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PROCEEDINGS OF THE NINTH CONFERENCE ON ENVIRONMENTAL TOXICOLOGY 28, 29, and 30 MARCH 1979

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TECHNICAL REVIEW AND APPROVAL

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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

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

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Major technical areas discussed included methodologies of shale oil retorting, the acute and chronic toxicity of these materials, and occupational health studies on shale oil workers. The toxicology of hydrazine compounds was presented and the areas of general toxicology and plant toxicity were discussed. Animal models used in genetic toxicology were reviewed, and specific techniques for examination of genetic damage were discussed.

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PREFACE

The Ninth Conference on Environmental Toxicology was held in Dayton, Ohio on 28, 29, and 30 March 1979. 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. Colonel Vernon L. Carter, Jr., USAF, VC, Deputy Director, Toxic Hazards Division, 6570 Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio served as Conference Chairman, and Mrs. Lois Doncaster, University of California, Irvine served as Conference Coordinator.

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INTRODUCTORY REMARKS

Colonel Roy L. DeHart, USAF, MC

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Wright-Patterson Air Force Base, Ohio

I have the privilege today to welcome you to this Ninth Annual Conference on Environmental Toxicology on behalf of the Air Force Systems Command, the Aerospace Medical Division, and the Aerospace Medical Research Laboratory. I am not an expert, by definition, I do not have 35 mm slides, I am not from out-of-town, and I don't have a briefcase. And, in fact, as a Commander of a laboratory, I do not have a Ph.D. I am, rather, a physician, an M.D. And in addressing groups such as this and other groups as well, I am often asked, "What kind of doctor are you?" And I make the point that I am not an internist and that, of course, is an individual who knows everything and does nothing. I am not a surgeon, the individual who knows nothing and does everything. Nor am I a psychiatrist, the individual who knows nothing and does nothing. Nor, in fact, am I a pathologist, the individual who knows everything and does everything but it's too late. I am a physician who specializes in caring for people.

I would like to commend the University of California for arranging a stimulating and intellectually challenging program for you. It is one which directly addresses Air Force needs both now and in the future as we attempt to cope with those problems associated with our fuels, propellants, and industrial chemicals in our inventory.

The session on environmental toxicology reflects our continuing concern with the possible adverse effects of missile exhaust products. It represents a commitment by the Air Force to prevent damage to the ecological environment surrounding our missile sites whether it be government or private property without unduly hampering launch operations.

In the near future, the MX, a new missile system, will perhaps be entering the Air Force inventory. And I can assure you that this will provide us an ongoing ecological challenge.

I have followed with interest the increased emphasis that is being placed on the short-term tests to evaluate adverse effects of chemicals on genetic materials. The guidelines from our regulating agencies more and more require such information in their procedures for setting standards. I am hopeful that this conference will help shed light on the interpretation and use of such methodologies.

I know from conversations with many of the past attendees that the inhalation toxicology session has been a highlight of these conferences. During this session, we will hear results of the first of a series of studies which attempt to establish a dose response relationship to the oncogenic potential of the propellant hydrazines when chronically inhaled. I certainly have more than a passing interest in these studies since they were designed and initiated under the sponsorship of the Aerospace Medical Research Laboratory.

These studies, coupled with the general session on hydrazine toxicology, fulfill a vital need in view of the expanded use of the mono propellant hydrazines in the Air Force. I don't know how many of you are aware that one of the new aircraft entering the Air Force inventory, the F-16, uses hydrazine as a propellant for an emergency power system on that aircraft. There will be approximately 1000 of these aircraft built. They will be used not only by our Air Force but also by our allies. We are already experiencing concern for exposure to ground crews with this hydrazine system.

Finally, the update in shale oil toxicology is certainly timely. In view of the 9% increase in the price of crude oil and with surcharge being added or the possibility of reduction in production in the Middle East to 30% of current levels, it appears appropriate that our federal government take this opportunity to pursue alternate fuels.

With the Navy as the lead agency, the Department of Defense, in cooperation with the Department of Energy, is embarking on a rather ambitious program to develop alternate sources for military fuel. In the past, much of the toxicology needed to assess the hazards associated with the introduction of chemicals into the Air Force system has developed after field deployment of that system. For a change, we are now developing a proper data base during the early development of shale derived fuels for Department of Defense use. This will allow a rational decision and trade-off to be made with the introduction of the fuel for operational purposes.

These studies will be conducted by the University of California at the Aerospace Medical Research Laboratory and will be cost shared with our Navy colleagues.

Again, I extend to you a very warm and cordial welcome to Dayton. The weather is delightful, at least for now. Should your schedule permit, I would be delighted to provide you with a tour of the Aerospace Medical Research Laboratory.

I trust that these three days will prove most profitable.

SESSION I

TOXICOLOGY OF SHALE OIL MATERIALS

Chairman

Roy L. Gibson, Jr., M.D.
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Houston, Texas

PAPER NO. 1

SHALE OIL RECOVERY PROCESSES - ABOVE GROUND AND IN SITU RETORTING

R. Merril Coomes, Ph.D.

Tosco Corporation
Los Angeles, California

Retorting oil shale is not a modern discovery, although it has received considerable attention in recent years. In 1350, Austria produced medicinal oil from shale. The English recorded a patent in 1694 to distill "oyle from a kind of stone" (Mountain Empire Publisher, 1974).

Australia, Brazil, Canada, France, Germany, New Zealand, Scotland, and the United States all had produced oil from oil shale before 1900. The lean oil shales of Appalachia were retorted to produce lamp-oil lubricants and some medicines. The Mormon pioneers retorted oil shale from the Green River Formation in a similar fashion during the last half of the 1800's. During the early 1900's, there were more than 200 oil-shale retorting corporations formed. While several actually got into production, discovery of the East Texas oil fields made such operations unprofitable. Today Russia, China, and Brazil have substantial oil shale operations.

The United States has the world's largest known oil shale deposits. Figure 1 shows the location of the Green River Formation located in Colorado, Utah, and Wyoming, and includes about 17,000 square miles. The Piceance Creek Basin, a 1300 square mile area, could produce about 500 billion barrels of oil from mining horizons at ten feet or more that contain at least 25 gallons of oil per ton of shale (Atwood, 1973). This reserve is enormous - equal to 170 years of domestic crude production at the July 1978 rate. An evaluation of the current energy situation makes it clear that the United States is interested in developing this valuable resource.

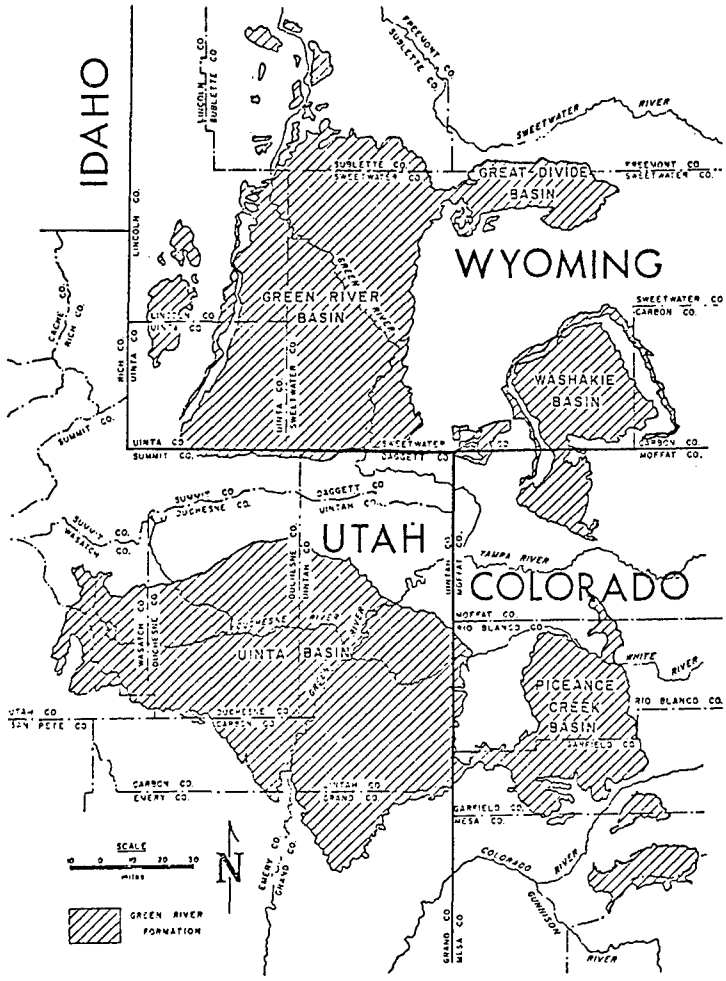

Figure 1. Extent of the Green River Formation in Colorado, Utah and Wyoming.

RETORTING OIL SHALE

The objective of all oil shale retorting techniques is to convert Kerogen found in the oil shale into oil and to isolate that oil from the retorted, or heated, solid. Kerogen is a three-dimensional, high molecular weight polymer that is insoluble in conventional organic solvents (Schmidt-Collerus, 1971). The conversion of Kerogen into oil requires temperatures of 800-900 F. The heating process is called retorting and can take place above ground or below ground (in situ). Two basic mechanisms are used to heat oil shale, indirect heating and direct heating. Indirect heating is defined as heat supplied from outside the retort, while direct heating is defined as heat produced in the retort by combustion. These heating methods can be used independently or together and can be applied to above ground or in situ retorting.

ABOVE GROUND

The basic types of retorting can be categorized into the four types based on the method of heating (Cattell et al., 1950; Atwood, 1977). These four major retort types are shown in Figure 2. In Type 1 retorts, which are the simplest in design, heat passes through the retort walls into the oil shale. In Type 2 retorts, combustion of oil shale within the retort provides the necessary heat. Type 3 retorts are heated by contact with circulated externally heated gases. Type 4 retorts obtain heat by contacting externally heated solids.

