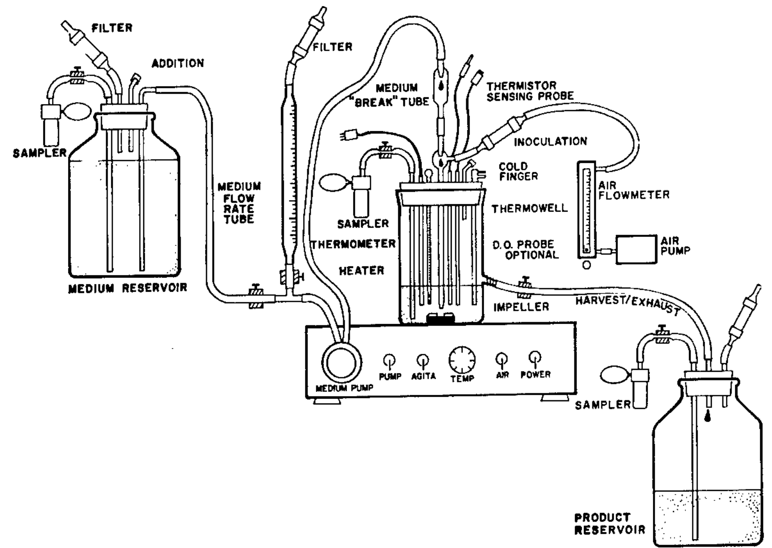


Figure 3. Functional diagram of the BioFlo (redrawn from Operating Manual, BioFlo, Model C30).

Aeration is provided up to 800 ml/min by a contained vibrator pump that is controlled by a needle valve and metered through a flowrater. The air is sterilized by passage through a cotton or glass wool filter, entering the growth chamber via an inlet tube in the medium inlet tube. This keeps the incoming medium under slight positive pressure, thus helping to prevent backgrowth in the medium inlet line. An additional design feature included to eliminate backgrowth are two medium breaks in the incoming medium line. These break the continuous flow at two points by forcing the medium to drop to the next lower stage. These are both shown in Figures 3 and 4.

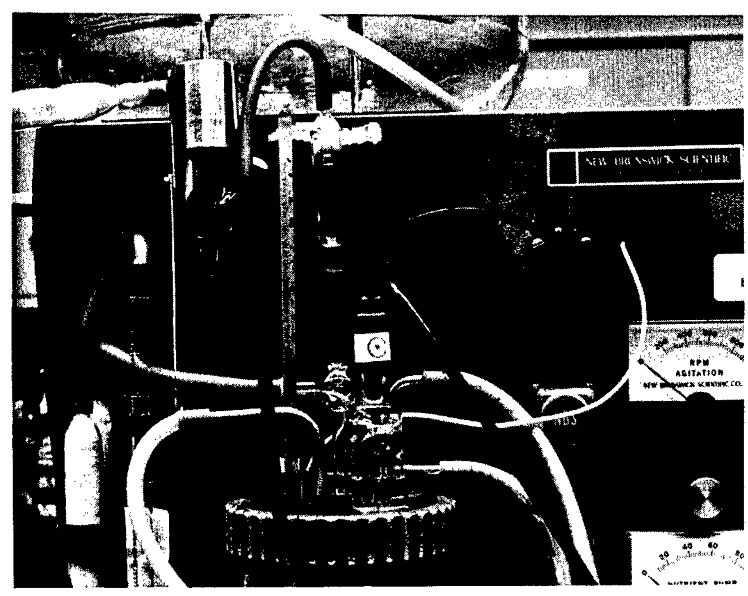


Figure 4. Close-up photograph of the BioFlo showing medium breaks, sampler and other growth chamber penetrations.

The medium reservoir supplied with the BioFlo was replaced with a larger 18.9 liter (5 gallon) Pyrex carboy. The closure provided by the manufacturer was a rubber stopper with various penetrations to allow medium sampling, additions, etc. This closure was not satisfactory for several reasons, primarily being a poor system for changing medium reservoirs. A different closure shown in Figure 5 was developed after several designs were tried. It consists of a stainless steel plate and neoprene gasket fixed to the carboy opening which has been ground flat by eye bolts held in place by a collar around the neck of the carboy (the eye bolt retainers and collar were used on the original closures). Mounted to the plate are a cotton filter of anodized aluminum, a port for nutrient addition, and the medium supply line. The end of this stainless steel line terminates in a Whitey stainless steel valve (SS-14DK-S4, Cincinnati Valve and Fitting Co., Cincinnati, Ohio). The valve accepts Swagelok fittings and during sterilization is covered by a stainless steel plug (SS-400-P). The latex rubber medium inlet line on the BioFlo terminates in a short piece of 1/4" stainless steel tubing with a Swagelok fitting. Thus medium reservoirs may be changed rapidly and with minimal chance for contamination.

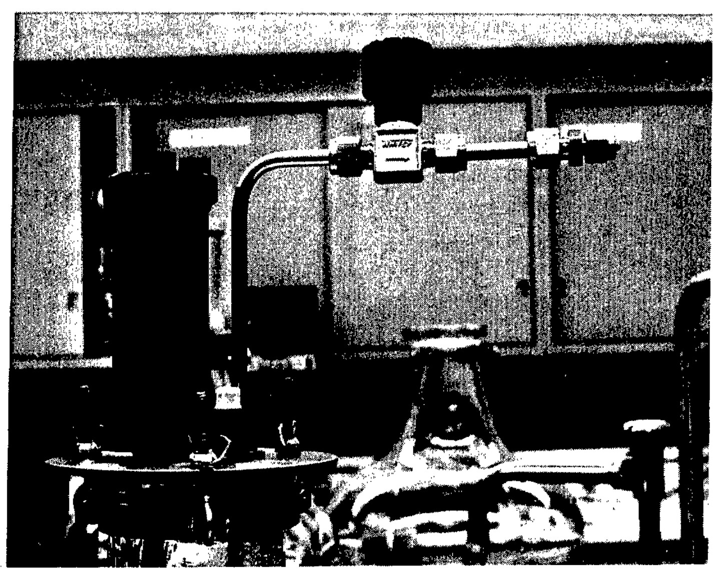


Figure 5. Closure for medium or pollutant reservoir.

Medium is transferred from the reservoir to the growth chamber at the required rate (from 25 to 500 ml/hr) by a peristaltic pump contained in the BioFlo. Pump speed is controlled by a solid state device that maintains flow rate within $\pm$ 2%. The pump operates through a side-mounted cam that applies pressure to the side of the tubing loop rather than centrally, thereby reducing tubing wear considerably.

It is possible to measure medium flow rate during operation of the unit by virtue of a flow rate tube that is connected to the medium transfer line and is calibrated in cubic centimeters. This is a valuable capability since electronic instability and change in tubing elasticity can induce change in flow rate. Unfortunately, the flow rate tubes supplied by NBS on all 5 units were highly inaccurate. They were replaced with 10 ml pipettes that had been modified to fit the BioFlo. It was also necessary to recalibrate all of the control functins of all the units, since factory calibration was not sufficiently accurate.

The major modification made on the BioFlo was to the hooded sampler. This attachment enabled obtaining a sample of the culture aseptically by applying a vacuum with an attached rubber bulb to the sample vial (see Figure 3). The sampler is also shown on the medium and product reservoirs. Since a capability to continuously monitor cell concentration by turbidimetry was mandated by the experimental design, it was prudent to incorporate the facility for sampling with this system. The continuous monitor requires a closed loop for circulating the culture from the growth chamber through the turbidity sensing device and back to the growth chamber. The sampler was introduced into this loop in the sequence growth chamber ⟶ circulating pump ⟶ turbidity flow-through cell ⟶ sampler ⟶ growth chamber. The sampler consists of a stainless steel fitting (as originally supplied with the BioFlo) and a threaded test tube or glass vial that has been modified with (1) a fitting in the bottom to which rubber tubing (approximately 1/4" ID) is attached, and (2) a side extension that is capped with a small (5 x 9 mm) rubber serum bottle stopper. The sampler is shown in Figure 4. The culture flows through the sampler by virtue of the circulating pump and returns to the growth chamber by gravity. To obtain a sample, the rubber tubing connected to the bottom of the glass sampler is pinched off until the liquid level rises sufficiently in the vial to allow withdrawal by syringe through the side port. Thus sampling is accomplished aseptically and without disturbing the continuous culture system.

Of the many techniques used to quantitate bacterial mass or numbers, the determination of cell density by optical properties of a liquid culture is certainly the most rapid, if not the most accurate. The majority of such observations have usually employed transmission instruments at selected wavelengths. Measurements were recorded of optical density or its reciprocal, percent transmittance. This approach does not account for absorption phenomena and lacks sensitivity.

Turbidimetric techniques are most reliable when instruments designed as nephelometers are used. In this approach, light scattered at an angle of 90° to the incident beam by the suspended particles/cells is measured, the amount of light scattered dependent upon the number, size and shape of the suspended particles. The instruments may be calibrated with Formazin in Formazin Turbidity Units (FTU) or with some measurement of cell mass (number, nitrogen content, DNA content, etc).

The HF Instruments Turbidimeter Model DRT-1000 (HF Instruments Ltd., Bolton, Ontario, Canada) was designed essentially to measure water quality. We selected this instrument because of its simplicity, operation as a true nephelometer with two photosensing devices, continuous flow-through capability, wide response range (from 0 to 1000 FTU) and recorder outlet. The flow-through cell consists of a stainless steel fitting with entry and exit ports and a threaded glass scintillation vial as the sensor cell. The flow-through unit is autoclaved along with the circulating loop. Figure 6 shows the DRT-1000 and the flow-through cell. To reduce the noise inherent in the signal to the recorders, a filter was included in the output to the recorder consisting of a 5000 to 6000 μf capacitor.

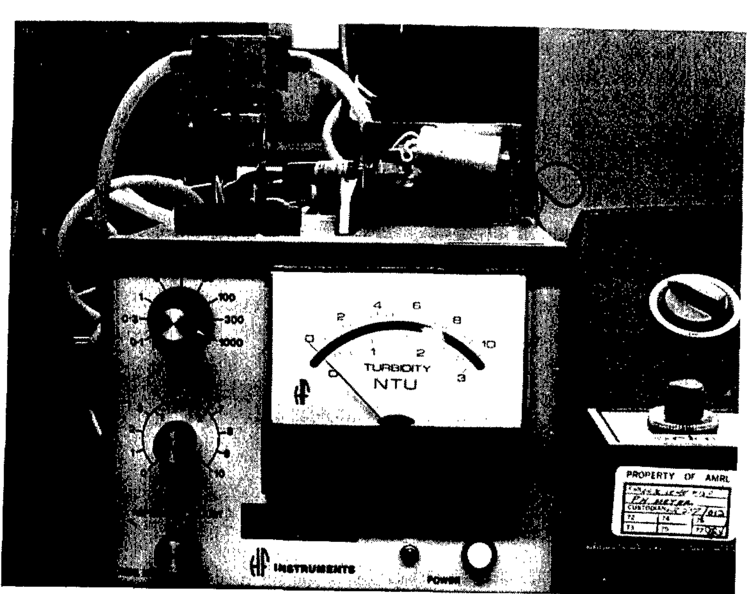


Figure 6. Turbidity monitor showing flow-through cell and electrical filter circuit.

Circulation of the culture through the DRT-1000 flow-through cell is accomplished with a peristaltic pump, Masterflex Pump Drive Model 7568 with Masterflex Pump Heads Model 7017 (Cole-Parmer Instrument Co., Chicago, Illinois). This versatile unit can accommodate up to 10 pump heads for 10 channel operation, any mixture of the six available pump head sizes being possible. The continuously variable speed control and the range of pump heads enables a pumping capacity range of 0.3 to 380 ml/min. We operate the flow through the turbidimeter at approximately 100 ml/min which is sufficient to obtain adequate mixing in the sensing (flow-through) cell and minimizes bacterial adherence to the wall. To further minimize bacterial deposition on the walls of the sensor cell as well as on all other glass surfaces, all glass materials in contact with the culture were siliconized by treatment with Siliclad (Clay Adams, Parsippany, New Jersey) according to the directions provided. The appropriate sized silicone tubing for pump head 7017 was used as a 24 in. loop. Stainless steel Swagelok reducing unions (3/8 in. to 1/4 in.) were used to connect the tubing in the pump head loop and the smaller latex rubber tubing used in the remainder of the circulating loop.

The pH of the culture medium is a useful monitor of the culture status and is a readily obtainable value. To enable the sterilization of the entire BioFlo working unit, NBS selected the Ingold Autoclavable pH Electrode, No. 6070-01 (Ingold Electrodes Inc., Lexington, Maryland). This repeatedly autoclavable unit is a combination electrode, compatible with all standard pH meters, and may be purchased with any one of the usual connectors (Beckman type, BCN, etc.). We are using two pH meters: the Corning Model 125 (Corning Glass Works, Corning, New York) and the Beckman Model 76 Expanded Scale (Beckman Instruments, Fullerton, California). The former instrument has a recorder output with a span control; the older Beckman unit was modified by addition of a variable resistor to the recorder circuit. The pH meter output can thus be adjusted to enable pH to be indicated directly on the recorder and, with balancing of the recorder, pH 7 registering at mid-range with a recording capability of from pH 2 to pH 12.

Each recorder provides a permanent record of the pH and turbidity of the culture in one BioFlo. The recorders operate at 2 cm/hr (Linear Model 385, Linear Instruments, Irvine, California) or at 1 in/hr (Varian Model G-2000, Varian Associates, Palo Alto, California), both types using Z-fold paper. The combination of slow chart speed and Z-fold paper enables both easier data evaluation and more efficient storage of records.

Addition of pollutant to the growth chamber at a constant rate commensurate with the growth of the bacteria was accomplished with a highly accurate and stable peristaltic pump. We selected the Cole-Parmer Deluxe Ultra Masterflex Drive System with Masterflex Pump Heads Model 7015 (Cole-Parmer Instrument Co., Chicago, Illinois) because of its excellent reliability. It can operate with up to four channels and over a wide flow rate range (0.1-135,000 ml/hr) because of the number of available pump head sizes, the 100:1 gear box, and the continuously variable rate control. The solid state controller maintains constant pump speed despite torque changes by virtue of a feedback circuit and provides indication of both of these operational variables on two separate meters.