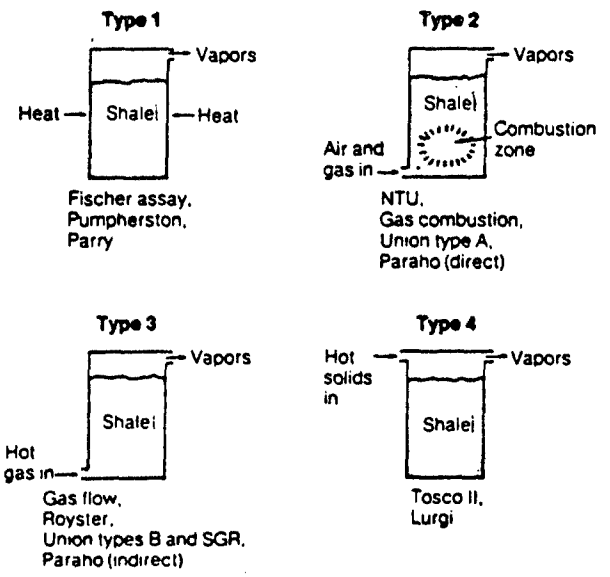

Figure 2. Retort types.

The Type 1 retorts are the most simple design and many early retorts were of this type. The Fischer Assay retort, shown in Figure 3, is a Type 1 retort used for the laboratory analysis of oil shale (Goodfellow and Atwood, 1974). In the Tosco variation of Fischer Assay, the product gases are totally captured and subsequently analyzed by gas chromatography. The gas analysis and volume measurement allow a complete material balance to be made around the retorting operation. The shale is heated electrically to 932 F following a specified temperature-time profile. Other Type 1 retorts include the Pumpherston Retort, used in Scotland (Scottish Oils Ltd., 1948), and the Parry Retort, which was evaluated by the U.S. Bureau of Mines in 1945 with coal.

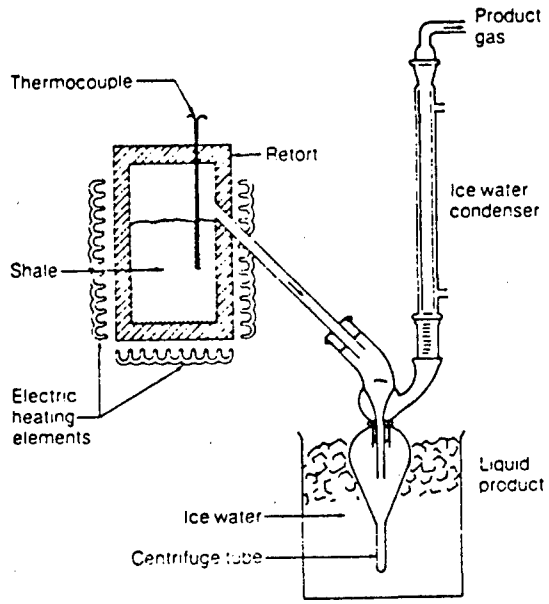

Figure 3. Fischer assay (Type 1).

A schematic of the Type 2 direct heating retort is shown in Figure 4. Examples of this type of retorting are the NTU (Nevada-Texas-Utah) retort which was a batch process, and the Union Type A, gas combustion, and Paraho direct-fired retorts which are continuous processes. As shown in Figure 4, this type of retorting process requires that small oil shale particles (less than 1/4 inch) be removed from the shale feed before retorting. Finer materials tend to restrict gas flow through the retort.

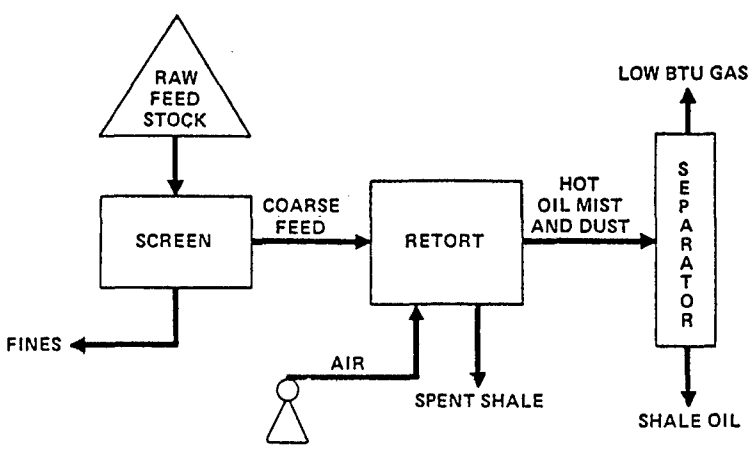

Figure 4. Schematic of direct heating retorting process.

The NTU retort was developed at Anvil Points, Colorado by the U.S. Bureau of Mines and retorted 37,500 tons of oil shale in 920 tests between 1947 and 1951 (Ruark et al., 1956). The U.S. Bureau of Mines also developed a continuous process, the gas combustion process, that was extensively tested by the Bureau and later by a lease agreement with the Colorado School of Mines Research Foundation, which was supported by six major oil companies: Mobil Oil Corporation, acting as project manager; Humble Oil and Refining Company; Pan American Petroleum Corporation; Sinclair Research, Incorporated; and Phillips Petroleum Company. Tests progressed through 6, 25, and 150 ton per day retorts (Jones, 1976).

The Paraho retort was developed by Paraho Oil Shale Demonstration, Incorporated formed in 1973. More than 15 participants supported this program at Anvil Points, Colorado. The Paraho retort resembles the gas combustion retort, but has the addition of a continuous feeder and a moving grate discharge mechanism which assures a smooth, more uniform flow of shale through the retort. Continuous operation has shown that problems plaguing the gas combustion retort have been solved (Jones, 1976).

Union Oil Company developed a unique version of the NTU retort, the Union Retort A, in which oil shale was continuously fed into the bottom of the retort using an oscillating rock pump (Reed et al., 1952). A large retort was successfully operated on Union's Colorado oil shale property during the 1950's. Air is injected at the top of the retort and is heated by the hot spent shale. Below this area the spent shale carbon residue is burned and the resulting flue gases move down through the retort, heating the upward moving oil shale. Below the retorting zone, the incoming oil shale is preheated by the gases and hot condensing oil vapors.

A schematic of a Type 3 is shown in Figure 5. This type of retort uses an externally heated gas to heat the oil shale. Fuel for heating the recirculated gas is obtained from the retorting process. The spent or processed shale and hot oil mist and dust are removed from this retort. A separator recovers the shale oil and a high BTU gas as products.

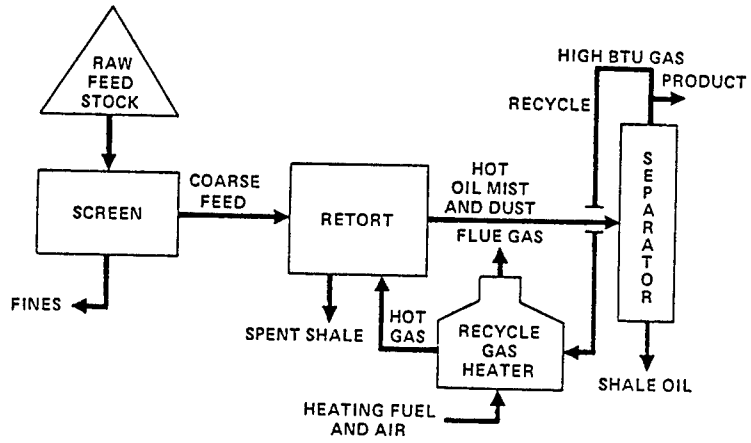

Figure 5. Schematic of indirect heating/recycle gas retorting process.

Union and Paraho have modified their direct fired retorts and, using the same basic hardware, use the indirect heating mode. Because there is no contamination of the product gas stream by nitrogen from the air or carbon dioxide from combustion, the BTU value of the product gas approaches that of natural gas (Hopkins et al., 1976; Pforzheimer, 1976).

Other indirect fired recirculated gas retorts are the gas flow retort, the Royster retort, and Superior Oil Company's circular grate retort. The gas flow retort did not advance beyond the pilot stage and consisted of a cylindrical retort containing a series of louvers through which the shale passed. Hot gases were introduced through the side of the retort. The Royster retort used a batch operation and a pebble stove to heat the recycled gases. The pebble stove contained pebbles that were previously heated by product gas combustion. Heat from the pebbles was carried into the retort by the recycled gas. Two pebble stoves were used, and while one was being heated, the other containing heated pebbles was used to supply heat to the retort. Superior's retort is a circular moving grate which carrying crushed oil shale travels between a hood above the grate and wind boxes below the grates. Retorting is by contact of oil shale with hot gases.

A Type 4 retort, indirect heating-solid heat carrier, is shown schematically in Figure 6. There are two prominant Type 4 processes, the TOSCO II (Whitcombe and Vawter, 1975) and the Lurgi IR (Schmalfeld, 1975). Heated ceramic balls supply heat to the TOSCO II retort where they are mixed with preheated oil shale and flow together through a rotating retort drum. The processed or spent shale has a small particle

size at the retort exit and is separated from the half inch ceramic balls by passing the mixture over a trommel screen. The balls are recirculated to the ball heater and then to the retort. The TOSCO II retort can utilize oil shale fines, and in fact, the feed must be crushed to minus one half inch before retorting. The product gas has a high BTU content and product yields are normally 100% of those expected from Fischer Assay. About 200,000 tons of shale have been processed at the Parachute Creek 1000 ton per day TOSCO II retort facility.

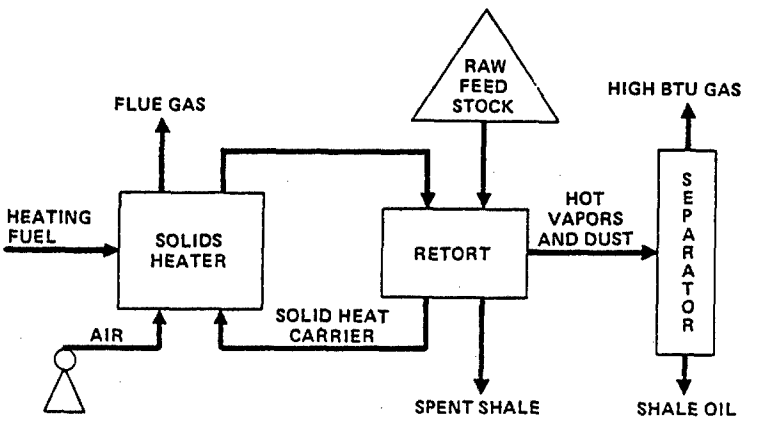

Figure 6. Schematic of indirect heating/solid heat carrier retorting process.