The pollutant reservoir is a Pyrex glass carboy with a closure identical to that used with the nutrient reservoir. In practice, 10 liters of glass distilled water is sterilized by autoclaving. The pollutant (in our experiments to date, hydrazine) is added to the reservoir with an accurate syringe to the desired concentration along with sufficient sterile 10% HCl to maintain the pH in the reservoir at 5 for stability. Prior studies have shown hydrazine to be quite stable at pH 5 in good quality water for some time; in our experiments this is a period of about four days - the time to deplete the pollutant in the reservoir. The pollutant reservoir is connected to the continuous culture system through a Swagelok fitting, as with the nutrient reservoir, and thence through the pollutant feed line of silicone rubber, the loop of larger bore silicone tubing around the pump head, and finally to the culture via a 1/8" stainless steel tube that penetrates the rubber stopper of the growth chamber. This entire pollutant entry line is autoclaved as an attachment of the BioFlow growth chamber. The Swagelok fittings of both the pollutant line and medium line are carefully covered with cotton during autoclaving to maintain sterility.

The most serious disadvantage associated with peristaltic pumps of any design is the stretching and abrasion of the tubing. This obviously results in changes in delivery rates and, ultimately, tubing failure. We have minimized these problems by using the appropriate size (both inner and outer dimensions) of silicone tubing if available and by periodically moving the portion of tubing directly in contact with the pump head. These simple precautions have enabled experiments to proceed for as long as one month. However, in the event of tubing failure, a tray placed under each pump head will contain any spilled liquid (medium, pollutant, or culture) and allow its contents to enter a suitable trap by gravity flow.

SYSTEM OPERATION

The major laboratory activity associated with continuous culture research is that devoted to preparation of materials, media, pollutant, and sterilization of wastes (spent culture medium, count plates, dilution tubes, etc). We have attempted to minimize this manpower expenditure by using larger sized reservoirs, simplified dilution series, disposal pipette tips, and by preparing concentrated stock solutions of medium constituents for direct volumetric addition to precalibrated (volume) medium reservoirs. The medium composition was based upon the requirements of the selected bacterial strain used in our studies to date.

Three criteria were used in the selection of a suitable test bacterial strain: (1) it must be a normal soil inhabitant and not a spore-forming species; (2) it must exhibit moderate sensitivity to hydrazine compounds in acute, batch experiments; and (3) it must be capable of reasonably rapid growth in a minimal medium, i.e., one comprised of only mineral salts and glucose. This third criterion is mandatory to insure negligible interaction between the medium constituents and the pollutant. An additional benefit of studying a phototrophic organism is that it lends itself to investigations concerned with screening for carcinogens and teratogens in which mutation to auxotrophy occurs and can be readily ascertained.

The composition of the medium we use is shown in Table 1. The concentrations of stock solutions and volumes used to prepare one medium reservoir (13 liters) are also shown. After addition of the various salts, the reservoir is autoclaved for one hour and allowed to cool overnight. Filter-sterilized glucose solution is then added aseptically with a syringe and the reservoir incubated at room temperature for at least 24 hours as a check on sterility. Final pH of the medium is 7.1.

TABLE 1. COMPOSITION OF MINIMAL MEDIUM

Constituent	Stock Conc. g/ml	ml Stock/ Reservoir-13 L	Final Conc. g/L
KH_2PO_4	0.095	130.0	0.95
$K_2H\ PO_4$	0.488	65.0	2.44
$(NH_4)_2\ SO_4$	0.500	26.0	1.00
$Mg\ SO_4 \cdot 7H_2O$	0.200	13.0	0.20
$Fe\ CL_3 \cdot 6H_2O$	0.010	1.3	0.001
$Ca\ Cl_2$	0.150	1.3	0.015
α-D-Glucose	0.500	52.0	2.00

The growth chamber of the BioFlo and the various accessories and penetrations are autoclaved as a complete unit for at least 20 minutes. Upon cooling, usually overnight, the chamber is positioned on the control section of the BioFlo and the various connections made; water line to the cold finger, heating element and thermistor probe in wells, air line to filter, pH electrode to pH meter, turbidity flow-through cell in the DRT-1000 and circulating loop in the pump head, and waste overflow line to waste receiver jug. The final and two most critical connections are then performed: (1) the medium transfer line is aseptically connected to the medium reservoir via the Swagelok fitting; and, (2) the section of medium transfer line is placed in the head of the peristaltic pump and the head is carefully tightened to insure the same degree of tension on the tubing used during medium flow calibration. The growth chamber is then filled with medium from the reservoir and all control functions actuated. The unit is operated for at least 24 hours to allow all parameters to reach equilibrium and as a final check on total system integrity and sterility. Our experience with the 5-unit system has indicated our design choices and operational procedures have resulted in a highly reliable system.

Previous studies have shown that glucose at 2 g/liter is rate limiting for our experimental conditions. An inoculum is prepared by growing the soil isolate in the minimal medium plus 2 g/liter glucose (SMS + G) for 16 hours at room temperature on a reciprocating shaker. The growth chamber is inoculated with 0.5 ml of this culture through the rubber capped port in the sampler. The usual operating conditions are: temperature = 25 C, agitation rate = 300 rpm, and aeration = 0.8 scf/hr or 0.38 liter/minute. The culture volume is 350 ml in the growth chamber plus 150 ml in the circulating loop. The viable count of the inoculum is determined and is generally on the order of 5×10^8 cfu/ml. Thus, the inoculum concentration is approximately:

$$\frac{5 \times 10^{8}\ \text{cfu/ml}}{0.5\ \text{ml} \times (350 + 150\ \text{ml})} = 2 \times 10^{6}\ \text{cfu/ml}$$

The culture attains maximal growth after 16 hours, at which time glucose is exhausted, and the nutrient pump is turned on for the initiation of continuous culture. The medium is added to the growth chamber at a rate of 20 ml/minute. The dilution rate, D, is then:

$$D = \frac{120\ \text{ml/hr}}{375\ \text{ml} + 150\ \text{ml}} = 0.24\ \text{hr}^{-1}$$

Continuous culture can then be maintained indefinitely, at least theoretically, requiring only that medium reservoirs be changed when empty and waste collection jugs exchanged when full.

Results observed during our first complete system evaluation are shown in Figures 7 and 8. The viable counts, given as cfu/ml x 10^8 in Figure 7 stabilized reasonably well after approximately 36 hours continuous culture operation. The turbidity values shown in Figure 8 suggest dynamic equilibrium was attained in all 5 units at the same level. (These values were obtained from overflow waste samples and read in the Coleman Junior Spectrophotometer Model 6D (Coleman Instruments, Oak Park, Illinois) at 570 nm since all the DRT-1000 units had not been completely calibrated at this time.) The pH of the cultures was also an excellent indicator of culture status: starting at pH 7, the value decreased to approximately 5.3 as culture density increased and then stabilized at 6.1 as the culture reached equilibrium.

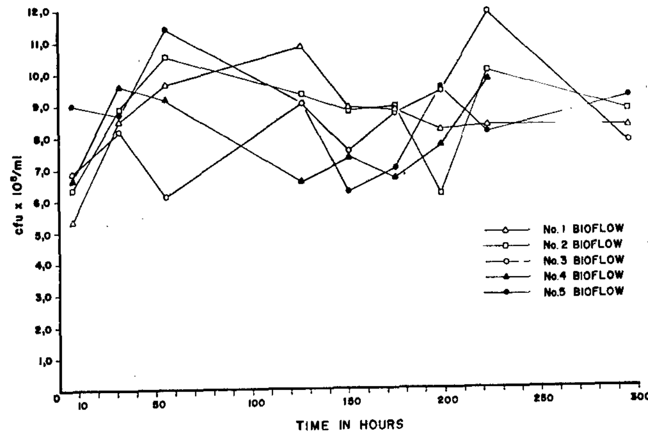


Figure 7. Viable counts obtained during initial system evaluation.

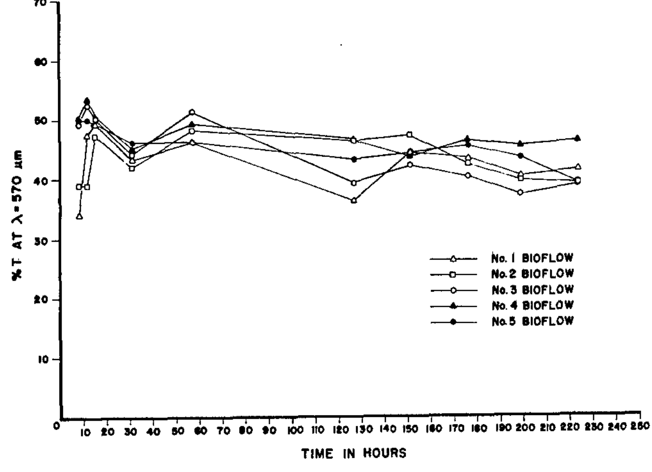
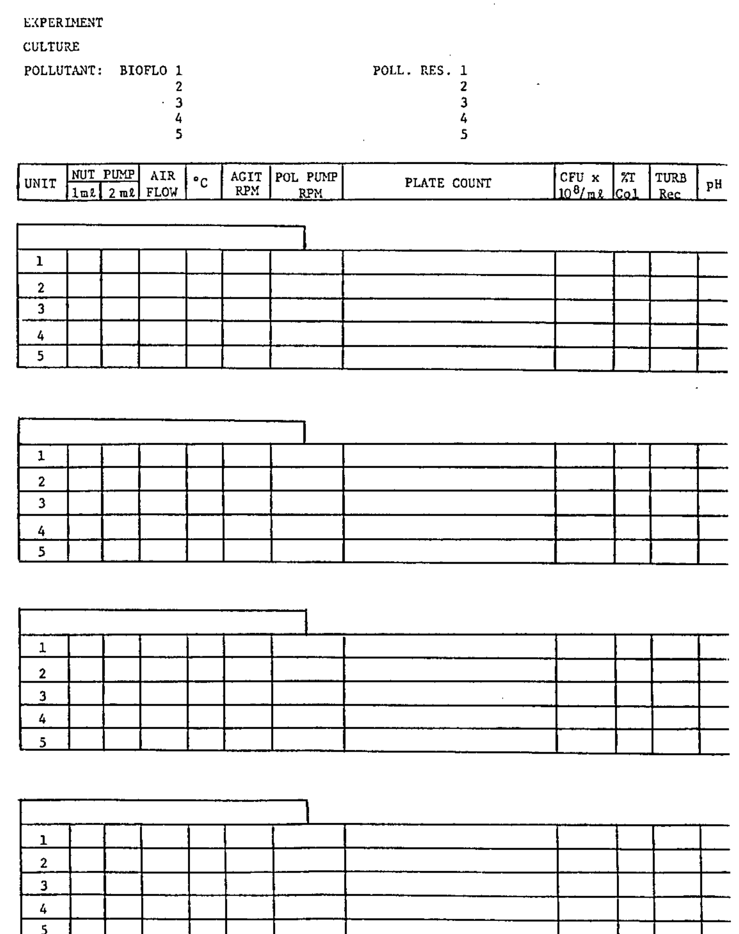


Figure 8. Turbidity values obtained during initial continuous culture operation.

The addition of a pollutant to the system requires several modifications of the procedure described. The pollutant reservoir is connected to the system with the Swagelok fitting in the pollutant feed line and the silicone tubing placed in the pump head. Several different approaches can be taken for pollutant addition; we elected to deliver larger volumes of dilute solutions as a more accurate method since the pump is not working at an extreme of its capability. To most easily accomplish this, we changed the rate of medium addition from 2 to 1 ml/minute and doubled the concentration of all constituents. The concentration of pollutant in the reservoir is twice that desired and it, too, is delivered at a rate of 1 ml/minute; therefore, we achieve a total flow rate of 2 ml/minute at the desired concentrations of both nutrients and pollutant. To start the phase of the study with pollutant, the following sequence is employed after equilibrium has been achieved, usually 3 days: (1) double strength medium and double strength pollutant reservoirs are connected; (2) medium pump control setting changed to 1 ml/minute; (3) growth chamber inoculated with an amount of neat pollutant to establish desired concentration; and (4) medium and pollutant pumps turned on. The addition of pollutant directly to the growth chamber can result in a small transient change in pH which reverts to the normal value of 6.1 in a few hours.

During an experiment, the operational parameters of the BioFlo are recorded at least once per day, viable counts are made, and turbidity of the overflow determined as a check on the DRT-1000 turbidity monitors. Figure 9 shows the data work sheet used for recording daily operations. Total cell counts are performed with the Coulter Counter Model F (Coulter Electronics, Inc., Hialeah, Florida) using a 30 µm aperture and a 50 µl manometer. The attentuation control is set at 0.250, aperture control at 8, and threshold at 8.0.


EXPERIMENT
CULTURE
POLLUTANT: BIOFLO 1 2 3 4 5 POLL. RES. 1 2 3 4 5

UNIT	NUT PUMP 1mℓ	NUT PUMP 2 mℓ	AIR FLOW	°C	AGIT RPM	POL PUMP RPM	PLATE COUNT	CFU x 10^8/mℓ	%T Col	TURB Rec	pH
1											
2											
3											
4											
5											
1											
2											
3											
4											
5											
1											
2											
3											
4											
5											
1											
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5											

Figure 9. Data work sheet for pollutant runs.

Several continuous culture experiments were conducted at concentrations of hydrazine ranging from 1 to 1000 ppm. Figure 10 predicts the results of an experiment in which 100 ppm was studied in three chemostats. The turbidity values shown were obtained with the Coleman spectrophotometer, as indicated previously, and hand plotted for ease in presentation. A steady decline in turbidity occurred immediately upon addition of the hydrazine. After somewhat greater than 20 hours exposure to the hydrazine, the density of the culture was reduced significantly. The culture medium appeared visually to be devoid of cells. Viable counts obtained during the experiment indicated that the growth rate was inhibited resulting in a lengthened mean generation and hence washout of the cells. Since approximately 5 complete volume exchanges occurred in the growth chamber over the 20 hour period, a bacteriostatic or bacteriocidal effect would have resulted in sterility (or practically so) via complete

washout. Since the viable count remained approximately 200-5000 cfu/ml from 21.5 hour of hydrazine exposure to 70 hour (experiment termination), growth must have occurred but at a considerably lower rate. Future experiments will be concerned with quantitating this change. This experiment indicates that 100 ppm hydrazine can consequentially alter growth kinetics of the test organism and that the no-effect level of chronic exposure is lower than anticipated.