IN SITU

In situ oil shale retorting is basically a method of heating the oil shale under the surface of the ground and recovering the oil without the necessity of removing the shale for retorting. The process leaves retorted shale in place after the retorting is complete. There are two basic types of in situ retorting; true in situ and modified in situ. The modified in situ technique is shown schematically in Figure 7. Shale is mined from the retort to create a 20% void volume in the underground retort. The 20% void provides the necessary permeability required for retorting. True in situ uses other methods to create a permeable zone in the shale. There are several different modified in situ technologies, for example: Occidental and rubble in situ extraction developed by Lawrence Livermore Laboratory and described in a report by the Department of Interior.

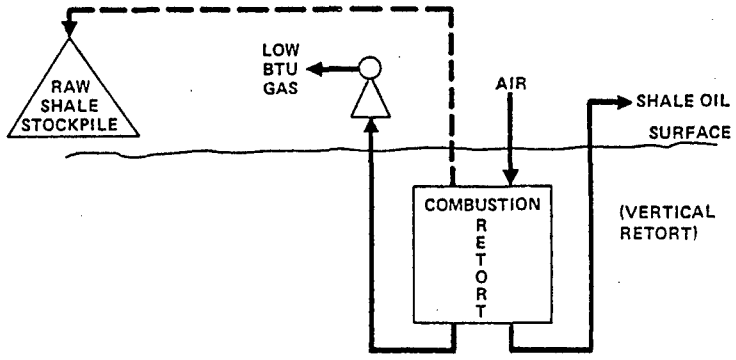

Figure 7. Schematic of modified in situ reporting process.

The Occidental process includes three basic steps: (1) mine a 20% void at the top and/or bottom of the retort zone; (2) drill vertical longholes into the shale layer from the mined-out room, load holes with explosives and detonate; and (3) complete connections at top and bottom of retort to add air and remove oil and ignite. Occidental has been testing its process since 1972 and currently plans to use the process on the Cb oil shale tract in a 57,000 gallon per day shale oil production facility. Retorting is performed under a small positive pressure.

The rubble in situ extraction process is similar to the Occidental process except that the 20% void is created by a continuous mining process utilizing a sublevel caving technique, and retorting is performed under a slight negative pressure. This process is proposed for the Ca oil shale tract and will be used in combination with TOSCO II above ground retorting of the mined oil shale.

The true in situ retorting method (no mining) has been examined by the Laramie Energy Technology Center (LETC), Geokinetics, Tally Frac, and Equity Oil Company. LETC has operated a series of test retorts near Rock Springs, Wyoming during which a rubblization procedure has been developed. The latest procedure uses hydraulic pressure in closely spaced wells to create about three horizontal factures 3.5 to 4 feet apart with a radius of approximately 75 feet from each well. The lower fractures are filled by pressurized injection of a slurried explosive that creates a rubblized zone of oil shale on detonation. A horizontal flame front retorts the shale and the oil is recovered from a series of production wells that are drilled into the retort before ignition.

The Tally Frac process is similar to the LETC process except that Tally Frac plans to use microsecond delays between explosive detonations within the hydrofractures, while LETC uses simultaneous detonations.

The Geokinetics process is applicable to thin rich shale deposits having little or no overburden. Experiments to date have used 25 foot thick shale deposits covered by 40 to 75 feet of overburden. Series of wells are drilled into the deposit, loaded with explosive and detonated. A surface upheaval of about five feet generally results from the explosion. Injection and production wells are drilled into the rubblized shale and horizontal retorting is initiated. Shale oil is collected at the bottom of the retort and pumped to the surface.

All of the previous in situ retorts have been Type 2 retorts. The Equity Oil Company developed a Type 3 in situ retorting procedure during the early 1960's which utilized a heat carrier gas (natural gas or methane) to retort oil shale. The process is suitable for naturally permeable shale deposits. Basically, the process consists of drilling an injection well into the deposit through which hot gas is injected. This relatively low temperature in situ process yields a higher quality product than combustion in situ methods but has not proven to be economical.

POTENTIAL OCCUPATIONAL EXPOSURE

The previous information on oil shale retorting technology is necessary in order to make an evaluation of potential occupational and environmental exposure to oil shale materials. Occupational exposures can be determined by examination of Colony Development operations facility (Colony Development Operation, 1974), which is shown schematically in Figure 8 and is the one with which I am the most familiar. Colony is a joint venture of Tosco Corporation and Atlantic Richfield Company, with Arco as operator, which has prepared detailed plans to construct a 50,000 bbl/day facility on private land in Colorado. The fundamental oil shale materials for which there would be potential exposure include: raw oil shale, crude shale oil, upgraded shale oil, process atmospheric effluent, process or retort water, and processed oil shale. Exposures for modified in situ retorting would include all of the above materials except processed oil shale.

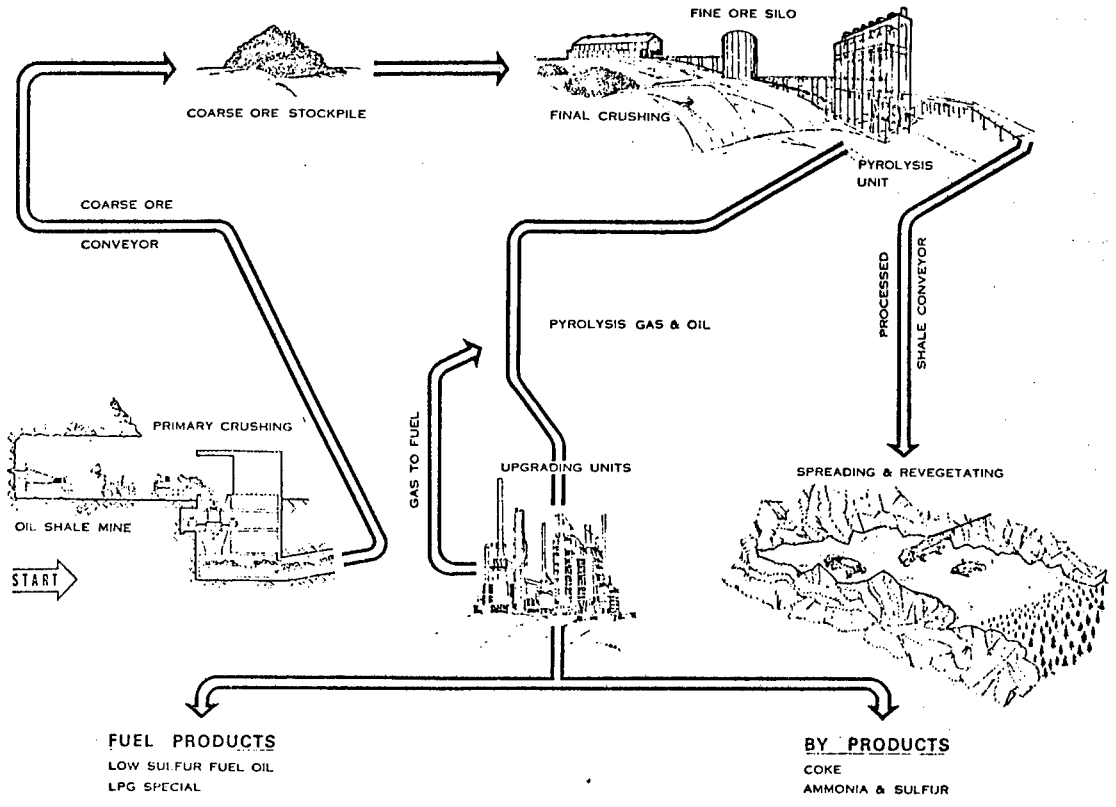

Figure 8. Schematic diagram of an oil-shale processing operation.

Exposure to raw oil shale dust would occur during mining and crushing operations. The amounts of shale to be mined are large; Colony (TOSCO II) 66,000 tons per day, Ca (in situ and TOSCO II) 40,000 tons per day, and Cb (in situ) 44,000 tons per day. The equipment for oil shale mining has been demonstrated in prototype full scale mines. The mining technique for above ground retorting is the room and pillar method which is shown schematically in Figure 9. A mining horizon is 30 feet high and 50 feet wide. Pillars left in place for roof support are about 50 by 50 feet. To complete the mining operation, a second 30 foot horizon is removed from the mine floor which gives 60 feet between the floor and roof of the completed mine.

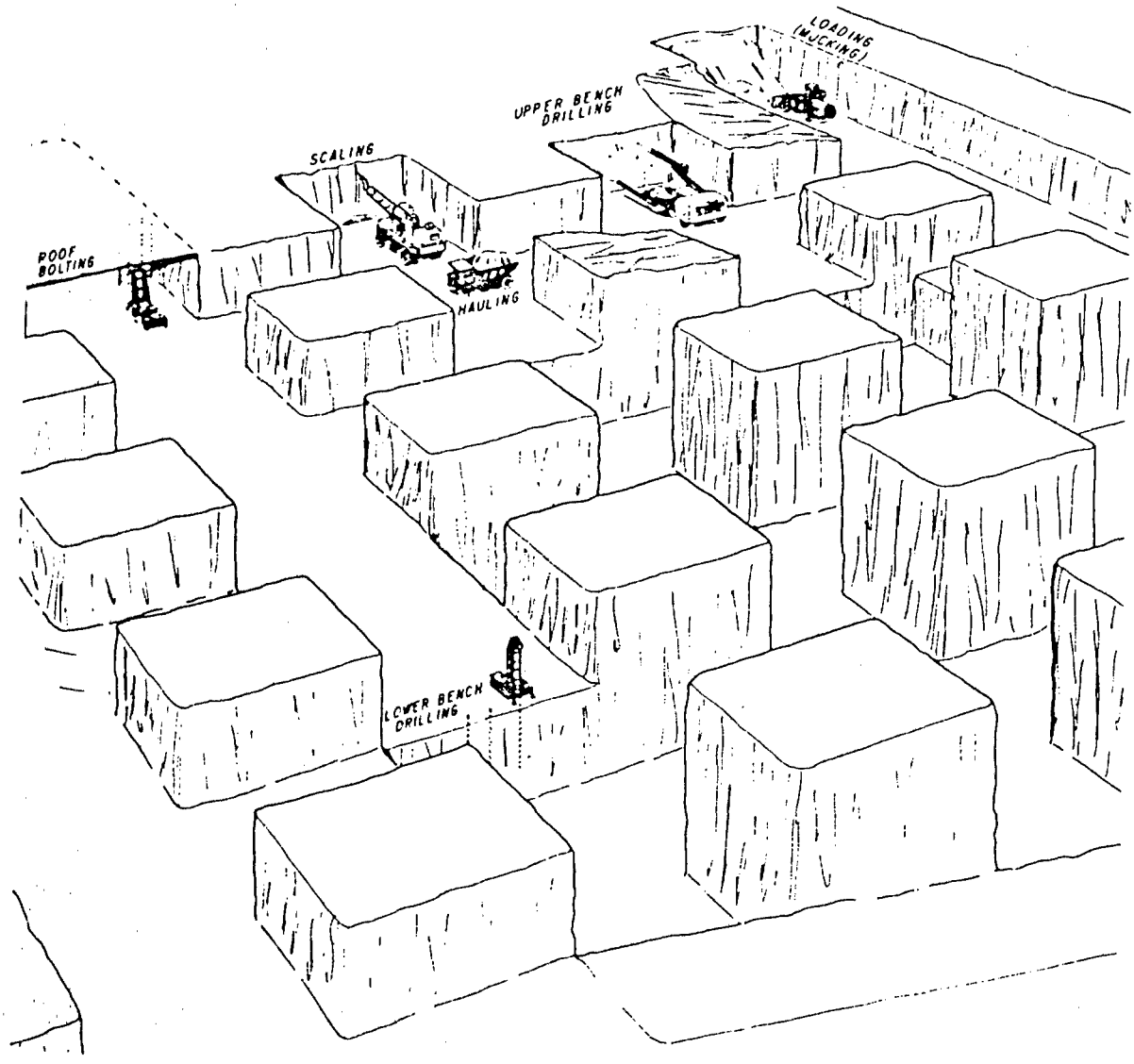

Figure 9. Cutaway drawing of oil shale room-and-pillar mining.

Workers in the retorting complex may be potentially exposed to oil mists and vapors in addition to dusts from raw and processed oil shale. The control of oil vapors and mists should not present any situations that are not currently experienced in modern petroleum refinery operations.

Exposure to processed shale dust will occur during processed shale disposal. In Colony's operation, the processed shale is moisturized to about 12 weight percent water in order to minimize dust levels and to aid in compacting the processed shale in disposal embankments. After the processed shale is compacted, using normal earth moving and compaction equipment, the surface is prepared for revegetation. The surface is loosened and the salts are

leached with water from the surface of the shale. Revegetation experiments at the Colony site since 1968 have demonstrated that revegetation is an acceptable method for surface stabilization of processed shale embankments. Even in the arid Colorado areas, revegetated processed shale does not require maintenance after 3 years.

HEALTH EFFECTS

Colony has been concerned with the potential health effects of oil shale processing and contracted our first health-related studies to outside laboratories in 1965. Since that time, over $500,000 has been invested in chemical characterization and biological testing of TOSCO II oil shale materials (Atwood and Coomes, 1974; Coomes, 1976; Coomes, 1978). We have tested raw oil shale, processed shale, crude shale oil, hydrotreated shale oil, and atmospheric effluent for their toxicity and carcinogenicity. It is concluded from these experimental results with TOSCO II materials that crude shale oil presents a carcinogenic hazard that is similar to or less than that of currently used refinery materials, that hydrotreating dramatically reduces this carcinogenicity, and that solid oil shale materials do not present a carcinogenic hazard.

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PAPER NO. 2

ACUTE TOXICITY OF SEVERAL RAW SHALES, SPENT SHALES AND SHALE OILS

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D. B. Disbennett

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This report describes the acute toxicities of representative samples of shale oil and spent shale as determined by the Cannon Laboratories, Inc., Reading, Pennsylvania, in studies conducted by Stuart Todd Associates of Wayne, Pennsylvania, for the American Petroleum Institute of Washington, D.C.

The materials studied are listed in Table 1. The oils and the raw shale from which they were derived and the spent shale resulting from the extraction process are grouped together. The shales come from different geographical areas and were subject to some variation in processing; however, the differences between the oils and between the shales are quantitative rather than qualitative in nature.

TABLE 1. TEST MATERIALS

Shale Oils	Raw Shale	Spent Shale
--	RS 101	--
RO 1	RS 102	SS 201
RO 2	RS 102	--
RO 3	--	--
RO 4	--	SS 202
--	RS 103	--
--	--	SS 204

Standard techniques were employed to determine the acute toxicities of these materials. Determinations were made of both their oral and percutaneous toxicities, eye and skin irritation, and of their sensitizing potential. A Coulter S® was used for the blood counts; differentials were done manually. Blood chemistries were determined using analytical procedures employing the SMA 12® system. In order to increase the fluidity of the shale oil samples, they were heated to and maintained at 100 F during their application to test animals. The solids were ground to 100 mesh size (<150 micron).

Estimates were made of the acute oral toxicities of the seven dry and four liquid samples.

The raw and spent shales, finely ground, were suspended in corn oil to make a 20% suspension. Ten g/kg of each material was administered to five male and five female rats, and the animals were observed for a total of 14 days. All surviving animals were sacrificed at the end of the 14th day, autopsied and observed for gross organ changes. Clinically, the animals showed central depression as evidenced by decreased locomotor activity and ptosis. Pilo-erection was also recorded by the observer. Recovery took from 4-14 days, and 4 deaths were noted among the 70 animals dosed. No specific organ pathology was noted on postmortem examination. Organs grossly examined included the liver, spleen, stomach, intestine, heart, lungs, pancreas, and kidneys.

Five doses of each shale oil ranging from a little above 2 grams to a little above 14 grams were administered to five male and five female Sprague Dawley rats weighing between 200-300 grams. The animals were followed for 14 days and observed for signs of toxicity daily. All animals were necropsied and gross organ examinations made. The 14-day LD_{50} was calculated for each of the oils according to the method of Miller and Tainter. The calculated LD_{50}'s are shown in Table 2.

TABLE 2. LD_{50}'s OF REPRESENTATIVE SHALE OILS (g/kg)

	LD_{50}
RO 1	10.05 ± 0.62
RO 2	10.25 ± 0.96
RO 3	9.63 ± 0.62
RO 4	8.00 ± 0.49

Orally, dosed animals showed clinical signs of depression, the severity of which was dose related. Specifically, there was observed decreased locomotor activity, ptosis, respiratory depression, and death. Pilo-erection was observed in all animals. The recovery rates of those animals that did not die were generally dose related. No deaths occurred on the day of dosing; those animals that did die did so between days 1 and 7, and the day of death appeared to be directly related to the dose administered.

Acute dermal effects were determined on abraded and non-abraded albino rabbit skin. The animals were observed for effects 24 and 72 hours after a 24-hour application of the various materials. Evaluation for edema and a combination of erythema and eschar formation was performed as described in the Code of Federal Regulations, Section 1500.41. The maximum irritation score possible is 8 units. A concentration of 0.5 ml of the oils or 0.5 gram of the solids was applied to each rabbit. Of the materials tested, only the shale oils themselves showed primary irritant effects with indices calculated for the oils ranging from 2.5 to 3.37. Irritation scores are shown in Table 3.

TABLE 3. RABBIT SKIN REACTION

	24 Hours	72 Hours	1° Irritation Index
RO 1	2	3	2.5
RO 2	1.8	3.5	2.7
RO 3	2.3	4.3	3.4
RO 4	2.7	2.5	2.6

The acute effects of these materials on the eye were examined in New Zealand albino rabbits according to methods described in the Code of Federal Regulations (Fed. Reg. 38:187, September 27, 1973, page 27019). The test material (0.1 ml of each oil or 0.5 g of each of the dry substances) was placed directly into the left eye conjunctival sac. The right eyes of the rabbits receiving the shale oils received 0.1 ml of USP white mineral oil also warmed to 100 F; the right eyes of the animals receiving the solid materials served as untreated controls. None of the eyes were washed out after instillation of any materials. Observations were made daily for the first four days and at 7 days until the treated eyes returned completely to normal.

Tables 4 through 7 show the effects of the materials tested. All of the oils, one of the raw shales, and the spent shales were reversibly irritating to the conjunctiva. There does not appear to be a significant relationship between the raw shale, shale oil, or spent shale. Compare RO 1, RO 2, with RS 102 and SS 201. SS 201 and SS 204 were reported to produce minor corneal irritation and iritis. These same two spent shales were observed to produce the greatest degree of conjunctival irritation as well. All of these effects were reported as reversible by 7 days.

TABLE 4. CONJUNCTIVAL IRRITATION
(Maximum Score = 20)

Test Material*	1	2	3	4	7	8	9
[a]R 01	4.0	2.7	0.7	0.0			
[a]R 02	4.0	4.0	3.0	1.7	1.3		
[b]R 03	4.0	1.7	1.7	1.3	1.3	0.7	0.0
[c]R 04	4.0	3.3	2.0	2.0	0.0		
RS 101	0.0						
[a]RS 102	0.0						
RS 103	4.0	0.0					
[a]SS 201	7.3	6.3	4.3	2.0	0.0		
[c]SS 202	2.7	0.0					
[b]SS 203	1.3	0.0					
SS 204	12.0	8.7	7.7	6.7	0.0		

*Superscript letters refer to related materials, i.e., oil and spent shale derived from raw shale.

TABLE 5. CORNEAL IRRITATION
(Maximum Score = 80)

Material Tested	1	2	3	4	7
SS 201	26.0	25.0	16.7	6.7	3.3
SS 204	29.2	29.2	27.5	8.3	0.0

TABLE 6. IRIS IRRITATION
(Maximum Score = 10)

Material Tested	1	2	3	4	7
SS 201	5.0	5.0	0.0		
SS 204	5.0	4.2	2.5	1.7	0.0

TABLE 7. EYE EFFECT OF SPENT SHALE

	Cornea Irritation (Max = 80)	Iris Irritation (Max = 10)	Conjunctival Irritation (Max = 20)
SS 201	26	5	7.3
SS 202			2.7
SS 203			1.3
SS 204	29.2	8	12.0

The acute percutaneous toxicities of the various materials were observed following their 24 hour application to rabbits with and without abraded skin. The shale oils were applied at rates of 5, 10, 15, and 20 ml/kg and the solids were applied as an aqueous paste at 20 g/kg. Excess material was held in place with gauze and all covered by an elastic sleeve. After 24 hours, the test areas were wiped clean of any remaining test material. Observations were made frequently for the first 24 hours and daily thereafter.