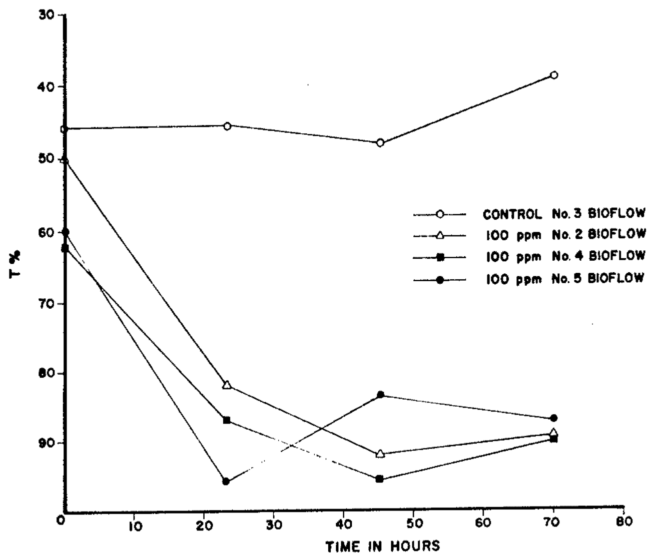

Figure 10. Effect of 100 ppm hydrazine on continuous culture of D-31.

This rather simple and direct utilization of continuous culture illustrates how effective a research tool it can be. Instructive information on the degree of biological effect as well as the pollution consequences of a given substance can be rapidly ascertained by manipulation of the many environmental variables available to the investigator. In conjunction with other "simple system" bioassay and screening techniques, it offers the environmental toxicologist a unique and highly flexible tool for unraveling problems concerned with both man and his environment.

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PAPER NO. 20

MULTIPLE TOXICANTS IN THE ENVIRONMENT*

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INTRODUCTION

An extensive methodology has been developed for evaluating the effects of discrete environmental toxicants on a variety of test organisms; however, when environmental pollution does occur several toxicants are usually present simultaneously. The recognition of this situation by environmental toxicologists and those responsible for assessing the potential hazards of manmade pollutants has generated considerable interest in developing approaches for evaluating the effects of mixtures of environmental toxicants. Sprague (1970) in his series of papers on the measurement of pollutant toxicity to fish reviewed some of the approaches and the results of previous studies assessing the joint toxicity of aquatic pollutants.

Several years ago, we became interested in this problem and initiated a program to develop and empirically evaluate an approach for studying the effects of multiple toxicants on the whole organism performances of fish.

We recognized as others have (Plackett and Hewlett, 1948) that only pharmacological studies on the modes of action of toxicants applied separately and jointly can definitively determine the type of interaction between them. However, the primary actions (the underlying processes by which toxicants initiate alterations in some preexisting physiological or biochemical process) of toxicants has been

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elucidated in only a few cases. Even in these cases it can probably be expected that the more a presumed action is studied the more likely it will be found to be an effect, the sequence of biochemical and physiological events that are initiated by the action of a compound (Fingl and Woodbury, 1965).

Given the difficulty and uncertainty in determining the primary mechanisms of action of toxicants, the classical pharmacologic approach for evaluating the toxicity of compounds involves studying the relationship between the concentration of a toxicant and the effects it produces. The selection of an appropriate effect for evaluating the toxicity of a compound depends on the objectives of the toxicologist. Lethality is often used as a starting point for studying the toxic properties of a pollutant. Therefore, it is not surprising that most studies on the joint toxicity of environmental toxicants have been on quantal responses (all or none) - primarily death. However, to insure the success of organisms in nature, it is also necessary to study the effects of toxic substances on such whole organism performances as growth, reproduction, and behavioral responses.

Plackett and Hewlett (1948) suggested that the mathematical examination of the concentration mortality curves for individual toxicants may indicate the types of combined effects that occur when the toxicants are present simultaneously. As a first step for evaluating the effects of multiple toxicants on whole organism performances, we based our approach on aspects of various models originally presented by Bliss (1939) and Plackett and Hewlett (1948) for quantal response data. Using their approach, Anderson and Weber (1977) were able in most cases to predict the effects of mixtures of selected environmental toxicants on the survival of guppies (Poecilia reticulata). Based on these results we designed a series of experiments to evaluate the applicability of the approach to graded (sublethal) responses.

The primary objective of this paper is to discuss the rationale of the proposed approach for studying both the quantal and graded responses of whole organisms to mixtures of environmental toxicants. Hypothetical dose response curves with their associated isobole diagrams are presented to illustrate the different types of toxicant interaction discussed. The results of preliminary experiments evaluating the effects of the chlorides of copper, nickel and their mixture on the growth rate, food consumption, and gross growth efficiency of juvenile guppies are presented.

RATIONALE

Using Bliss's paper (1939) as their point of departure, Plackett and Hewlett (1952) described rather general biological models for toxicant interactions and deduced mathematical models for each based largely upon statistical considerations. They proposed general types of toxicant interaction based on the following two-way classification scheme:

	Similar	Dissimilar
Noninteractive	Simple similar (concentration addition)	Independent (response addition)
Interactive	Complex similar	Dependent

They defined toxicant mixtures as "similar" or "dissimilar" according to whether the toxicants acted upon the same or different biological systems and as "interactive" or "noninteractive" according to whether one toxicant influenced the "biological action" of the other toxicants. "Simple similar" and "independent action" were regarded as special cases in a continuum of biological possibilities and the mathematical models proposed for complex similar and dependent were generalizations of the models proposed for "simple similar and independent action", respectively.

Their mathematical models, particularly for the quantal responses to mixtures of "interactive" toxicants, are very complex and require the knowledge of certain parameters which are normally unattainable when evaluating the effects of toxicant mixtures on whole organism performances. However, Hewlett and Plackett's models for "joint action" are useful for elucidating the limitations of and the assumptions required for the special cases of "simple similar and independent joint action". As a first approach to evaluating the effects of toxicant mixtures on the whole organism performances such as survival and growth, the present discussion only considers the special cases of "noninteractive" toxicant mixtures.

A multitude of terms have been suggested to describe the various types of combined toxicant effects. Ariens (1972) and Fedeli et al. (1972) reviewed the various terminologies that have been used. As Sprague (1970) and Warren (1971) point out, the nomenclature is confusing particularly since certain terms have been defined in more than one way by different authors. Furthermore, terminology describing

mechanisms of toxicant action is not appropriate for studies evaluating the effects of toxicant mixtures on whole organism performances without knowledge of the action of the individual toxicants. To avoid both ambiguities in terminology and assumptions implying knowledge of sites and mechanisms of toxicant action, Anderson (1973) introduced the terms concentration and response addition which are mathematically analogous to the "simple similar" and "independent action" defined by Plackett and Hewlett (1952).

Concentration addition is mathematically defined as the additive effect determined by the summation of the concentrations of the individual constituents in a mixture after adjusting for differences in their respective potencies. The primary assumption governing this type of addition is that the toxicants in a mixture act upon similar biological systems and contribute to a common response in proportion to their respective potencies. Bliss (1939) and others have assumed that if two toxicants act similarly the variations in susceptibility of individual organisms to the toxicants are completely correlated. As a consequence, the dose response curves for the components and the mixture are parallel. This has been observed for some toxicant mixtures; however, Plackett and Hewlett (1952) presented examples of chemically related insecticides which gave nonparallel lines. They and other toxicologists (Ariens and Simonis, 1961; Casarett, 1975) have stated, and we believe rightfully so, that parallelism and hence complete correlation of individual susceptibilities is not a necessary prerequisite for this type of addition.

In cases where the dose response curves for the individual toxicants in a mixture are parallel, a dose response curve for the mixture can be calculated based upon the assumption of concentration addition. With the regression equations for the individual toxicants in the form of $y = a + b \log x$ (where y is the % response to each toxicant and x is its concentration), the regression equation for a binary mixture can be represented by (Finney, 1971):

$$y_m = a_1 + b \log (\pi_1 + p\pi_2) + b \log Z \qquad (1)$$

where,

y_m = % response to the mixture
a_1 = y intercept of the first toxicant
b = common slope
π_1 = proportion of the first toxicant in the mixture
π_2 = proportion of the second toxicant in the mixture
p = potency of the second toxicant relative to the first
Z = concentration of the mixture.

This equation can be readily adapted to represent mixtures containing more than two toxicants. It should be noted that equation (1) for concentration addition is similar in principle to the toxic unit method used by Lloyd (1961), Brown (1968) and others. Whereas the toxic unit method measures the toxicity of mixtures only at particular levels of response (LD_{10} , LC_{50} , etc.), equation (1) incorporates the entire dose response curve.

Response addition is the additive effect determined by the summation of the responses of the organism to each toxicant in a mixture. This form of addition is based on the assumption that the toxic constituents of a mixture act upon different biological systems within the organism. Each organism in a population is assumed to have a tolerance for each of the toxicants in a mixture and will only show a response to a toxicant if the concentration exceeds its tolerance. Consequently, the responses to a binary mixture are additive only if the concentrations of both toxicants are above their respective tolerance thresholds. However, for quantal responses the tolerances to the toxicants in a mixture may vary from one individual to another in a population; therefore, the response of the test animals depends also upon the correlation between the susceptibilities of the individual organisms to the discrete toxicants. For example, in order to predict the proportion of organisms killed by a binary mixture, it is necessary to know not only the proportion that would be killed by each toxicant alone but also to what degree the susceptibility of organisms to one toxicant is correlated with their susceptibility to the other toxicant.

Plackett and Hewlett (1948) recognized this statistical concept and developed mathematical models that accounted for the correlation of individual tolerances ranging from total negative to total positive correlation. If the correlation is completely negative (r = -1) so that the organisms most susceptible to one toxicant (A) are least susceptible to the other (B), then the proportion of individuals responding to the mixture (P_m) can be represented by:

$$P_m = P_A + P_B \text{ if } (P_a = p_b \leq 1) \qquad (2a)$$

where P_A and P_B are the respective proportion of organisms responding to the individual toxicants A and B. With no correlation (r = 0) in susceptibility the relationship is expressed by:

$$P_m = P_A + P_B (1 - P_A) \qquad (2b)$$

In the limiting case of complete and positive correlation ($r = 1$), individuals very susceptible to toxicant A in comparison with the population will be correspondingly very susceptible to toxicant B. In this situation the proportion of animals responding to the mixture is equal to the response to the most toxic constituent in the mixture. Mathematically this is represented by:

$$P_M = P_A \quad \text{if } P_A \geq P_B$$

$$P_M = P_B \quad \text{if } P_B \geq P_A \tag{2c}$$

For response addition, no significance can be placed on the slope of the dose response curves because the toxicants in a mixture are acting primarily upon different biological systems with varying degrees of susceptibility between organisms. Even if the regression equations for the constituents in a mixture are parallel for toxicants acting in this manner, the dose response curve for the mixture will not be linear (Finney, 1971). This will be illustrated later for two hypothetical toxicants whose dose response curves are parallel. Although the mathematical equations (2a,b,c) representing response addition are relatively simple, the statistical consequences of this type of addition are more complicated than those of concentration addition (Finney, 1971).

Terms such as supra- and infra-addition are used to describe toxicant interactions which are greater or less than those predicted on the bases of either concentration or response addition.

QUANTAL RESPONSE STUDIES

Hypothetical Dose Response Curves

To illustrate graphically the relationship between concentration and response addition, hypothetical dose response curves for two toxicants (A and B) are plotted in Figure 1 expressing percent response in probits as a function of the logarithm of total concentration. In this example the dose response curves for the discrete toxicants are parallel with A being 100 times more toxic than B. We could have also chosen nonparallel curves; however, for these cases equation (1) for concentration is not appropriate. Hewlett and Plackett (1959) have developed a more generalized model (from which equation (1) can be deduced) which does not depend on the assumption of parallel dose response curves.

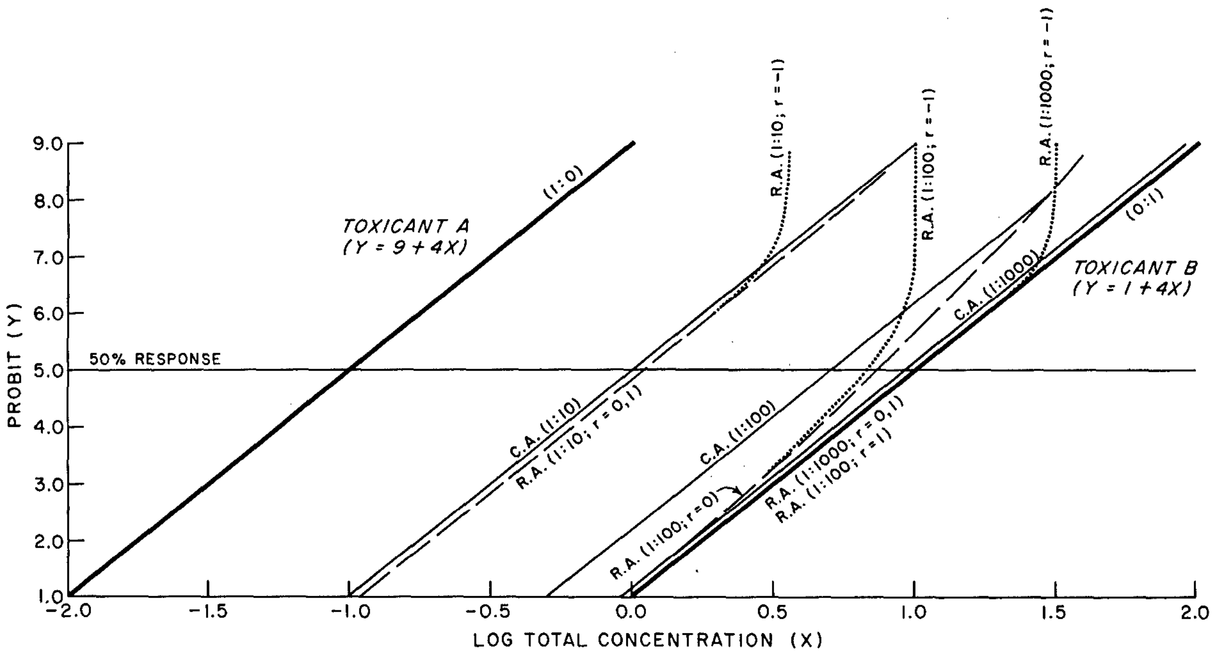

Figure 1. Hypothetical dose response curves for toxicant A (1:0), toxicant B (0:1) and their mixture containing the fixed proportions (1:10, 1:100, 1:1000). See text for explanation.