The results with the solid materials will be described first. No systemic toxicity was observed with any of the raw or spent shales. Only SS 201 produced some erythema at the site of application in three out of the five rabbits with abraded skin; none was observed in rabbits with intact skin. SS 201 was not observed to produce skin irritation when applied in lesser amounts to rabbits with abraded and nonabraded skins in a standard skin irritation test but did produce eye irritation in an assay for that activity (Table 8).

TABLE 8. PERCUTANEOUS TOXICITY OF RAW AND SPENT SHALES: RABBITS

None of the raw or spent shales were systemically toxic.

SS 201 Erythema in 3/5 (abraded skin only)

Nonirritating in standard skin irrition study

Was primary eye irritant (score 5/10)

The oils were examined in two series of studies as outlined in Table 9. Only rabbits with intact skin were used in these studies. In the first study (A), the indicated doses of each oil were applied to the backs of groups of four rabbits. In a few instances, an additional group of four rabbits was treated and these rabbits had collars around their necks to prevent

ingestion of any of the test materials. In an attempt to obtain a better estimate of the LD_{50} of these materials, additional groups of four male and four female rabbits were similarly treated and observed, and these animals are indicated under the replication (B). Similar results were observed in both parts of the study and the results of both parts will be reported together.

TABLE 9. PERCUTANEOUS TOXICITY STUDY

Dose (ml/kg)	RO 1		RO 2		RO 3		RO 4	
	A	B	A	B	A	B	A	B
5	1/4	0/8	1/4	0/8	-	0/8	-	0/8
10	1/4	0/8	2/4	1/8	0/4	0/8	0/4	0/8
15	1/4	1/8	2/4	1/8	2/4	0/8	2/4 1/4	1/8
29	3/4 3/4	3/8	2/4	3/8	2/4	1/8	0/4 3/4	3/8

In the expanded part of the study, we elected to add histologic examinations of the liver, kidney, skin, spleen, and brain and to include hematology and clinical chemistry on days 0, 1, 8 and 14.

Table 10 shows the observations recorded for one dose level of one oil and is pretty typical for the observations made for all the oils. This table is a copy of part of a page recording the observations made during the 14 days following the application of 15 ml of RO 4 per kg to two male and two female rabbits during part "A" of the study. The marked cutaneous effects of erythema and edema were noted throughout the study; weight loss over this period was also observed; decreased locomotor activity was reported later in the study and may be a reflection of secondary toxicity since its appearance seems to be related to the animals' deteriorating condition and subsequent death.

TABLE 10. OBSERVATIONS FOR THE ACUTE DERMAL LD_{50} OF RO 4 ON RABBITS

Dose (ml/kg B.W.)	Animal Number	Hour 24	48	72	Day 4	5	6	7	8	9	10	11	12	13	14
Repeated 20	13 M	E	E	E	E	E	E	E	E	E	E	E	E	E	E
												D	D	D	D
	14 F	E	E	E	E	E	E	E	E	E	E	E	E	Death	
												D	D		
	15 M	E	E	E	E	E	E	E	E	E	Death				
									D	D					
	16 F	E	E	E	E	E	E	E	E	E	E	E	E	Death	
								D	D	D	RR	RR	RR		

D = Decreased locomotor activity.
E = Edema/Erythema.
RR = Loss of righting reflex.

Similar effects were observed in part B of the study with the following exceptions. The association between decreased locomotor activity and death was not as apparent as in the part A. Depressed activity was observed in animals that did not die and its onset was observed earlier in the study.

One discrepancy between the two groups of animals was noted. Pupillary constriction was described in all of the rabbits treated with all of the oils in the second part of the study. Such observations were not reported in part A of the study nor was it observed when the materials were placed directly in the eye in eye irritation studies or following oral administration.

Postmortem examinations of the major viscera and skin indicated tissue changes reflecting the irritating properties of the oils and indicated that the toxic effects of the shale oils were upon the liver.

Gross discoloration of the liver was noted and histologic examinations indicate a gross dose response relationship as shown in Table 11. The data on this table show an increase in the degree of liver pathology as one increases the dose of the shale oils.

TABLE 11. LIVER TOXICITY OF SHALE OILS IN RABBITS (RO 1, RO 2, RO 3, RO 4)

Dose (ml/kg)	Normal	Fatty Changes	Focal Necrosis	Diffuse Necrosis
5	16/32	12/32	4/8	0/8
10	10/32	9/32	12/32	1/32
15	7/32	15/32	5/32	6/32
20	3/30	7/30	7/30	12/30

Hematologic observations showed no distinct compound related effects. All the variations seen in the white blood cell count and differential are probably secondary to alteration in the integrity of the skin secondary to the irritant properties of the oils themselves.

Significant increases were reported for the serum cholesterol, BUN, and SGOT with varying recovery during the 14 day observation. Bilirubin and uric acid levels were increased as well. In contrast, phosphorus, alkaline phosphatase, and LDH levels were lowered; good dose response relationships were not observed. (Although the Laboratory has data on all the rabbits prior to exposure to the oils and experience using this technique in similar studies, it should be pointed out that concurrent controls were not used.)

Examination of the individual data values showed a great deal of variation from animal to animal and from time period to time period which did not correlate well with dose, time, or apparent clinical condition of the animal. I think one could conclude, however, the clinical chemistries probably do reflect the systemic toxicity of the shale oils particularly those effects on the liver.

All of the materials were evaluated for sensitization potential using guinea pigs. Table 12 shows the amount and concentration of the material applied to the skin of the guinea pigs six hours a day, three times a week for three consecutive weeks. The animals were subsequently challenged six weeks later. None of the materials produced sensitization in this test. Some deaths were observed in the treated animals, but these were thought not to be solely compound related. No significant findings were observed on gross autopsy of the animals that died and no systemic effects were observed during compound administration or challenge.

TABLE 12. SENSITIZATION TEST (GUINEA PIG)

	Volume of White Oil Suspensions in ml	% Suspension
RO 1	1.0	0.5
RO 2	1.0	1.0
RO 3	1.0	0.5
RO 4	1.0	1.0
RS 101	0.5	40
RS 102	0.5	30
RS 103	0.5	40
SS 201	0.5	40
SS 202	0.5	30
SS 203	0.5	40
SS 204	0.5	30

SUMMARY

The acute toxicities of several raw and spent shales and shale oils were examined using standard techniques. The rat acute oral LD_{50}'s of raw and spent shales were above 10 g/kg. The rat acute oral LD_{50}'s of the shale oils were on the order of 8 to 10 g/kg. The dermal LD_{50} for shale oil in rabbits appears to be above 10 ml/kg. Systemic toxicity observed after oral and percutaneous administration includes decreased locomotor activity, pilo-erection and ptosis. The shale oils are moderately irritating to the skin and eye; both effects would appear to be reversible. The target organ of toxicity appears to be the liver systemically and the skin and mucous membranes locally. None of the oils or shales tested appear to be sensitizing to the guinea pig.

PAPER NO. 3

CARCINOGENESIS STUDIES - OIL SHALE

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Text of this presentation is not available for publication.

PAPER NO. 4

TOXICOLOGY OF SHALE MATERIALS - CHRONIC INHALATION STUDIES

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INTRODUCTION

As previous speakers have indicated, shale rock may be made to yield a raw shale oil by any one of several retorting processes. It is evident that the rock itself may have to be mined, be crushed to some extent, transported, and crushed further for above-ground retorting; and, in the case of in situ retorting, some mining and rubblizing operations are also required. Since shale rock contains siliceous materials, the question of a pneumoconiotic effect as the result of long-term inhalation arises. The kerogen and other organic constituents of the rock contain condensed ring compounds, so a possible carcinogenic action may also be envisioned under the same circumstances of dust inhalation.

Raw shale oil is exceedingly viscous and of low volatility, so it is unlikely to present any kind of inhalation hazard under anticipated plant conditions.

Once the shale rock has been retorted, there is left behind a spent or processed shale containing some fine particles. In above-ground processes, the spent shale must be loaded and transported to some disposal site. Again, siliceous materials are present, and also residual organic material not removed by retorting.

The considerations examined above suggested the value of conducting long-term inhalation studies with raw shale rock dust and the dust of spent shale. On the other hand, there seemed to be little reason to conduct such studies on raw shale oil. The end points in the dust studies could be pneumoconiotic or carcinogenic effects.

EXPERIMENTAL DESIGN

In 1976, fairly standard protocols were developed for the proposed long-term inhalation studies, utilizing a representative raw shale rock and a processed or spent shale. It was decided to expose two species, rats and cynomolgus monkeys, in the trials. The design called for 5 groups, each containing 100 rats and 8 monkeys, both species equally divided as to sex, to be exposed as follows:

Group 1 - Common control, air-exposed,
Group 2 - Raw shale dust, low concentration,
Group 3 - Raw shale dust, high concentration,
Group 4 - Spent shale dust, low concentration,
Group 5 - Spent shale dust, high concentration.

The target concentrations selected for the two levels of dust were 10 and 30 mg/m^3. The exposure pattern was 6 hours per day, 5 days per week, for a total of 2 years.

The program of biological measurements and observations being conducted on the animals includes the following:

Body weights, weekly for 13 weeks and monthly thereafter.
Clinical signs of toxicity on the same schedule.
A check for deaths twice daily, with a policy of sacrifice of any animal that appears moribund.
For rats, palpation for tumors at each weighing.
For monkeys only, a series of pulmonary function tests comprising respiratory rate and tidal volume (hence minute volume) in unanesthetized animals; airway resistance on inspiration and expiration, dynamic compliance, forced expiratory flow-volume curve, diffusion capacity of the lung, functional residual capacity, and distribution of ventilation in animals anesthetized with pentobarbital; measurements of arterial blood oxygen and carbon dioxide tensions and pH in tranquilized animals.

These pulmonary function tests are conducted in accordance with the following schedule:

a. Twice preexposure two weeks apart
b. After 4 weeks of exposure
c. After 3 months of exposure
d. Every 3 months thereafter to termination.

Hematological and biochemical determinations are also being carried out on the monkeys at 8, 16, and 24 months. A standard hemogram is obtained (RBC, WBC, differential, hematocrit, and Hb). The clinical biochemical measurements include Na, Ca, K, Cl, SGOT, SGPT, alkaline phosphatase, total bilirubin, total protein, BUN, albumin, CO_2, and fasting glucose.

Moribund animals and those sacrificed at termination are subjected to a gross necropsy, with weights of major organs being recorded, and some 35 tissues and organs fixed in formalin for histopathologic examination.

Consideration was given to the use of a test for collagen formation in lung tissue at termination by means of hydroxyproline estimations, but the value of this test is now under question.

RESULTS

These studies are in progress at one of the major life sciences contracting companies and are now in their 23rd month. In other words, surviving animals will be sacrificed next month.

Dust concentrations in the chambers, as determined from gravimetric samples taken daily, are as follows:

Group 2	10.62 ± 4.28 (±S.D.)
Group 3	29.55 ± 8.97
Group 4	9.97 ± 3.28
Group 5	27.93 ± 8.29

Particle size distributions, measured biweekly, are as follows:

Group 2	3.93 (MMD in microns)
Group 3	3.97
Group 4	4.34
Group 5	4.54

In the early weeks of the study, 5 monkeys died as a result of trauma from fighting. The first to die was replaced. Of the 4 missing animals, there is one each from Groups 1, 2, 3, and 5. Because of an intercurrent infection, a large number of rats have died, the numbers being fairly evenly distributed among the five groups. None of the deaths in either species is attributed to effects of the dust inhalation.

None of the various measures of response - weights, hematology, clinical biochemistries, pulmonary function tests and blood gas analysis - have shown any consistent or statistically significant changes in any of the groups that can be attributed to the dust inhalation to which the animals were subject. The presence of dust has been demonstrated microscopically in the lungs of animals that died in the course of the study or were sacrificed because moribund.

The results of the histopathological examinations should be available within a few months and the completed studies will be reported in due course.

PAPER NO. 5

TOXICOLOGY OF SHALE OIL - MUTAGENESIS AND TERATOGENESIS STUDIES*

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MUTAGENESIS

A battery of assays for the determination of potential mutagenic and carcinogenic activity of raw shale, shale oils, and retorted shale has been performed. The tests were designed to evaluate the genetic properties of these materials in a variety of organisms and included the following (Litton Bionetics, Inc., 1977c): assays in Salmonella typhimurium, using several strains of histidine requiring mutants, i.e. the Ames test, using both plate (Ames et al., 1975) and suspension (Malling, 1971) cultures, the yeast Saccharomyces cerevisiae strain D_4 gene conversion test (Loprieno, 1978), the thymidine kinase L5178Y mouse lymphoma cell assay (Clive and Spector, 1975), and the rat bone marrow in vivo cytogenetics assay for aberrations of mitotic chromosomes (Dean, 1969).

In the Ames Salmonella tests, maximum doses of oil samples for 50 percent killing of bacteria and 0.5 mg/ml doses of raw shale or retorted shale were employed. The samples were dissolved in DMSO (1:1) and diluted for 5 or 6 dose levels. Each dose level was administered to the 5 tester strains of bacteria. In identical experiments, the tester strains were activated with a rat liver post mitochondrial supernatant and a NADPH generating system to stimulate the metabolic conversion of potential mutagens. The dosed inoculla were incubated for approximately 48 hours and the revertant colonies scored. Appropriate solvent and tissue controls as well as positive chemical controls exhibiting either frameshift or base-pair substitution activity - ethyl methanesulfonate, methyl nitrosoguanidine, 2-nitrofluorene, quinacrine mustard, 2-anthramine, 2-acetyl aminofluorene, 8-aminoguinoline, and dimethylnitrosamine, were utilized.

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*This work was performed by Litton Bionetics, Inc., Kensington, Maryland under contracts with the American Petroleum Institute, and the report has been designated as Publication No. 2299 by Texaco, Inc.

Under activation conditions, significant mutagenic activity for samples RO-1, RO-3, and RO-4 was observed (Litton Bionetics, Inc., 1977a, 1978a, 1978b). The strain TA-98 demonstrated the greatest number of revertant colonies, but TA-1537 and TA-1538 were also active. On the basis of these experiments, a frameshift mechanism for the observed mutagenic activity of the shale oils was postulated.

The tester strain TA-1535 did not show significant mutations by oil shale in these experiments. Strain TA-100 was also not affected or only very slightly affected by the shale oil or shale samples. In the plate assays under non-activation conditions, oil sample RO-1 showed significant mutagenic activity in the strain TA-98. In no other Salmonella assay was significant mutagenic activity detected for shale oil samples without microsomal activation.

In the suspension culture experiments, similar results were obtained. The samples were not mutagenic in nonactivation tests, but strains TA-98 and TA-1538 were reverted in the presence of microsomes. Increases in the mutation rate of the other strains were generally not observed (Litton Bionetics, Inc., 1977a, 1978a, 1978b).

The yeast D_4 strain showed no mutagenic activity in any experiment.

Samples of raw shale or retorted shale were not active in any of the microbial assays (Litton Bionetics, Inc., 1978c, 1978d).

The mouse lymphoma assay was performed at 5 dose levels of shale oils ranging from 0.01 µl/ml to 0.32 µl/ml of Fischer's culture medium. Experiments with raw or spent shale were conducted at 5 dose levels up to 5 mg/ml. In these experiments, cells heterozygous for thymidine kinase were scored for forward mutations to TK^-/genotype by cloning the treated cells in soft agar with bromodeoxyuridine (BUdR), which kills all cells not undergoing mutations. The positive control chemicals were ethyl methanesulfonate and dimethylnitrosamine.

Shale oil RO-1 exhibited a significant increase in mutations at 0.32 µl/ml in nonactivation tests, but increases in mutations observed in activation experiments using a post mitochondrial fraction from mouse liver were not significant. The data are regarded as weakly positive (Litton Bionetics, Inc., 1977a). Considerable cell killing was observed which may account for the results obtained. Experiments with sample RO-3 were negative under nonactivation and activation conditions (Litton Bionetics, Inc., 1978a), while experiments with sample RO-4 showed that the

cells were mutated to some degree at the high end of the dose range. No clear dose response was observed, but the increases were statistically significant from solvent or negative controls (Litton Bionetics, Inc., 1978b).

In the experiments conducted with raw shale, a slight but not significant increase in mutations was observed at the higher dose levels under both nonactivation and activation conditions (Litton Bionetics, Inc., 1978c), while experiments with retorted shale were clearly negative (Litton Bionetics, Inc., 1978d).

To determine the effect of raw shale, shale oils, or retorted shale on mitotically active cells from rat bone marrow, male Charles River rats were administered by gavage 0.5 ml/kg, 1.7 ml/kg and 5.0 ml/kg of the test materials mixed with corn oil. The rats were sacrificed 6 hours, 24 hours, or 48 hours after dosing in acute studies, and 6 hours after the last of five identical daily doses in subacute experiments. The dividing cells were arrested in metaphase by injection of 4 mg/kg colchicine 2 hours prior to killing the rats. Cell spreads were prepared by dropping a suspension of marrow cells on a glass slide and staining with 5 percent Giemsa. In each test, about 250 cells were scored for chromosome breaks, gaps, fragments, and unusual chromosome rearrangement figures. A negative control group administered corn oil and a positive control group given triethylene melamine were provided.

In the experiments withraw shale, the subchronic dose group of 5000 mg/kg showed a significant increase in chromosomal abnormalities; the abnormalities of the intermediate dose group were high, but the increase was not significant (Litton Bionetics, Inc., 1978c). All other experiments conducted on shale oils, raw shale, or retorted shale did not show significant increases in chromosomal abnormalities in the rat bone marrow cytogenetics assay.

TERATOGENESIS

Teratology investigations were conducted on the same test samples to ascertain potential effects on developing fetuses. Pregnant female Charles River CRL:COBS-CO(SD)BR rats, 25 per group, were exposed to raw shale dust or retorted shale dust on day 6-15 of gestation 6 hours per day. The concentrations of the dusts in the inhalation chambers were 25 mg/m^3 and 100 mg/m^3. On day 20 of gestation the contents of the uterus were examined and the fetuses taken for skeletal analysis or soft tissue evaluation. Identical protocols were utilized in studies with shale oils at chamber concentrations of 5 mg/m^3 and 100 mg/m^3.

In the shale dust studies, no departures from the expected number of viable pups per implantation site were observed. The occurrence of fetal deaths was not abnormal, and inordinate numbers of resorptions of fetuses did not occur. In the studies with shale oils, the samples RO-1, RO-3, and RO-4 were associated with a decreased live fetus per implantation site ratio which was statistically significant only for the 100 mg/m^3 dose level when compared to control values (Litton Bionetics, Inc., 1977b, 1979a, 1979b). The occurrence of fetal deaths was not abnormally high when compared to controls in any study. The incidence of commonly observed abnormalities and unusual skeletal variations was within the range of those observed in control litters.

In these studies, no deaths occurred in the dams during exposure to shale dusts or shale oils. The concentration of 100 mg/m^3 shale oil aerosol caused significant decreases in food consumption and body weight on day 15 and day 20 (Litton Bionetics, Inc., 1979b). Examination of the females subjected to the retorted shale dusts and shale oils revealed lung changes in some rats, primarily discoloration and mottling, which was attributed to the deposition of the test material in the lungs (Litton Bionetics, Inc., 1978f, 1977b, 1979b).

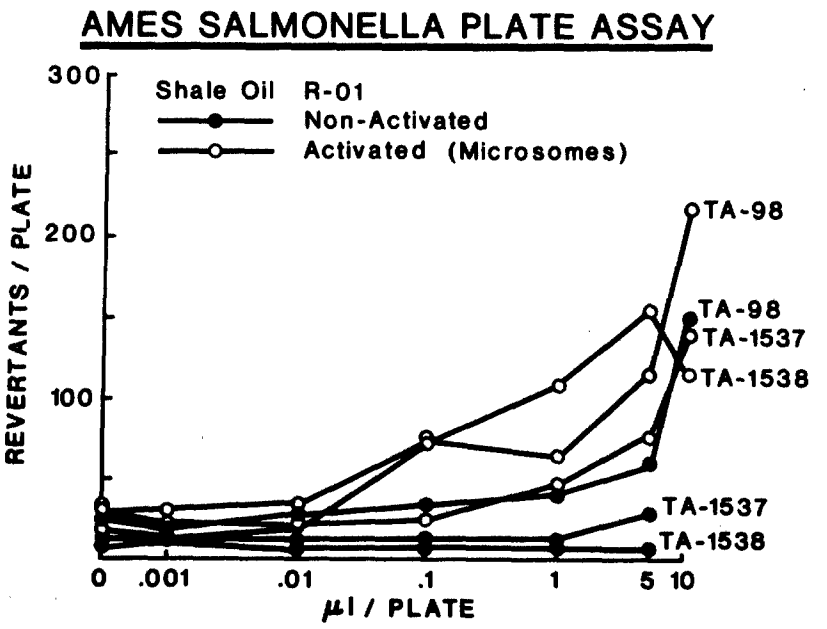

Figure 1. Induction of mutation by shale oil RO-1.