Dose response curves for mixtures of toxicant A and B are obtained when the total concentration is varied and the ratio of the concentrations for the individual toxicants is kept constant. Using the equations (1 and 2a,b,c) for concentration (C.A.) and response addition (R.A.), dose response curves were calculated for different mixtures containing fixed proportions of toxicants A:B (1:10, 1:100, 1:1000). In Figure 1, the responses to the mixtures are shown graphically in relation to the dose response curves of toxicants A and B.

Several observations can be made from the relationships between the dose response curves in Figure 1. As should be expected, the relative toxicity of the mixture depends on the ratio of its constituents. In Figure 1, a 1:10 mixture is more toxic than the other mixtures depicted because of the greater proportion of the more toxic component - toxicant A. At certain ratios, regardless of the correlation of susceptibility (r), the relative potencies of the mixtures acting in either a concentration or a responsive additive manner are very similar. This is observed in Figure 1 for fixed proportions of 1:10 and 1:1000. Furthermore, for any one ratio the relative potency of the dose response curves for concentration and response addition (r = 1, 0, -1) depends on the level of response. Focusing on the dose response

curves for mixtures in the ratio of 1:100, it can be noted that at low levels of response (i.e., at the probit of 2 which corresponds to approximately a 0% response) the mixtures acting in a concentration additive manner are considerably more toxic than those acting by response addition regardless of the degree of correlation (r). This is due to a fundamental difference in the two types of addition. At threshold or below threshold concentrations of toxicants A and B, a mixture acting in a concentration additive manner can elicit a measurable effect because both toxicants are acting upon similar biological systems. Therefore, their concentrations can sum to produce a concentration for the mixture which is above the threshold level. However, the responses to toxicants acting upon different biological systems (response addition) are only additive if each toxicant in a binary mixture is present in concentrations above their respective threshold levels. For similar reasons, as the concentrations for the toxicants in a 1:100 mixture increase, the dose response curves for response addition (except in the special limiting case where $r = 1$) become progressively more toxic relative to the dose response curve for concentration addition. It is even possible that at high levels of response (in this example, for responses greater than 84% probit of 6.0) mixtures acting in a response additive manner with negative correlation of susceptibility ($r = -1$) can be more toxic than those acting on the basis of concentration addition.

These factors (the type of interaction, the ratio of the toxicants in a mixture, and the level of response) must also be considered along with the toxic properties of the individual toxicants in assessing the relative toxicity of a mixture. The failure to recognize these factors can potentially lead to erroneous conclusions concerning the nature of the interaction of multiple toxicants.

Isobole Diagram

It is difficult to visualize the relationships between the dose response curves in Figure 1 primarily due to the number of curves presented. However, the relationships between the hypothetical curves in Figure 1 can be readily conceptualized with isobole diagrams, a technique introduced by Loewe (1928, 1953). Isoboles are lines of equivalent response. They are constructed by plotting on a two-dimensional diagram the concentrations of a binary mixture of toxicants that produce a quantitatively defined response, i.e. a 10%, 50% or 90% lethal response. It should be noted that an isobole diagram can be constructed for any level of response and the relationship between the isoboles may vary depending upon the response level selected.

The isobole diagram for the 50% level of response of the hypothetical dose response curves in Figure 1 is present in Figure 2. The x and y axes in this diagram represent the concentrations of toxicant B and A respectively. The radiating dashed lines or mixing rays correspond to a series of mixtures (A:B) of fixed proportions. If the 50% response is produced by combinations of two toxicants represented by points inside the square area, the toxicants are additive. Antagonistic interactions are represented by combinations of concentrations falling outside the square.

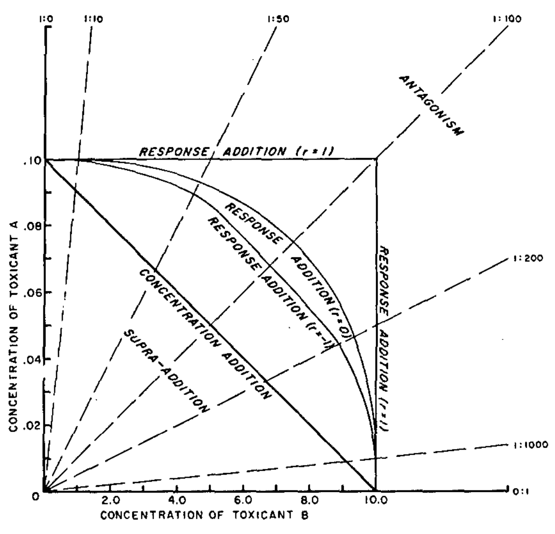

Figure 2. Isobole diagram for quantal response data. Isoboles for concentration and response addition were determined from hypothetical dose response curves in Figure 1.

The isoboles for concentration and response addition are determined from the concentrations of the two toxicants which correspond to the points of intersection between the 50% response line (Figure 1) and the respective hypothetical dose response curves. These concentrations are plotted in Figure 2 on the appropriate mixing ray. The lines connecting these points define the course of the isobole. Concentration addition is represented by the diagonal isobole. For quantal data, response addition is defined by the curved isoboles for complete negative (r = -1) and for no correlation (r = 0) in susceptibility. The upper and right boundaries of the square correspond to the limiting case of response addition with complete positive correlation (r = 1).

The term "no interaction" had been used by other authors (Sprague, 1970; Warren, 1971) to describe the response additive isobole in Figure 2 corresponding to complete positive correlation of susceptibilities. We recognize that the equation (2c) used to determine this isobole is not additive in a strictly mathematical sense. For example, in lethality studies, organisms whose tolerances to the individual toxicants are positively correlated (r = 1) die in response to the most toxic constituent in the mixture; therefore, there is no addition of responses. However, in experimental situations it is unlikely that complete positive or for that matter complete negative correlation will often be observed. Consequently, we have chosen to represent complete positive correlation as a limiting case of response addition to be consistent in our terminology and more importantly to emphasize that the isobole for response addition will for most toxicant mixtures fall between the extreme cases of r = -1 to r = 1 depending upon the degree of correlation.

For reasons similar to the one presented by Warren (1971), we have chosen to use the terms supra- and infra-addition to describe interactions that are greater or less than expected on the basis of either concentration or response addition. It is important that these terms be used in reference to a particular type of addition. For example, an isobole falling between the isoboles for concentration and response addition (r = -1) could be designated as both infra- and supra-additive depending on the nature of the interaction. This potentially confusing situation is avoided by using the terms in the manner we have suggested.

The term antagonism in Figure 2 refers to a physiological or functional antagonism. In the present discussion, we do not consider toxicants which can chemically or physically react in the external medium of an organism to form an inactive or less toxic product (chemical antagonism). Some investigators have used the term antagonism to describe interactions that are less toxic than strict additivity (concentration addition) but whose mixture still has a combined effect greater than either constituent applied alone. We prefer to use the term infra-addition to describe these cases and to reserve antagonism for those cases where the presence of one toxicant necessitates that a higher concentration of another toxicant be present to obtain the defined level of response.

GRADED RESPONSE STUDIES

A consideration of the nature of the dose response curves for quantal and graded responses shows that the effects they express are quite different. Quantal dose response curves express the incidence of an all-or-none effect (usually death) when varying concentrations are applied to a group of organisms. The curve is derived by observing the number of organisms which respond or fail to respond at various concentrations. Consequently, the slopes of these curves primarily express the individual variation of the population to a particular toxicant. Graded dose response curves characterize the relationship between the concentration of a toxicant and the magnitude of the effect under consideration. The dose response curve can be derived by measuring on a continuous scale the average response of a group of organisms at each concentration.

As Clark (1937) and others have pointed out, it is possible to represent any graded response as a quantal response provided that the response of each individual organism can be measured. However, this procedure if adopted is at the expense of some "loss of information" (Gaddu, 1953). Quantal response data reveal only the number of organisms that respond or fail to respond at some particular concentration. On the other hand, graded response data not only tell us whether or not a group of organisms respond but also how much they respond.

The mathematical equations (2a,b,c) for the response addition are not appropriate for graded effects for two reasons. First, there is a difference in the way the two types of data are measured. For quantal responses, the proportion of organisms responding to any concentration is determined by the ratio of number of organisms showing the response to the total number subjected to the concentration. For graded responses, the mean response to each dose is measured but in general the maximum possible response is not known. In cases where the maximal effect is not known, no proportional response can be calculated. This is particularly true for growth experiments where an organism's response can potentially range from growth enhancement to negative growth depending on the concentration of a particular toxicant. Secondly, the statistical concept of correlation between the susceptibilities of the organisms to the discrete toxicants in a mixture is not appropriate for graded responses measured in the manner described earlier. Graded response data representthe average response of a group of organisms.

Therefore, the response of each individual organism to the toxicants is not known. To be sure, the tolerances of the individuals in the group will vary for the different toxicants in a mixture; however, this factor will not alter the relative toxicity of the mixture because the range of tolerances of the population is theoretically represented in the sample of organisms from this population.

For graded response data, we have represented the combined response to a mixture of toxicants acting in a response additive manner as simply the sum of the intensities of response which each component toxicant produces when administered alone. A similar relationship was defined by Loewe (1953). Concentration addition can be predicted for a toxicant mixture using equation (1) if the component toxicants exhibit parallel dose response curves. Figure 3 represents an isobole diagram for a graded response. The isoboles for concentration and response addition were determined with the appropriate mathematical equations discussed.

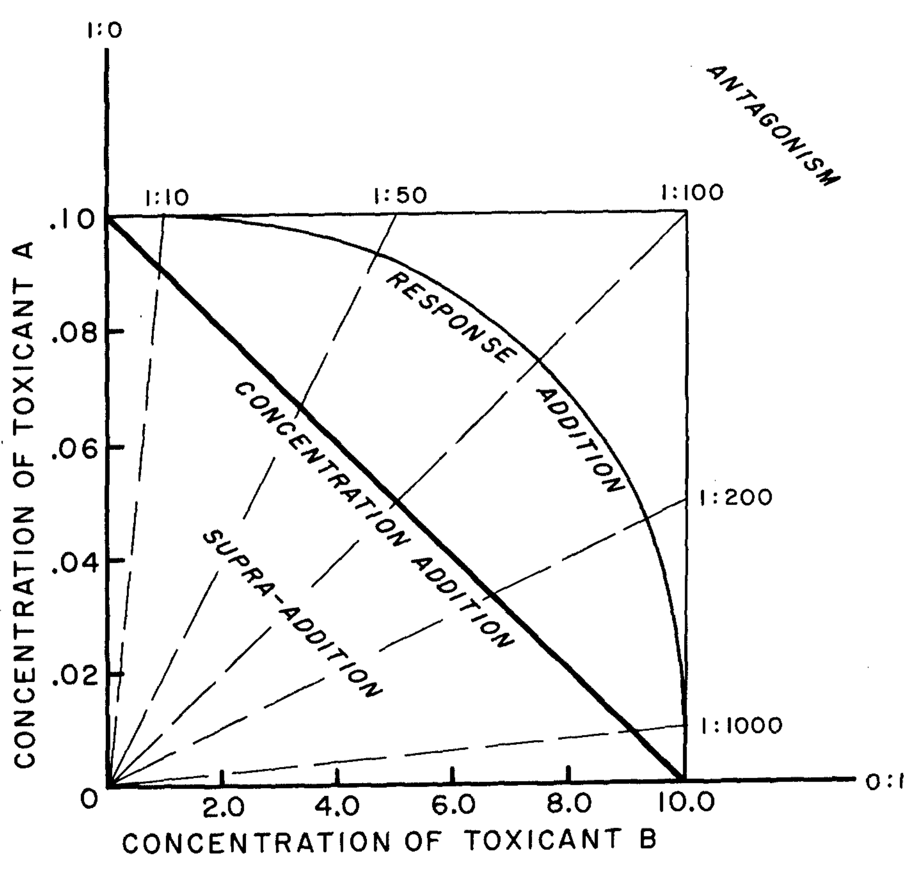

Figure 3. Isobole diagram for graded response data.

The relatively simple types of isoboles represented in Figures 2 and 3 should only be expected for relatively simple in vitro systems or in situations where there is a clear-cut relationship between dose and effect. Given the complexity and interdependency of physiological systems, it is reasonable to suppose a priori that the special types of additivity as represented by strict concentration and response addition will be approximated only occasionally in the responses of whole organisms to mixtures of environmental toxicants. Furthermore, as mentioned earlier, the relative toxicity of a mixture depends on several factors which include the level of response (i.e., 10%, 50%, 90% response), the ratio of the toxicants in a mixture (i.e., 1:10, 1:100, 1:1000), and the nature of the response itself. It should be noted that the type of addition can only be described in relation to the response under consideration. With the same mixture of toxicants, different types of toxicant interaction might be expected for different responses (i.e., survival, growth, reproduction). However, these special types of toxicant interaction do provide a frame of reference for evaluating the effects of toxicant mixtures on whole organism performances.

Isobole diagrams are useful for visualizing the relationship between different types of toxicant interactions and for delineating the various factors which can influence the relative toxicity of multiple toxicants. However, in practice, isoboles are difficult to derive requiring a series of dose response curves for the mixture at different ratios of the component toxicants. Furthermore, there are no statistical criteria which might be used to distinguish between one form of interaction and another (Plackett and Hewlett, 1952). Following the procedures of Anderson and Weber (1977), we empirically studied the interaction of copper and nickel by deriving a dose response curve for the mixture at one fixed proportion. The dose response curve determined for the mixture was statistically compared to curves predicted on either the basis of concentration or response addition. This approach, utilized by Anderson and Weber (1977) for lethality studies, was adopted in the present study in order to test its applicability to graded response data.

EXPERIMENTAL STUDIES

LETHALITY STUDIES

Anderson and Weber (1977) conducted a series of 96-hour bioassays, studying the effects of copper, nickel and their mixture on the survival of male guppies. Statistical tests suggested that the individual dose response curves derived for copper and nickel were parallel. Based upon this observation, it was assumed that the mixture would be concentration additive. To test this prediction, Anderson performed experiments exposing test organisms to a series of mixtures of the two toxicants at a fixed proportion. A statistical comparison of the observed dose response curve to the regression equation calculated on the basis of equation (1) indicated that the assumption of concentration addition adequately described the joint toxicity of the mixture. Using a similar experimental procedure, he demonstrated that a mixture of copper and zinc was supra-additive relative to concentration addition. Further studies showed that separate binary mixtures of dieldrin and potassium cyanide and potassium pentachlorophenate and potassium cyanide were response additive.

GROWTH STUDIES

Growth was selected as the graded response for this study because it represents a performance of the integrated activities of the whole organism and as such is often a sensitive indicator of the suitability of the environment (Warren, 1971). Two of the ways environmental toxicants can affect the growth of an organism are: (1) alter its ability to assimilate and convert food material into body tissue, and/or (2) change its rate of food consumption. To determine the manner in which toxicants affect the growth of an organism, both processes were investigated separately. The methodological and statistical procedures along with the complete results of this study will be published at a later date; however, the results of a preliminary analysis of this data are discussed.

Juvenile guppies were fed daily a restricted ration of tubificid worms to determine the effect of the toxicants on the gross growth efficiency and relative growth rate (as defined by Warren, 1971) of the fish. The effect of the individual toxicants and their mixture on food consumption was investigated by feeding groups of fish an unrestricted ration and measuring the amount of worms consumed.

Statistical tests comparing the slopes of the individual dose response curves for copper and nickel derived for each response suggested that they were parallel. On the basis of the mathematical model for concentration addition, equations for the predicted dose response curves were calculated and statistically compared to the regression equations experimentally determined for the mixture. The results indicate that the effects of the toxicant mixture on the gross growth efficiency of the fish subjected to both the restricted and unrestricted feeding regimes are predictable on the basis of concentration addition. However, the dose response curves for the mixture representing the effects of the toxicants on the food consumption of the fish was supra-additive relative to the dose response curve predicted on the basis of concentration. Because of the relationship between growth, gross growth efficiency, and food consumption, the effects of the mixture on the relative growth rate are similar to the ones observed for gross growth efficiency at the restricted ration (concentration addition) and for food consumption at the unrestricted ration (supra-addition).

CONCLUSIONS

The results indicate that the assumption of concentration addition adequately predicts the effects of a copper-nickel mixture on both the survival and gross growth efficiency of guppies. The dose response curves for the mixture representing the effects of the toxicants on the food consumption of the fish was supra-additive relative to the dose response curve predicted on the basis of concentration addition. An explanation for the differences in these two responses to the mixture is beyond the scope of the present study. However, it is possible that the effects of the toxicants on the metabolic processes involved in the conversion of food material into body tissue might be somewhat different than their effects on the biological processes regulating the consumption of food.

In our studies we found that the mathematical model for concentration addition predicted the responses of guppies to both lethal and sublethal concentrations of a copper and nickel mixture. However, it should not be inferred from these results that the type of joint toxicity observed when organisms are subjected to high, rapidly lethal concentrations of mixtures will necessarily occur in cases where animals are subjected to low concentrations of the same toxicants. Furthermore, the nature of toxicant interaction can only be meaningfully described in relation to the particular response under consideration. For example, we found that mixtures of copper and nickel

were concentration additive in experiments evaluating their effects on the gross growth efficiency of the guppies; however, in the food consumption studies, the same mixture at similar concentrations produced a more toxic response than was predicted on the assumption of concentration addition.

To insure the success of a species in nature, it is necessary to evaluate the effects of potentially hazardous toxicant mixtures on the performances of whole organisms. The proposed approach provides a methodology for assessing the toxicity of mixtures of environmental toxicants at this level of biological organization. However, to offer explanations as to why mixtures of environmental toxicants interact in a particular manner requires knowledge of the effects of combined toxicants on underlying chemical processes and physiological functions. Such studies will be useful for evaluating the assumptions of the proposed approach and in suggesting other possible types of toxicant interaction.

ACKNOWLEDGEMENTS

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PAPER NO. 21

MULTITEST PROFILES OF ENVIRONMENTAL TOXICANTS

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INTRODUCTION

Potentially toxic, mutagenic, or teratogenic materials have been, and continue to be, discharged or spilled into terrestrial, aquatic, and aerial environments. Since environmental effects, including those on human health may be far removed from the sources of these discharges, cause-effect relationships between discharge and environmental effect have rarely been documented. This may be in part because some effects may be caused by low, almost immeasurable levels of toxicants or mutagens. It was assumed for many years that dilution and biogeochemical processes were able to render toxic substances harmless, and an occasional author reaffirms the infinite capacity of some environments, such as the ocean, to absorb the wastes of civilization. In the past, certain microbiologists claimed that given enough opportunity for recruitment of microorganisms, natural selection and the presence of favorable environmental conditions, any organic material could be biodegraded. Unfortunately, although this doctrine of "microbiological infallibility" cannot be disproved, it is now well known that numerous organic materials are recalcitrant to biodegradation. Some materials may even undergo initial biodegradative steps which release breakdown products more toxic than the parent compound. Emerging information on distribution of certain types of cancer suggests that these cancers may be related to geographically localized industrial outputs of carcinogens. Data on DDT and its related compounds form the most cogent basis for tracing and determining the effects of potential environmental toxicants.

Passage of the Toxic Substances Control Act of 1976 pointed out the popular and governmental interest in regulation of usage of potentially toxic chemicals and mixtures, and put a massive burden of testing into the hands of the EPA and the chemical manufacturers. As yet, no official criteria have been promulgated for testing of materials to ascertain their environmental impact. Those charged with environmental protection are faced with a bewildering variety of substances to be regulated. Manufacturers and users are apprehensive about the possibility of the need for extensive test and evaluation programs before new chemicals and mixtures may be brought to market.

In order to develop standards to be met for the discharge of materials into the environment, a good deal of basic information is required concerning parameters to be measured and the magnitude of changes in parameters which are significant. Obviously there is a multitude of potential effects measurable, considering the large numbers of species of plants and animals throughout the United States and its territories. On one hand, there are arguments that only those species of measurable importance to man be tested for toxic effects, while the opposing view holds that an effect on any given species is of importance. Certainly, these opposing views cannot be resolved in the present context. However, since a beginning must be made in decisions regarding environmental toxicology of potential waste effluents, we have begun to select bioassay tests and bacteriological procedures supported by analytical chemical expertise which evaluate the effects of potential wastes on lethal toxicity, biodegradation, mutagenesis, bioaccumulation, and reproduction of selected organisms. Although most of the testing deals with aquatic organisms, the results obtained are probably highly relevant to other organisms, including man.

Multiple test profiling, described below, can be carried out within a reasonably short period of time (30-60 days) to provide an initial estimate of environmental toxicity to the selected "indicator" species, and provide valuable insights into areas in need of detailed testing. Conversely, if results of the multiple testing are generally negative, little further work is required. The test profiles described below have arisen from the need to know the characteristics of ordnance and propellant compounds unique to Naval use, with particular regard to indirect impacts on man.

A battery of five general test areas has been established, supported by one or more bioassays as follows:

a. Lethal bioassays
b. Biodegradability and alterations
c. Mutagenesis
d. Bioaccumulation
e. Life history impacts.

Bioassays

Acute toxicity bioassays have been used as the lead test to develop effluent level guidelines on a preliminary basis. These assays include the algae bottle test (EPA, 1971), a *Daphnia* test (Crosby et al., 1966; APHA, 1975), a test using the marine littoral copepod *Tigriopus* similar to the *Daphnia* test, a test using oyster larvae (APHA, 1975) and a test using stickleback fish (EPA, 1975).

Both seawater and freshwater species of algae are used, and usually prove to be the most sensitive species to the toxicants. Oyster larvae represent test organisms of commercial importance. As generally prescribed (EPA, 1971), results obtained with the most sensitive species are taken as a basis for estimation of effluent guidelines. As seen in the enclosed evaluation sheet for picric acid, bioassays on algae, *Daphnia*, and the fish gave results in the same order of magnitude. Following the suggestion of Sprague (1970), a level of 0.01 of the most sensitive organisms' $LC_{50/96}$ is used to establish the effluent guideline. In the case of picric acid, the algae were the most sensitive, yielding a guideline which paralleled the California effluent standard for phenols (0.5 mg/ℓ, McKee and Wolf, 1971).

Biodegradability

Disappearance of organic chemical wastes from the environment occurs via spontaneous chemical reactions, photolysis, and biodegradation by bacteria and fungi. Toxic chemicals, when released untreated into municipal waste systems, may not be degraded and may even have deleterious effects on treatment processes. When released in effluent streams, toxic organic materials may persist due to lack of biodegradability, or may degrade to form breakdown products of higher toxicity which are more resistant to breakdown than the parent compounds. Intractable compounds may percolate through groundwaters and contaminate drinking water supplies.

Our laboratory testing for biodegradation is carried out by subjecting known amounts of test materials to different environmental regimes including aerobic and anaerobic conditions, with inocula of anaerobic sewage sludge, aerobic and anaerobic natural aquatic sediments, or other substrates suspected of containing microorganisms capable of degrading the material. The bacterial species which carried out the first step in the decomposition of picric acid was a strain of *Pseudomonas aeruginosa* obtained from sludge in TNT waste treatment ponds.

Results of biodegradability testing can forewarn of potential long term persistence of chemicals in the environment, provide information on potential biological treatment of wastes, and provide breakdown products for further testing in the multitest scheme. Picric acid was very difficult to biodegrade and has been known to contaminate groundwater supplies in the past, after passing through the soil.

Mutagenicity Testing

A basic mutagenicity test scheme is carried out to screen potential mutagenic compounds. The test, developed by Ames et al. (1973), is gaining wide acceptance as a screening test for carcinogenic and mutagenic agents, and is of relatively low cost and undemanding of special equipment. Since this test is not definitive, particularly using water insoluble compounds, substances that show positive results by the test should be additionally tested using mammalian systems. (Negative results in the test do not necessarily indicate lack of mutagenicity).

Picric acid and its breakdown product, picramic acid, were only slightly mutagenic by the Ames test, and this only after activation with mouse liver enzyme treatment.

Bioaccumulation

Although effluent levels may meet water quality standards as judged by acute toxic bioassay criteria, there remains the danger of low level scavenging and bioaccumulation through aquatic food webs, with incorporation of toxic or mutagenic substances into animal tissues. Such materials may harm the organisms themselves, perhaps interfering with valuable commercial resources. Biological accumulation of toxic substances may be of public health significance if the concentrating organisms are taken for human consumption.

We have incubated the freshwater clam *Corbicula* for 30 days in levels of Naval wastes judged safe by bioassay toxicity tests. Water containing the toxicants was replaced every three days, at which time the clams were fed

suspensions of microscopic algae (Selenastrum). In the case of picric acid, analytical methodology was only sensitive down to 500 ppm dry tissue weight; this level was not exceeded in the tissue. It was concluded that no major bioaccumulation of the picric acid occurred, particularly since no yellowing of the clam tissue was in evidence. Further research is needed to improve detection levels of picric acid in tissue. There are no standard methods available for bioaccumulation assay, and currently this testing falls within the realm of applied research.

Life Cycle Testing

Reproduction and development of organisms may be impaired by low levels of chemical toxicants in the environment. A salient example has been the specific case of eggshell thinning in birds caused by DDT and its residues. This type of assay is of importance in relation to specific organisms as well as to groups of organisms which may exert ecosystems effects, such as the phytoplankton.

Growth curves of algae in the bottle test (EPA, 1971) was one type of observation in our testing in this area; another was observation of the life cycle of *Tigriopus californicus*. *Tigriopus* testing was of great interest, as previously described for tests on heavy metal effects (D'Agostino and Finney, 1974). The life cycle of *Tigriopus* was observed over a 30 day period in which this organism goes from egg to adult through five larval stages. At suggested effluent levels of picric acid there was apparently no impairment of egg development, formation of larval instar stages, and development of fecund adults. This type of research is also in its early stages, but has proven to be a valuable tool in our testing program.

ENVIRONMENTAL TOXICOLOGICAL EVALUATION

Material tested picric acid

Analytical Methods: gas chromatography, thin layer chromatography

Chemical composition (2,4,6, trinitrophenol)

1. **Bioassay testing:**

Organisms	$TC_{50/96}$	Recommended effluent levels
S. capricornutum (alga)	50 ppm	0.5 mg/1 (Same as California standard
Daphnia sp.	65 ppm	for phenols
Stickleback (fish)	75 ppm	

2. **Biodegradability testing:**

Methods: Laboratory fermenter/anaerobic /aerobic Mineral salts medium, seeded with TNT pond mud.

(numerous inocula from natural substrates tested with no effect)

Results: biodegradation with difficulty in presence of Pseudomonas aeruginosa to yield picramic acid (2,4 dinitrophenol), anaerobic conditions.

Remarks: probably a persistent environmental contaminant

3. **Mutagenicity testing:**

Test: Ames', using 4 tester strains picric acid and picramic acid showed weak activity, only with mammalian enzyme activation.

Remarks: Suspected mammalian mutagen, mammalian tests recommended.

4. **Bioaccumulation and chronic effects:**

Species: Corbicula, sp. (freshwater clam).

Methods: Incubation in aquarium water containing 0.5 mg/1 picric acid

Results: No apparent concentration, no yellowing of tissue.

Remarks: (detection limit for tissue is only 500 ppm)

5. **Life cycle tests:**

Species: Selenastrum, Tigriopus (marine littoral copepod)

Methods: Laboratory culture in effluent levels.

Results: No interference with life cycles.

SUMMARY RECOMMENDATIONS: Comparatively non-toxic material, adherence to established guidelines for phenol recommended.

Analytical Chemical Support

The multitest profiling system is currently only within the realm of the well equipped research laboratory, requiring highly adaptive analytical chemical support for biodegradability work (isolation and identification of breakdown products), and in bioaccumulation studies (recovery of test chemicals from tissues). New methods and new applications of established methods are routinely required. With the continued appearance of new chemical compounds and mixtures potentially released, multitest profiling of environmental toxicants will probably always require close cooperation between the analytical chemist and biologists who carry out various test procedures.