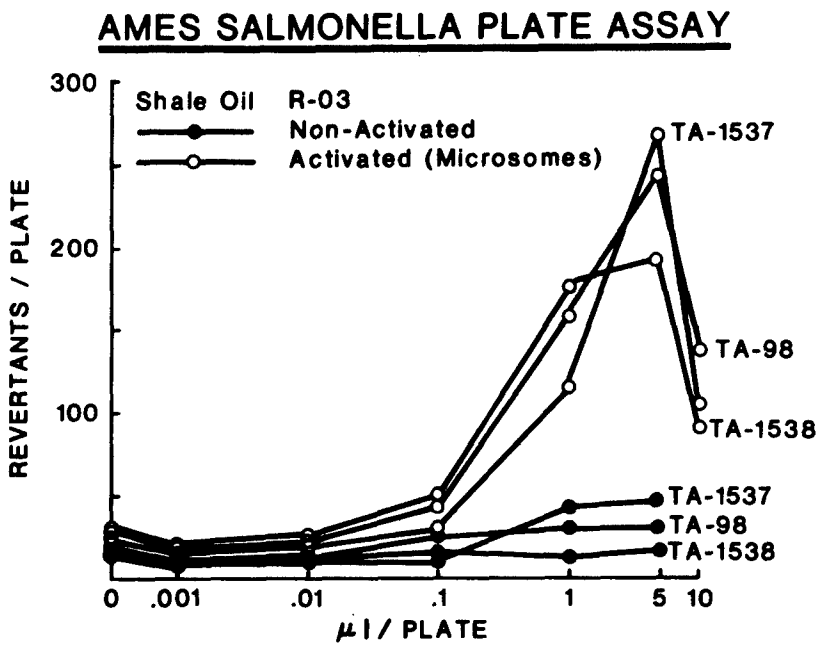

Figure 2. Induction of mutation by shale oil RO-3.

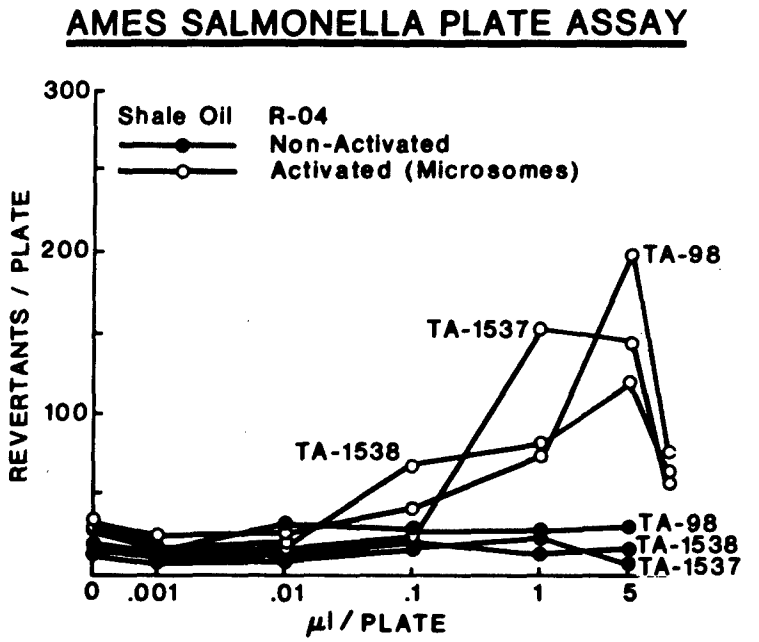

Figure 3. Induction of mutation by shale oil RO-4.

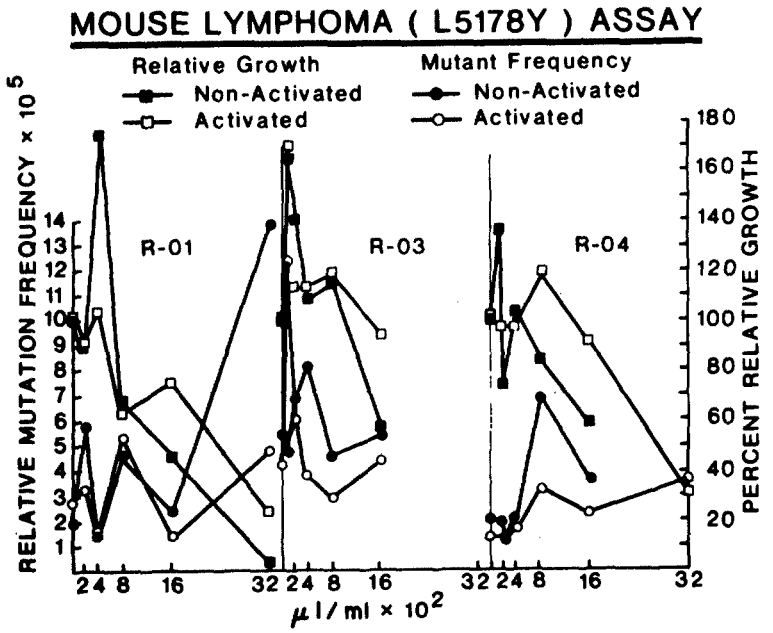

Figure 4. Induction of mutation in a mammalian cell line.

MUTAGENESIS ASSAYS

	Microbial		Mouse Lymphoma
Oil Sample	Plate Non/Act	Suspension Non/Act	Non/Act
R-01	±/+	−/+	±/±
R-03	−/+	−/+	−/−
R-04	−/+	−/+	±/±

− Negative
± Weakly positive or mixed results
+ Positive

Figure 5. Summary of in vitro mutagenesis assays of shale oils.

RAT BONE MARROW CYTOGENETICS

Sample	Dose(mg/kg)	Percent Cells with 1 or More Aberrations	
		Acute [a]	Subchronic [b]
Raw Shale	5000	1.1	5.0•
	1670	1.4	3.1
	500	0.6	0.0
R-01	5000	1.8	2.6
	1670	0.5	0.4
	500	0.8	1.2
R-03	5000	1.1	0.0
	1670	0.7	0.8
	500	0.1	1.2
R-04	5000	0.5	0.0
	1670	0.0	0.4
	500	0.6	0.5
Retorted Shale	5000	0.1	0.0
	1670	1.3	2.7
	500	0.4	2.8

a Average of 3 det. at 6,24, and 48 hr. after dose, respectively.
b Determination 6 hr. after last of 5 daily doses.
• Significantly higher than normal spontaneous range limit.

Figure 6. Rat bone marrow cytogenetics assay.

TERATOLOGY

	Live Fetuses/ Implantation Site	Malformations
Raw Shale	Normal	Negative
R-01	Normal	Negative
R-03	Normal	Negative
R-04	Normal	Negative
Retorted Shale	Normal	Negative

Figure 7. Summary of teratology experiments with shale oils and shales.

CONCLUSION

The mutagenic activity of the shale oil samples correlates well with carcinogenic activity observed in the skin painting tests (Suskind, 1979). In microbial tests, all shale oils were mutagenic in activation experiments; the oils were weakly mutagenic or negative in the mouse lymphoma cell assays, and negative in rat bone marrow cytogenetics tests. The Salmonella results have been confirmed in assays recently reported by Epler and coworkers (Epler et al., 1978a, 1978b). The principal mutagenic activity appears to reside in fractions containing polynuclear aromatic hydrocarbons (Guerin et al., 1979, 1978) and, possibly, aromatic amines and heterocyclics (Epler, 1979).

Shale oils or dusts were not teratogenic in rat studies although some fetal toxicity was observed at the high dose levels of shale oil.

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PAPER NO. 6

AN OCCUPATIONAL HEALTH STUDY OF OIL SHALE WORKERS

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INTRODUCTION

Oil shale is one of our largest undeveloped fossil energy resources. Research and development on efficient mining, processing and refining techniques are continuing in an effort to tap this enormous potential source of crude oil. During this developmental period, occupational health studies can make important contributions to our knowledge of the working conditions and potential for health problems related to oil shale work. This knowledge can be applied to future facilities and plants as the industry develops.

In late 1977, a proposal was initiated for a joint Department of Energy (DOE) and American Petroleum Institute (API) project to undertake a medical and industrial hygiene study at the Paraho oil shale facility. This facility is located near Rifle, Colorado and is operated by Development Engineering, Inc. (DEI). The occupational health study included: (1) field industrial hygiene surveys and sampling to assess possible environmental hazards in the workplace, and (2) medical examinations of the workers to determine current health status and to establish a data base for prospective epidemiologic studies.

This report describes the results of the medical evaluations conducted on the Paraho employees.

STUDY LOCATION AND PLANT SITE

The Paraho/DEI oil shale facility is located on a Naval Oil Shale Reserve at Anvil Points, Colorado, about seven miles west of Rifle at the edge of the Colorado Plateau. The present Paraho/DEI operations were started in 1974, first with construction and operation of a 4-1/2 foot diameter pilot scale retort, and later with a 10-1/2 foot diameter semi-works scale retort. The current installation consists of the mine, crushing and storage areas for the shale rock, the two retorts, spent shale disposal area, and laboratory, administration, maintenance and warehouse buildings. Most of the buildings date from the 1940's Bureau of Mines operations.

The mine is in the Green River formation of the Piceance Basin. The oil shale rock is mined using a conventional room-and-pillar technique. In the present system, the rock from the mine is hauled from mine to plant site in ore trucks. The road drops more than 2000 feet in its 5-1/2 mile length. The rock is then crushed in a series of two jaw and one roll crushers to a size range of 1/2 inch to 2-1/2 inches. The crushing equipment consists primarily of converted road building equipment.

The crushed rock is fed continuously into vertical retort vessels through a revolving seal mechanism. The retorts have self-sustaining combustion which burn the free carbon contained within the shale rock. This heating process reaches temperatures in the 900 F to 1400 F range. The heat breaks down the kerogen, the naturally occurring solid organic material, within the matrix of the rock and forms a vapor and mist within the upper regions of the retort chamber. The oil-laden mist is then conducted away from the retort, condensed, and the heavy oil shale crude formed.