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PAPER NO. 22

PREDICTIONS OF THE CHRONIC FISH TOXICITY OF TEST MATERIALS USING DAPHNIA MAGNA

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INTRODUCTION

The use of laboratory toxicity tests with aquatic species is the backbone of current efforts to develop aquatic safety assessments and establish water quality criteria for new chemicals and materials that could potentially reach the aquatic environment. In constructing laboratory dose/response relationships for aquatic species, one of the more important testing end points has become the determination of the maximum acceptable toxicant concentration (MATC) originally proposed by Mount and Stephan (1967) and defined as that test concentration at which no effects were observed on survival, growth, or any of the reproductive parameters examined during the full life-cycle exposure.

This testing concept was originally developed with the fathead minnow, *Pimephales promelas*, and subsequent investigators have come to rely heavily on the fathead as the species of choice for chronic toxicity tests. Its advantages are generally listed as relatively short life cycle (10-12 months), readiness to spawn within the laboratory, and ease of handling eggs and fry. However, these same features can also be listed as disadvantages, depending on one's frame of reference. For example, the Cladoceran, *Daphnia magna* offers an extremely short life cycle, reaching reproductive maturity within only 8 days. The species is then extremely prolific, producing as many as 20 to 30 young per day from a single mature individual. Life cycles and reproductive parameters are extremely consistent between individuals, creating a good statistical basis for description of effects. The additional advantages of small water volumes and miniaturized testing apparatus make this invertebrate an extremely desirable test species for aquatic toxicologists.

Daphnia magna has been recognized for many years as a useful test species (Anderson, 1944), and their sensitivity to chemical substances has been recognized as generally representative of other widely distributed zooplankton species (Anderson et al., 1948). Information extending these comparisons between *Daphnia* MATC values and those reported for fish is limited to a series of five metal ions (Beisinger and Christensen, 1972), indicating that estimated full life cycle no-effect concentrations for Cr^{++}, Cu^{++}, and Zn^{++} to daphnids were similar to those for gammarids and fish, even though different metal salts, water hardness, and dilution water quality were used. However, Ni^{++} and Cd^{++} toxicity was found to be greater to *D. magna* in soft water than to fathead minnows in hard water, and comparative assays in waters of similar quality are not available.

The objectives of this investigation were twofold: (a) to develop a laboratory procedure for the continuous-flow toxicity testing of *Daphnia* that would allow the maintenance of expected nominal concentrations of highly degradable surfactants throughout the 21-day chronic test; and (b) to develop a data base for several representative surfactant materials to serve as a comparison of observed *Daphnia* effect concentrations with existing chronic values for fish species.

MATERIALS AND METHODS

TEST SYSTEM

With respect to the initial objective, a system has been developed and evaluated in our laboratory which provides for the delivery of one chamber volume every 2.5 hours or less with no perceptible current-induced effects on the test individuals and allows the automatic introduction of known concentrations of food at each diluter cycle (Maki, 1977).

The test system employs a standard 0.5 liter proportional diluter (Mount and Brungs, 1967), the operation of which has been published in numerous locations (Jackson, 1973) and the description of operation, therefore, is here limited to specific modifications.

The system operation is controlled by a 60 minute automatic reset interval timer, Dayton No. 2E335. The timer is set for a 20 minute interval and will cycle the water and food supply to the diluter accordingly when the double-pole double throw (DPDT) switch is in the automatic mode. The normally closed solenoid (Asco Co. 0.64 cm orifice, stainless steel) is energized, allowing water flow to the diluter. Simultaneously, a magnetic stirring motor is mixing a one-liter flask of food

suspension and a six-channel peristaltic pump (Manostat Cassette Pump) is adding a 5 mg/liter concentration of food from the flask to each mixing cell of the diluter. The water and food flow continue until the last cell (W-6) empties to a small container which depresses the normally closed microswitch (Micro Switch BZ-2RW80-A2), thus closing the water supply solenoid and terminating food addition.

The diluter delivers the predetermined concentrations of test material to the mixing cells where the newly added food is mixed and the cell contents drained through the siphon tube to the flow-splitter box (Figure 1). The flow-splitter box, constructed of glass (35 x 7.5 x 2.5 cm) on a 10 x 40 cm glass base, receives the 0.5 liter volume from the mixing cell and delivers 125 ml to each of four replicate test chambers. The flow splitting box is drained through 2.5 cm sections of 1 mm O.D. capillary tubing to restrict rate of flow to the test chambers, which are 1-liter beakers with a 2.5 x 5 cm notch covered with a portion of 60 mesh stainless steel screen to prevent escape of newly-produced young. Daily cleaning of the capillary tube drains with a section of stainless steel wire will insure equal flow distributions among the four replicates. The food material consists of a 100 mg/liter suspension of ground trout chow and alfalfa after Biesinger and Christensen (1972) and is renewed daily. Dilution water used for all *Daphnia* tests was carbon and reverse osmosis filtered well water.

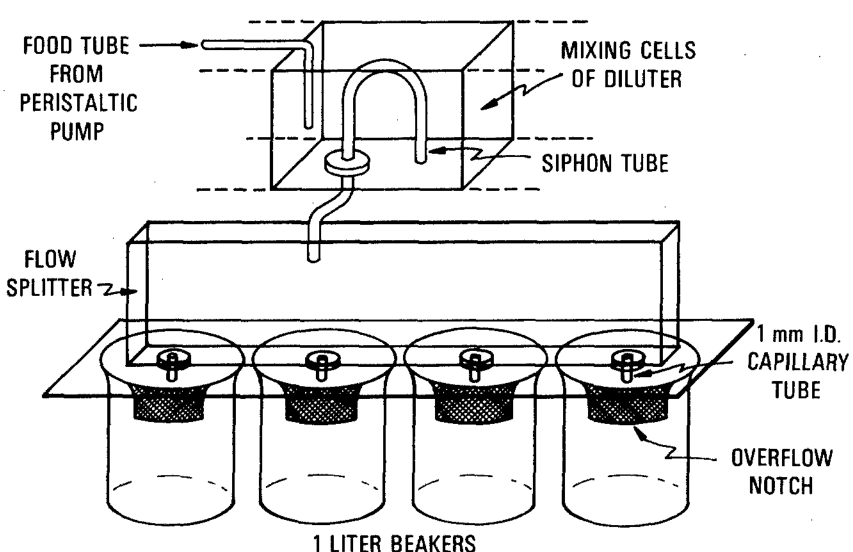

Figure 1. Flow splitting device operated in conjunction with a proportional diluter for the continuous-flow toxicity testing of *Daphnia magna*.

The control switch is provided with a manual setting which overrides the timer, allowing water and food to flow continuously for initial calibration of the diluter. In the off mode, no power is supplied to the timer and the system is inoperative. Dye tests have demonstrated excellent mixing of solutions with minimal "short-circuiting" of water flow through the overflow notch of the test beaker. The system routinely produces a mean daily brood size of 10-12 young per adult over the 14 day test period of maturity.

TEST CONDUCT

The test was set up with young Daphnia less than 12 hours old. The young were siphoned from culture units and randomly assigned to test containers. Five young were used in each of the four replicates, thus yielding 20 F_0 individuals per concentration. All test beakers were filled with the appropriate concentrations of test material by manually operating the diluter through consecutive cycles prior to adding young. Initial F_0 mortality is recorded after 24 hours, 96 hours, 7 days, and thereafter on a daily basis. On days 7 and 8, the individuals begin to produce the second generation and daily counts of young are made. All young produced are discarded following this daily count. Records were kept of the total number of F_0 surviving at each observation day, the total number of F_1 young produced by the F_0 individuals after reaching reproductive maturity, the mean brood size as the quotient of F_1/F_0, and the percentage of days that young were produced within each replicate beaker of all five test concentrations and controls.

Initial readings of pH, temperature, and dissolved oxygen were taken immediately after inoculation. Also at this time, a water sample was taken from the beaker and preserved with either 1% formaldehyde for anionic surfactants or H_2SO_4 to pH 2 for nonionics and kept at 5 C prior to analytical confirmation of the nominal test concentrations. A continuously reading probe provided a constant record of water temperature throughout the test. All tests were run at 21 $\pm$ 1 C and additional analyses of test material, pH, and dissolved oxygen were taken at intervals of 3 to 5 days for the remainder of the 21-day test.

Four anionic surfactants: $C_{11.8}$ LAS, C_{13} LAS, an alkyl ethoxylate sulfonate, and an amine oxide; two nonionics: C_{12-13} and C_{14-15} alkyl ethoxylates; and a detergent builder, type A zeolite, were selected for testing on the basis of previously existing full or partial chronic test data for the fathead minnow, Pimephales promelas. All test materials used in these investigations are listed and characterized in Table 1.

TABLE 1. CHEMICAL CHARACTERIZATION OF THE TEST SURFACTANTS AND SINGLE BUILDER. GENERIC NAMES ARE USED THROUGHOUT TEXT TO REFER TO THE TEST MATERIALS

Generic Name	Structure	Chemical Characterization
$C_{11.8}$ LAS	$CH_3-(CH_2)_X-CH_3$ with phenyl ring bearing SO_3Na^+	Anionic, mean alkyl chainlength = 11.8, range C_{10-14}; mean phenyl position = 3.76; mean molecular weight = 345
C_{13} LAS	(same structure)	Anionic, mean alkyl chainlength = 13.3, range C_{10-14}; mean phenyl position = 4.5; mean molecular weight = 367
Alkyl Ethoxylate Sulfate	$CH_3-(CH_2)_X-O(C_2H_4O)_YSO_3Na$	Anionic, X = 13.67; range = 13-15 Y = 2.25; molecular weight = 425
$C_{14.5}$ Alkyl Ethoxylate	$CH_3-(CH_2)_X-(C_2H_4O)_YH$	X = 7 to 19, mean = 13.5 Y = 0 to 12, mean = 7.0 Nonionic
$C_{12.5}$ Alkyl Ethoxylate	(same structure)	X = 7 to 19, mean = 11.5 Y = 0 to 12, mean = 6.5
Amine Oxide	$CH_3-(CH_2)_X-N^+(CH_3)_2-O^-$	Nonionic, alkyl dimethyl amine oxide Molecular weight = 243 X = mean 11.7
NTA	$Na-O-C-CH_2-N(CH_2-C(=O)-O-Na)_2$	Builder, trisodium nitrilotriacetate Molecular weight = 257
Type A Zeolite	$Na_{12}[(AlO_2)_{12}(SiO_2)_{12}]\cdot 27HOH$	Mean particle size = 3μ Al:Si molar ratio = 1:1

Water samples were analyzed by MBAS (APHA, 1977) for anionic surfactants and CTAS (Boyer et al., 1977) for non-ionics to confirm expected nominal test concentrations. Type A zeolite was analyzed by the silicate method (APHA, 1977).

STATISTICAL ANALYSIS

All results were calculated with a computerized probit analysis program (Finney, 1964 and 1971). Survival data for 96 hours and 21 days were used to calculate LC_{50} values and associated 95% confidence intervals. Data for the three measurements of reproductive inhibition were converted to percent effects considering unexposed controls for each test as 0% effects. Respective EC_{50} values denoting the test concentration where a 50% reproductive inhibition was observed were also calculated with the probit analysis program. All test results were calculated based on mean measured concentrations of test material taken from the test containers during the 21-day test period.

RESULTS

An example of the type of chronic data generated during a typical 21-day *Daphnia* test is summarized in Table 2. These data for the nonionic surfactant, C_{12-13} alkyl ethoxylate, indicate that 21-day survival of initial F_0 individuals is significantly reduced at concentrations in excess of 0.4 mg/liter. Measurements of reproductive inhibition indicate that the cumulative total young production and the mean daily brood size are both significantly reduced at concentrations in excess of 0.24 mg/liter. The percentage of days that reproduction occurred within each test concentration relative to controls was not affected until mean concentrations of the surfactant exceeded 0.81 mg/liter, indicating that this measurement is apparently a less sensitive indicator of chronic toxicity than are the other measurements of total young production and mean brood size.

TABLE 2. A SUMMARY OF TYPICAL SURVIVAL AND REPRODUCTIVE PERFORMANCE DATA FOR POPULATIONS OF *DAPHNIA MAGNA* EXPOSED TO THE NONIONIC SURFACTANT C_{12}-C_{13} ALKYL ETHOXYLATE.

NUMBER OF INDIVIDUALS SURVIVING

	Test Concentrations (mg/l)					
Day of Test	Control	0.15	0.24	0.4	0.81	1.45
0	20	20	20	20	20	20
1	20	18	20	19	20	16
2	19	18	17	18	19	7
4	19	18	17	16	18	3
5	19	17	17	16	17	1
7	19	17	17	16	15	0
9	19	17	17	16	15	0
12[1]	19	17	17	16	15	0
14	19	17	17	16	15	0
16	18	17	17	16	15	0
19	18	15	16	16	14	0
21	18	15	16	16	14	0

TOTAL YOUNG PRODUCTION

	Test Concentrations (mg/l)					
Day of Test	Control	0.15	0.24	0.4	0.81	1.45
9	42	117	129	58	5	0
12	174	222	282	92	0	0
14	134	156	98	117	0	0
16	451	434	383	166	0	0
19	771	434	683	436	0	0
21	238	209	249	436	0	0
TOTALS:	1810	1572	1779	1305	5	0

MEAN BROOD SIZE

	Test Concentrations (mg/l)					
Day of Test	Control	0.15	0.24	0.4	0.81	1.45
9	2.6	6.9	7.6	3.6	.03	0
12	10.9	13.1	16.6	5.8	0	0
14	8.3	9.2	5.8	7.3	0	0
16	28.2	25.5	22.5	10.4	0	0
19	48.2	28.9	39.9	27.3	0	0
21	15.9	13.9	15.6	27.3	0	0
Grand Means	19.0	16.3	18.0	13.6	$\sim$0	0

PERCENTAGE OF DAYS REPRODUCTION OCCURRED

	Test Concentrations (mg/l)					
Replicate	Control	0.15	0.24	0.4	0.81	1.45
A	83	83	67	100	17	0
B	83	100	67	100	17	0
C	83	83	83	83	0	0
D	83	100	100	83	0	0
Means:	83	91.5	79.3	91.5	8.5	0

A summary of the calculated 50% effect levels for survival and reproduction of Daphnia populations under continuous flow exposures to the six surfactants and one builder demonstrates similarities in responses to these materials (Table 3).

TABLE 3. THE CONCENTRATIONS IN MG/L OF SEVEN SURFACTANTS AND A DETERGENT BUILDER THAT AFFECT THE SURVIVAL, GROWTH AND REPRODUCTION OF DAPHNIA MAGNA UNDER CONTINUOUS FLOW TESTING CONDITIONS

Material	96 hr. LC50	21 Day LC50	Reproductive Inhibition EC50 (mg/l)[1] Total Young Production	Average Brood Size	Percentage of Days Reproduction Occurred
$C_{11.8}$LAS	3.94 (2.87 - 6.83)	1.67 (1.28 - 2.18)	1.50 (0.75 - 3.33)	2.30 (1.62 - 3.55)	2.31 (0.30 - 4.18)
C_{13}LAS	2.19 (1.85 - 2.82)	1.17 (0.96 - 1.39)	1.11 (1.03 - 1.18)	1.41 (1.33 - 1.50)	1.29 (1.21 - 1.37)
C_{12-13} Alkyl Ethoxylate	1.14 (0.96 - 1.31)	0.93 (0.75 - 1.13)	0.46 (0.43 - 0.53)	0.47 (0.44 - 0.53)	0.70 (0.51 - 0.76)
C_{14-15} Alkyl Ethoxylate	0.43 (0.37 - 0.51)	0.37 (0.32 - 0.44)	0.28 (0.27 - 0.29)	0.29 (0.28 - 0.30)	0.33 (0.30 - 0.36)
Alkyl Ethoxylate Sulfate	1.17 (0.82 - 1.66)	0.74 (0.56 - 0.94)	0.37 (0.22 - 0.54)	0.40 (0.23 - 0.60)	0.52 (0.31 - 0.76)
Amine Oxide	1.01 (0.85 - 1.21)	0.96 (0.90 - 1.03)	0.88 (0.77 - 1.04)	1.01 (0.95 - 1.07)	1.04 (0.98 - 1.11)
Type-A Zeolite	377.17 (315.30-464.83)	214.63 (174.27-256.17)	211.29 (177.89-255.66)	340.97 (281.79-412.57)	312.70 (272.89-369.04)

[1]Figures in parentheses denote confidence limits for p = 0.05

A comparison of acute effect data for 96 hours and 21 days demonstrates the expected directional increase in toxicity following the longer exposure period. However, the magnitude of this difference is not great for the majority of these materials, since the 95% confidence limits overlap for four of the compounds, thus indicating that cumulative toxicity is apparently not significant for surfactants. A comparison of the 21-day LC_{50} with reproductive inhibition data indicates that the 21-day LC_{50} yields a close approximation of the chronic effects indicated from the reproductive data.

An examination of the reproductive inhibition data demonstrates that the total number of young produced by *Daphnia magna* is the most sensitive indicator of sublethal or chronic toxicity. The EC_{50} values for total young production are uniformly the lowest effect concentrations measured for all seven test compounds. The average brood size, calculated as the number of young removed daily from each test container divided by the total number of adults present, yields EC_{50} values slightly higher than total young production and generally wider confidence intervals. This is due to the high variability in the day to day production of young from individual *Daphnia*. Whereas total young production integrates all young produced during the test to a single value, the measurement of daily mean brood size has a considerably higher variance, thus yielding higher effect levels and more variability.

Similarly, the measurement of the percentage of days that reproduction occurred, defined as the relative percent or frequency of days that young production was observed within the beaker compared to controls, yields still higher effect levels, due to the individual variance in brood production. On any given day, it is likely that at least one of the remaining test adults per container will produce a brood of young which is then recorded as a day upon which reproduction occurred. Thus, the EC_{50} values calculated for this parameter are generally higher than the other measurements of reproductive inhibition.

All 50% effect levels for both survival and reproductive inhibition are lower for C_{13} LAS than for $C_{11.8}$ LAS, indicating that the addition of two alkyl carbons creates an approximate 50% increase in toxicity. A similar comparison of test results between C_{12-13} and C_{14-15} alkyl ethoxylates demonstrates the longer chain length compound exerts a greater toxic effect for all life history parameters measured. The chronically toxic concentrations of the alkyl ethoxylate sulfate and amine oxide are comparable to the effects observed for C_{12-13} alkyl ethoxylate with the exception that the concentrations of amine oxide producing inhibitory effects on reproduction are significantly higher than the alkyl ethoxylates. Data for the builder, type A zeolite, indicate that suspensions of this insoluble compound are relatively nontoxic to daphnids at concentrations below 200 mg/liter.

DISCUSSION

The test results demonstrate that *Daphnia magna* is a sensitive indicator of chronic toxicity for various surfactants and the detergent builder, type A zeolite. A measurement of survival and total number of young produced following a continuous 21-day exposure to the test materials yields an accurate estimate of survival potential and reproductive inhibition. From these test results, it is possible to report MATC values for *Daphnia* based on 21-day no-effect concentrations. These values are then compared to MATC values for the fathead minnow, *P. promelas* (Table 4). A close agreement between these two columns is obtained for the seven test materials. A regression analysis of this relationship yields a correlation coefficient (r) of 0.96, indicating a strong relation exists (Figure 2). The straight line equation of this line is:

$$\ln(\text{Fhm MATC}) = .814 + 1.062 \ln(\textit{Daphnia}\ \text{MATC})$$

$$R^2 = .96$$

where ln(Fhm MATC) = ln of fathead minnow MATC value,

ln(*Daphnia* MATC) = ln of *Daphnia* MATC value.

TABLE 4. COMPARISON OF CHRONIC TOXICITY VALUES (MATC) FOR DAPHNIA AND FATHEAD MINNOWS FOR SEVERAL SURFACTANTS AND TWO DETERGENT BUILDERS

Test Material	MATC Values (mg/l)	
	Daphnia	Fathead Minnow
$C_{11.8}$ LAS	1.18	.90
C_{13} LAS	.57	.15
C_{12}-C_{13} Alkyl Ethoxylate	.24	.32
C_{14}-C_{15} Alkyl Ethoxylate	.24	.23
Alkyl Ethoxylate Sulfate	.27	.10
Amine Oxide	.70	.50
Type-A Zeolite	200.0	275.0
NTA	100.0[1]	75.0[2]

[1]Biesinger et al., 1974.
[2]Arthur et al., 1974.

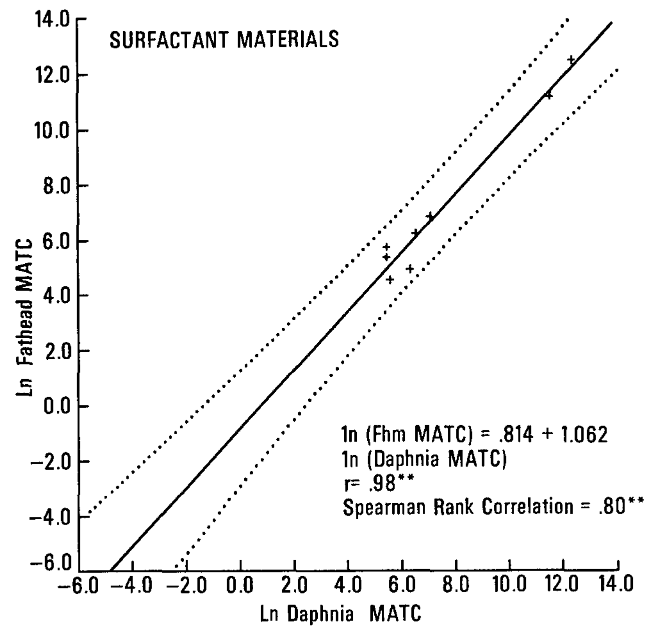

Figure 2. Regression equation relating Daphnia and fathead minnow MATC (Maximum Acceptable Toxicant Concentration) values.

Thus, by employing this equation, it is theoretically possible to calculate long-term chronic MATC values for the fathead minnow given the MATC value for Daphnia derived from a 21-day chronic exposure. It is important to emphasize that, in the present context, the method does not yield data for the assessment of effects of actual environmental forms of these test materials, since significant environmental biodegradation is known to substantially reduce the acute and chronic toxicity of the parent molecule. Although the relationship is based on only eight test compounds mostly of a similar nature, the relatively good agreement between chronic MATC values for these two species is encouraging. The development of chronic fish toxicity data currently represents a major obstacle for research personnel attempting to determine the relative hazard to aquatic life associated with the use of a new compound. If the cost and time commitment necessary to derive a chronically "safe" concentration to a fish population (i.e., MATC value) can be significantly reduced through the use of predictive correlations employing Daphnia as a representative of a lower aquatic trophic level, then it may be possible to develop chronic data for a wider variety of compounds for which previous chronic data were economically unjustifiable based on a low interest or low usage levels.

The correlation between Daphnia and fish toxicity values also serves to demonstrate that aquatic safety data developed for the protection of one trophic level may similarly extend to include representatives of a higher level.

CONCLUSIONS

1. A system was developed and evaluated for the chronic toxicity testing of Daphnia magna under continuous flow conditions. The system has advantages over existing methods by providing for the delivery of a test chamber volume every 2.5 hours with no perceptible current-induced effects on the test individuals.

2. The freshwater macroinvertebrate, Daphnia magna is a sensitive indicator of the toxic effects of surfactants. Reproductive inhibition calculated from the basis of young produced is the most sensitive indicator of sub-lethal toxicity.

3. Observed MATC values for Daphnia magna demonstrate a correlation coefficient, $r = 0.96$, with MATC values reported for the fathead minnow, P. promelas, for seven detergent components.

4. The relatively short life cycle and 21-day duration of the test, small water volumes, ease in handling, and high fecundity make Daphnia an attractive alternative to the conduct of longer-term chronic fish tests.

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OPEN FORUM

MR. WANDS (National Academy of Sciences): Dr. Maki, you have an overall correlation of .76 and correlations within those 35 tests ranging from .99 on down to something fairly low. Are there any significant outlying values? Are there any false negatives or positives here that would be of real environmental significance? Are we overlooking any important effect?

DR. MAKI (Proctor & Gamble Company): Well, by splitting out the pesticides as a general class of materials, we see that individual class has probably the highest percentage of outliers and that certainly reflects the large variety of chemical formulations and modes of action. We do have outliers in that correlation, which I believe was .22, giving a fairly good indication that we must be cautious about generalizing when developing pesticide information. *Daphnia* does not appear to be a good predictor of fish toxicity numbers for the general class of pesticides. By and large for the other materials, surfactants, metals and PCB isomers, the overall relationship between *Daphnia* tests and fish toxicity is encouraging.

MR. HASS (Argonne National Laboratory): I have two questions, one to Dr. London and one to the panel if I may. Dr. London, can your system be used in the field? Have you ever tried a field test with it? The question to the panel: Have you made any tests with compounds that are not water soluble? How do you get around this particular problem?

DR. LONDON (6570 Aerospace Medical Research Laboratory): The system is definitely not for field testing. One may attempt to duplicate field conditions such as an activated sludge system or something like that. The system is amenable to all kinds of manipulations, but it is not a field system.

MR. HASS: You have to sterilize it first, don't you?

DR. LONDON: One need not. Not, for example, if one wanted to run it as a activated sludge type of system. An activated sludge system is a kind of continuous culture receiving its feed in a back phase though. I will attempt to answer your second question. There are some ways that one can test a nonwater soluble material in a system like ours. Some people have made a water extract by one means or another, analyzed the extract in the aqueous phase and

then done a dose response based upon dilutions of the aqueous extract. It is very difficult to dump a mixture of non-soluble and soluble materials into any system because the material falls. Fish will actually eat some of the material and you have no control on the dosage that you are working with.

DR. MOUNT (U.S. Environmental Protection Agency): I might add, as a point of information, that there are at least two approaches in the published literature to that question, one for materials that are solid at room temperature and another for ones that are liquid at room temperature that involve the water saturation approach. I'd like to raise one question for you along the same line. I gather that your system also would not work for any waste material that had an inherent turbidity in it since that's what you're measuring as the output of the chemostat. Is that correct?

DR. LONDON: If one could get a zero time, a baseline turbidity, and then look for a change from that point which is the equilibrium state of the culture, it could be done as long as the turbidity of the feed remained constant. You can always see a change.

MR. CHRISTIAN (University of Cincinnati): Perhaps a comment on some of the mixture studies. We have been working with the mammalian cell bioassay system which follows many of the same parameters that we've discussed today. We use human and other mammalian cells. We have used this system to work on municipal and direct reuse water for the Army. We were quite interested, as you have been, in mixtures. We thought that perhaps we'd learn several things. We did see competitive inhibition in the case of zinc and cadmium. We found an additive effect with organics such as hydroquinone and dinitrobenzene. And when six phenolic compounds were mixed together, we found that the total toxicity was less than the toxicity of the most toxic substance. To date, we have not seen a clear cut synergistic or superadditive effect and we are a little bit more confident that two compounds being mixed together does not become so much more toxic. It doesn't seem to be a usual case, at least.

DR. WEBER (Oregon State University): In acquiring our quantal data, we did use up to four different compounds at the same time in our predictive model. Zinc and nickel together were highly superadditive in terms of lethality so I think superaddition is possible. Certainly pharmacologically we see synergism. Pharmacologically, we think of synergism as a situation where one of the compounds in fact

often doesn't elicit any response towards what we're looking at but will potentiate or cause a synergistic effect with the other material. One of the reasons I avoided using terms like synergism and potentiation is because they have such wide definitions.

LT. COL. TAYLOR (USAF Environmental Health Laboratory): My question is for you, Dr. Mount. You very casually dropped a bomb earlier when you said that biological monitoring may very well become a requirement for all discharge permits. I'd certainly like for you to expand on that and let me know what your crystal ball says on what these requirements are going to be and what kind of a time frame we're looking at.

DR. MOUNT (U.S. Environmental Protection Agency): I'll answer to the best of my ability. If it were next Thursday or Friday, I could probably do better. I think there is going to be almost certainly a very simple test required. I would guess it's going to be initially based on lethality. I think you are going to see it begin in the second round of permits which starts this year for many. I think my crystal ball would also suggest that as the third round of permits comes, it's going to be a much more complex testing requirement. I say it without any hesitancy, not with certainty that it's going to happen, but with optimism that it ought to happen. If we are indeed supposed to regulate the discharge of toxic materials and toxic amounts as required in the regulations, I don't know how else we're going to measure whether or not they are toxic unless we start measuring toxicity. I think it's high time that we quit depending on electronic analytical instruments to tell us what only an organism can tell us. I look at biological monitoring as being a very realistic move, a move which is going to be an order of magnitude or more cost effective than mass spectral analysis which is one of the alternate ways to go. As you may well have guessed, I have been an advocate of this approach and perhaps can be held responsible for the timing of it. My conversations with industrial people who have to live with this sort of regulation seem to indicate that they are favorable, that they feel this at least is going to be a meaningful test. Obviously, if misused or asked to do things that it can't do, then we're in trouble.

DR. LONDON: I have a question for Dr. Maki. What about the use of a similar system as *Artemia* for salt water testing of pollution? Have you done anything like that? Has anyone made the same kind of pollution effect analyses using *Artemia* and various types of marine fish?

DR. MAKI: We have done some studies on surfactants with marine species. We haven't used _Artemia salina_ but most of our work to date has involved commercially imported species. I'm talking about the blue crab, the pink shrimp, and the eastern oyster which are the three invertebrate species we study. We have done some work with fungilus, a top minnow estuarian species. The work that we've done to date with representative surfactants of various classes indicates that the susceptibility of these species is comparable to fresh water species. The blue crab seems to be able to tolerate levels of test materials considerably in excess of what we see for fresh water species. The data base is limited, however, to a few materials that we've done inhouse.

DR. WEBER: I had a similar question for Dr. Maki. I was wondering if you looked at any fast running water fish such as trout. Do you have any of that kind of data?

DR. MAKI: The salmon is typical of numerous other species. They are indeed slightly more susceptible in most cases. If you do statistical analyses of the data, you will find that LC_{50}'s are lower for salmon than for most warm water species. But confidence limits may, in most cases, overlap. There's not a great difference between salmon and representative warm water species. We are basically concerned with the blue gill as an intermediately susceptible species, representative of warm water environments such as receiving water areas that may be under the influence of domestic sewage effluent. This is where a biodegraded surfactant may ultimately reach surface water in communities. These typically are not trout streams or areas where you find salmon so we have emphasized basing our safety criteria on the temperate species.

DR. MOUNT: I might ask a question here of the panel or of the audience. Given an R^2 value of .22 for a group of compounds which we could categorize as synthetic organic chemicals but not necessarily all halogenated, I wonder how many people here would be comfortable with a regulatory system based on a test system in which that was the kind of correlation coefficient? I don't ask this in a frivolous or leading way. I just wonder what kind of confidence people who are on the other side of the fence than the regulatory agencies would place on that sort of statistic.

DR. CAIRNS (Virginia Polytechnic Institute and State University): It seems reasonable to me to use Dr. Maki's approach for those groups of chemicals for which the correlation is high and find another approach for the more dissimilar classes of compounds such as pesticides.

LT. COL. TAYLOR: We do a fair amount of this kind of work in our laboratories in San Antonio. Unfortunately, the things that we look at are perhaps a bit exotic but I don't believe you can simply look at each individual compound and have any way of predicting what the comparative lethality will be for Daphnia or fathead minnows or other species. It varies widely depending on the individual compound. I think perhaps in the area of pesticides you could have some confidence that they would not be similar. For any given compound that we have looked at, I think it would be very difficult to extrapolate from Daphnia and predict what the LC_{50} would be for the fathead minnow.

MR. WANDS: I'd like to add to Col Taylor's comment a little something out of the background of mammalian toxicology. It is very difficult to categorize chemicals and to say that all alcohols have a toxicity of this nature, and all acids, or all chlorinated aromatics fall in the same individual ballpark. There are, within any such broad category, extremely wide variations. That's the point I was seeking to make a few moments ago with Dr. Maki about the outliers. We know, for example, in alcohols that ethyl alcohol can be ingested in fairly large quantities with some impunity whereas similar quantities of methyl alcohol or butyl alcohol are much more toxic. That's just within one simple category. Similarly, you will find wide variations with any other category of organic compound or even with inorganic compounds unless the common feature happens to be a common ion or an interconversion as in the mercury to methyl mercury situation. It's a rather tenuous situation to attempt to find correlation within categories. I would approach it with a great deal of caution and look upon the concept of correlation and categorization of chemicals either for mammalian or nonmammalian species or plant life as a very, very first screening step for further follow up as described in Dr. DeSalvo's paper.

DR. MAKI: I'd like to make a comment here regarding the economic aspects of the comparison. Initially, the development of data for the intermediate trophic levels is strictly important in any integrated hazardous assessment scheme. It is certainly important to consider some of the fish food organisms. It makes little sense to develop protection criteria based solely on fish if we are going to be affecting fish food species at some lower level, so we should at least consider the intermediate trophic levels in any safety assessment program. Secondly, use of these invertebrates may provide us a method whereby we can at least get

a ballpark estimate for a chronic toxicity number earlier in a hazard assessment program. It may also allow us to obtain some predictive data for materials in cases where it was previously economically unjustifiable to run a full life cycle chronic test of one year duration. Perhaps we can develop information for more materials using at least a shorter term test. It gives us at least an estimate of chronic effect levels on materials in surface waters recognizing in some cases that the correlation is not as high as we would like to have. It's at least another bit of information to be added to an integrated or sequential hazard assessment program.

MR. WANDS: I'd like to ask a question of Dr. Cairns, please. I understood you to be talking about a basic biological concept of the assimilative capacity of an individual organism or a collection of identical species, or a mixture of species as in a total ecosystem. What you talked about were essentially nonmammalian aquatic type species. Do you feel that, since this is a basic biologic concept, it is also applicable to the mammalian, to plants or to other systems within the total phylogenetic spectrum as a common feature that man, too, has a total assimilative capacity?

DR. CAIRNS: I worry about thinking about a single species and applying this concept. The reason I used the McArthur-Wilson equilibrium model was to show that part of the dynamics of the system is the invasion pressure which will insure that a species that has no assimilative capacity will be replaced by one with a similar function in the system but perhaps slightly different tolerances. I have wondered about this not only in terms of mammalian species but in terms of rare and endangered species. I guess what you're getting at is a thing that has bothered me and that is where do rare and endangered species fit into the system I espouse. They don't fit in at all. I think it requires an entirely different strategy to protect individual species as opposed to protecting the integrity of a complex system. I suppose that the two concepts are partly in conflict because I'm assuming there will be sufficient invasion pressure for the system to come into a new equilibrium situation that really doesn't depend on the presence of a single species. When we come to things like the Tennessee Snail Darter or other such species, there are problems in strategy which may be in direct conflict with the other approach. Another area that I was going to explore originally and which I think a group like this should at least someday consider is a means of estimating recovery. Our research group is trying to do this in a very crude way. What we need to do is also develop a risk analysis that will assume that every now and then a serious mistake will be made like the kepone episode and work out ways of predicting the

recovery of the entire system to something approaching its original structure and function. That's something badly needed and in the very, very early stages of development.

MR. WANDS: Thank you, Dr. Cairns. I was concerned along the lines that you were. Man may well be an endangered species particularly as we do so much of our mammalian testing in rodents which are generally much more resistant to these materials. That's why we use safety factors in arriving at safe exposures for man. I just wanted to bring out the fact that your concept of assimilative capacity is a broad spectrum concept. It's a basic biological concept which must be taken into account as we deal with standards. Certainly these perturbations that flair up may be localized but not always.

DR. HOFFLER (NASA, John F. Kennedy Space Center): In defense of Dr. Maki's correlation, wasn't that an R^2 of variance which would make the correlation coefficient more like .45?

The statistical validity would be more a function of the sample size. Could a short-term organism like the Daphnia evolve a change in response over a long period of time so that you would be misled in its response or correlation with the larger species you were more interested in?

DR. MAKI: In response to your first comment, yes, the Pearson correlation coefficient and also the Spearman rate correlation coefficient which we did run for all the materials but was not shown on the slides, does prove to be statistically significant for all the materials, exclusive of the pesticides, at the 95% level. I'm not sure if you are talking here of short-term acclimation or actually some change in susceptibility of the invertebrate species. We are looking at acclimation to test materials for this individual species. There is some literature to indicate that Daphnia and several fresh water fish species can indeed acclimate to long term, low level exposures of test materials. Subsequently, when they are used in a toxicity test, you do get statistically significant differences in susceptibility of those previously exposed individuals versus unexposed controls. This may speak to problems using Daphnia or even fish species in a biomonitoring system. An individual exposed for a long period to an effluent or a particular test material may develop some acclimation ability and ultimately evolve so that they are no longer a very good indicator of toxicity for other species in the system further downstream.

MR. WANDS: Dr. London, in your experiment you did not totally wipe out your population. Was that a process of selection or was it a reduction in fertility as you suggested or was it adaptation as Dr. Maki has suggested? Really, what was going on there?

DR. LONDON: The basic mechanism was that the growth rate constant changed to result in a much lower population level. However, whether that occurred by introduction of a mutation or by selection out of a preexisting mutation or by a biochemical influence on the specific rotary constant is not clear. It could be any one of those three. In that experiment, there was no way of telling. You could go in and get the organism out and then determine under other conditions, for instance, in the absence of the hydrazine, what is its new value? That is one of the interesting features of the system and if you want a system that you know can mutate and indeed does mutate, and if you establish an environment to allow that mutation to be expressed, you will see it.

MR. WANDS: Did you generate the mutation with your compound?

DR. LONDON: That's what you can use the system for, to score mutation rate with time and with exposure to the compound of interest and see if that is increasing. And I didn't get a chance to mention it but one of the three things that one can look for in this system is mutagenesis, accumulation and degradation. And all three of them can be accomplished by selection out of a particular organism that has an increased capability for that parameter that you are looking for in the population and then you provide the environment which favors that particular capability.

DR. BACK (6570 Aerospace Medical Research Laboratory): Dr. London, the obvious experiment, you didn't show us follows what you demonstrated. What was the recovery of your system? You showed us the bottom of the line with about 40% reduction in population there. The obvious experiment now, if that's a mutant, is to let them recover and then do the experiment over. Are the test organisms now more resistant or less resistant? Was that done?

DR. LONDON: No, that wasn't done. That was a summation of the very first few experiments trying to find the ED_{50} of hydrazine which would not wipe the entire population out but yet cause a measurable change. The concentration turned out to be at 100 ppm and that's why we then ran it in triplicate to make sure it wasn't a quirk such as we had seen in previous experiments. That's where we are now and the next experiment that

we plan to do is to go down to essentially the no-effect level and precisely determine this amount and then go back to the 100 ppm and take those cells and determine if we have a more resistant population to hydrazine.

CLOSING REMARKS

AN ESSAY ON THE PHILOSOPHY AND STATE OF KNOWLEDGE OF THE SCIENCE OF TOXICOLOGY

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If one were to undertake to write an essay on what toxicology is all about, one could do no better than review the proceedings of this 8th Conference on Environmental Toxicology. The review would show that toxicology is a new scientific discipline focused primarily on promoting the health and welfare of our fellowman. It is primarily preventive in nature as it seeks to identify and delineate potential hazards of chemicals entering the environment before they can cause injury. Secondarily, but no less important, is the role of toxicology in the deliberate selection and development of toxic effects as in pharmaceuticals, pesticides and antibiotics. Toxicology is thus closely allied with the older disciplines of preventive medicine and public health. Although toxicology is focused on man it recognizes man is not independent of his biosphere.

Toxicology, like the best in the practice of medicine, is a multidisciplinary field. It is, however, an emerging science. It can trace its roots back to the death of Socrates and the practice of Caesar's high priests who selected campsites for the Gallic wars by examining the entrails of the local wildlife. Today's toxicology began 50-75 years ago and encompasses only 3 generations. It began with men like Henry Smyth and his father who established an industrial toxicology laboratory in Philadelphia and pioneered many of today's standard procedures. One of Henry's graduate students was on our program this week. Several of us, myself included, learned much from Dr. W. F. von Oettingen, one of the early directors of du Pont's Haskell Laboratories and the first toxicologist in the U.S. Public Health Service.

Our society today presents great needs and demands for toxicology. It asks questions of us which we are not yet able to answer. Fortunately, our military services have shown real foresight and leadership first in developing occupational toxicology and more recently in engaging in toxicology related to environmental and public health problems.

This Conference has been a dissertation of the state of knowledge of toxicology and the breadth of the discipline. Toxicology involves fundamental and basic science as shown by the exquisite biology studies reported by Dr. Kun who turns the inner envelope of a mitochondrium inside out and examines the transport enzymes. Also the outstanding genetic work described by Dan Nebert on the differences between sibling animals foretelling what we can expect someday in our understanding of the differences between thee and me. One of the earliest examples in toxicology of the importance of this kind of information were the studies by Paul Nees at WARF on the toxicology and metabolism of cyclamate. Gross examination of their data showed no significant differences between the controls and the experimentals but a detailed examination showed a bimodal distribution in which some of the rabbits metabolized the cyclamate to cyclohexylamine. This led ultimately to the present controversy over nonnutritive sweeteners which raises questions of our need for them and whether or not their benefits are worth the risk.

Toxicology, as any science, can only deal with defining the risk; it cannot make the ultimate social decision of how much risk is acceptable. In our Conference, we have the pharmacokinetics of John Young to help in such decisions. We saw the practical side in the applications of toxicology in the work of Weil 20 years ago. He painted the skins of mice paralleling the progress on emerging pilot plant technology. His results alerted Dr. Sexton, the plant physician, to watch for skin cancer and ultimately led to the termination of the project.

We heard about the TNM work at WPAFB/AMRL, the VBC studies by Dow, and the DMF tests at du Pont as examples of the application of toxicology to preventive medicine.

We learned of the neophyte field of fire toxicology which will ultimately save many lives but which today is still groping for reliable techniques. Yet, we also saw the advanced technology of semiautomated toxicity exposures and monitoring. We could have had papers on the automated data recording systems pioneered in the industrial laboratories of Dow and now at a highly advanced state of computer technology at NCTR.

Lest we forget that man is not an island we have this morning the reports by those looking at toxic effects in nonmammalian species. Here, we learned that basic biologic concepts such as assimilative capacity of an individual organism or a complex system of many species are applicable at any level of the phylogenetic spectrum.

One of the shortcomings of toxicology in its development to date is that it usually looks only at a single chemical whereas the real life situation involves simultaneous exposures to more than one toxicant at a time. Dr. Weber gave us today a simple technique for examining this issue.

We have had a rare privilege this week of seeing our discipline in perspective as well as hearing specific details. I thank each of you for your contribution to this Conference. We all owe a vote of thanks to the Air Force and its contractor, the University of California at Irvine, for a well planned and well conducted Conference. Goodby and God speed.

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