Processed or "spent" shale is removed from the bottom of the retort. In the present operation, the spent shale is disposed of above-ground in an adjacent canyon.

POPULATION

The DEI/Paraho oil shale operations employed a total of 92 persons in August 1978 when the medical examinations were conducted. Six of these persons spent all, or virtually all, of their time in the DEI offices in Grand Junction, Colorado. The remainder of the employees worked on-site at Anvil Points. Of the 92 employees, 87 (95%) volunteered to participate in the study. Seven of these 87 were females.

For the purposes of analysis of the results of the study, the 87 persons examined were divided into three groups (see Table 1).

TABLE 1.

	GROUP I "Highest Exposed"	GROUP II "Less Exposed"	GROUP III "Minimally Exposed"	TOTAL
Number	15	45	27	87
Male	15	44	21	80
Female	0	1	6	7
Mean Age (Years)	38	37	49	

Group I is comprised of 15 persons, all male, who were identified by DEI as having the greatest exposure to the shale oil production process, whether in mining, crushing, retorting or maintenance. This group, in a very rough estimation, was the "highest exposed" group.

Group II contains the remaining employees who had contact to an appreciable degree with any aspect of mining, transporting, processing, or analyzing the material. This group is comprised of 45 persons, including one female laboratory technician.

GROUP III is made up of those employees who, at the time of the study, were engaged in activities such as research or administration and had little or no contact with the shale or its processing. This group contains six females.

Mean ages in the three groups were: Group I, 38 years; Group II, 37 years; and Group III, 49 years.

Characterization of these groups of employees with respect to duration of employment in oil shale and in DEI/Paraho oil shale operations proved difficult due to the wide range in length of employment. Mean duration of employment at Anvil Points for Groups I and II was identical - just under three years. Many of the employees in Groups I and II had previous experience at other oil shale facilities as well, both above-ground and below-ground (in situ). In addition, some of the administrative personnel presently in Group III had oil shale exposure histories dating back as far as 30 years, but with negligible recent exposure (see Table 2).

TABLE 2.

Years in Oil Shale Industry	GROUP I	GROUP II	GROUP III
<1	0	7	4
1-5	11	30	18
6-10	3	6	3
11-15	1	1	1
>15	-	1	1
TOTAL	15	45	27

Another important area with regard to work history was previous mining or other similar dust exposure. This was felt to be of importance particularly in those individuals who had many years exposure in underground hard-rock or uranium mining prior to becoming engaged in oil shale mining or processing.

PROJECT DESIGN

Prior to conducting the medical evaluations of the DEI/ Paraho employees, extensive preparatory work was carried out.

Literature review showed no evidence of a similar comprehensive health evaluation in oil shale workers. One early study of dermatologic effects done during the late 1950's is currently being followed up under NIOSH sponsorship, but with examinations limited to pulmonary and urine cytopathology and dermatologic evaluation. Industrial hygiene and toxicology literature suggested that the skin and pulmonary organ systems would be the most likely to be affected by oil shale exposure.

Following planning visits to the area and enthusiastic responses from local hospital and medical personnel in Rifle, it was decided to utilize local resources as much as possible in carrying out the examinations. This approach, which made the study in part a local effort in support of a local industry, was well received and probably contributed to the extremely good response for participation in the study.

As a further aid to obtaining worker cooperation in the study, the examinations were conducted during working hours for each employee, with the study paying for one-half working day for each employee examined. DEI administrative personnel provided valuable assistance in scheduling of examinations.

Several weeks prior to the examinations, introductory meetings were conducted on-site at Anvil Points with small groups of employees. Written information regarding the nature and content of the study was given out following verbal presentations by the physicians on the study. Questions regarding confidentiality and DEI company involvement were carefully addressed. Employees were also made aware that each of them would receive a personal follow-up letter from a physician on the study regarding his or her individual findings and evaluation. Additionally, copies of all results would be made available to the employee's private physician if the employee so desired.

Following these meetings, greater than 95% of the employees volunteered for, and subsequently participated in, the study.

MEDICAL STUDY ELEMENTS

The comprehensive medical evaluations of DEI/Paraho employees were carried out during a three week period in August 1978. The evaluations consisted of:

1. Medical History: ROCOM Occupational Health History Questionnaire, plus physician interview.
   a. Family history,
   b. Social history, including education level,
   c. Habits, including detailed lifetime smoking history,
   d. Past medical history,
   e. Current systems review.
2. Occupational History: Detailed lifetime occupational and exposure history.
3. Accident History: Job-incurred accidents for previous 12 months.
4. Physical Examination: Complete examination, including rectal and optional pelvic examinations, with special attention to skin and pulmonary systems. Recorded on ROCOM Occupational Health Data Base form.
5. Laboratory and X-ray:
   a. Urinalysis,
   b. CBC,
   c. Chemistry profile (25 components),
   d. Pulmonary Function Testing (FVC, FEV-1, FEV-1/FVC ratio, FEF 25-75),
   e. Chest x-ray to meet standards for pneumoconiosis interpretation,

f. Sputum for cytology - read by Dr. Geno Saccomanno, Grand Junction, Colorado,
g. EKG - at the option of examining physician.

6. Special Studies:
   a. Ames Testing of urine - last evening and first morning specimens,
   b. Aryl Hydrocarbon Hydroxylase inducibility in peripheral lymphocytes done by Dr. M. Rasco, M.D., Anderson Hospital, Houston, Texas,
   c. Chromosome Karyology on peripheral lymphocyte culture preparations - done on Group I only (due to expense and time considerations).
7. Epidemiologic Record: Standard form completed of personal information useful in future epidemiologic tracking and studies; kept separate from medical records.

RESULTS

Results from this group of 87 DEI/Paraho oil shale workers revealed generally a healthy population with only a few serious acute or chronic medical problems. There appeared to be no indication of health problems or effects which could be attributed to or positively correlated with exposure to oil shale operations. What health problems existed were readily attributable to other contributing factors such as smoking or previous mining history, particularly hard-rock or uranium underground mining.

SKIN FINDINGS

Skin problems were few in number and all of rather benign character. Frequency was greater in Group III than either Group I or Group II (see Table 3).

TABLE 3.

	GROUP I	GROUP II	GROUP III
Number	15	45	27
Mean Age (years)	38	37	49
Skin Problems (all types)	1	4	6
Skin Cancer (at time of exam.)	0	0	0
Skin Cancer (past)	0	0	2
Pulmonary Problems (all types)	3	4	3
Opacities noted on Chest X-ray	1	5	4

The only skin problem of any sort in Group I was that of one case of dermatophytosis affecting the feet (athlete's foot). In Group II, only four persons had skin problems of any sort, all rather minor in nature. One individual had a photosensitivity reaction, probably to hydrochlorothiazide (a blood pressure medication), which was exaggerated on his left arm which received considerable sun exposure in the course of his work as a truck driver. The other skin problems found in Group II were mild atopic dermatitis in a recent employee, a long-standing patch of vitiligo in another, and mild psoriasis affecting the neck area of a third employee. The individual with atopic dermatitis had noted no change in the problem during his three months employment at Anvil Points. The patch of vitiligo was present on that individual prior to his entering the oil shale industry.

Thus, among the 60 employees in Groups I and II who were exposed to oil shale or its products, five had skin problems of any sort. None of these skin problems appeared to be attributable to oil shale exposure.

Six persons with skin problems were encountered in Group III, the group with least current exposure. The most significant of these were one actinic and one seborrheic keratosis in one person, a basal cell skin cancer which had been treated with radiation in another, and a history of a singular excised skin cancer of unknown type in a third person. Other skin problems found in this group included skin photosensitivity, mild atopic dermatitis, and past x-ray treatment for dermatitis of an unknown type. The somewhat higher prevalence of skin problems in Group III probably reflects the 11-12 year older mean age of this group.

PULMONARY FINDINGS

Pulmonary problems encountered appeared to be related not to shale or shale oil exposure, but rather to smoking and, in some cases, probably to long mining histories other than oil shale mining.

In Group I, the "highest exposed" group, we found chronic obstructive pulmonary disease and chronic bronchitis in smokers with 90 pack-year and 72 pack-year histories respectively (one pack year = one pack smoked per day for one year). Chest x-rays on these two individuals were interpreted by both "A" and "B" level readers as normal. The only other pulmonary finding of note in Group I was minimal diffuse interstitial change on x-ray in one male employed a total of 5 years at Anvil Points as a miner, but with a 40 pack-year smoking history and 11 years previous underground mining work which included 6 years uranium mining, 3 years molybdenum mining and 2 years coal mining.

In Group II (45 employees) two individuals had histories of asthma - one a laborer and the other a professional employee - both recently hired. The latter had a probably associated x-ray finding of prominent pulmonary outflow and vascularity. The only other pulmonary problems reported among this group were two cases of bronchitis in smokers and a vague history of "lung problems" in a recently hired middleaged mechanic/welder.

Chest x-rays on members of Group II showed 4 of the 45 to have minimal small rounded opacities or minimal interstitial changes. All of these findings were noted for the strict recording criteria of pneumoconiosis records, but fell within the realm of normal for routine interpretation. The only other x-ray finding reported for this group was irregular, rounded opacities in moderate number (but increased since 1970) in a 60 year male employed just over one year. This individual smoked heavily until 10 years ago and had engaged previously in 17 years underground mining, as well as having worked several years as a hot tar roofer.

Pulmonary problems encountered in Group III, the minimally exposed group, included two individuals, ages 69 and 71, with chronic obstructive pulmonary disease and long, heavy smoking histories. Two persons had mild interstitial changes on chest x-ray. One of these has a history of asthma. The other is a heavy smoker. The only other person with abnormal x-ray findings in this group was a 54 year old male, heavy smoker, with scattered miliary granulomata of unknown etiology, but consistent with old disease. This person also reported a 50 pound weight loss but had no active acute disease during an 8 month follow-up period.

Thus, chronic lung disease cases noted in all three groups had strong positive correlation with cigarette smoking. No discernible relationship of pulmonary problems with oil shale activities was evident. Some influence from past, non-oil shale mining appeared to be present in several cases.

SPUTUM CYTOLOGY AND PULMONARY FUNCTION TESTING

Pulmonary sputum samples were collected on all persons by the method of saline induction. All samples were analyzed in the laboratory of Dr. Geno Saccomanno, Grand Junction, Colorado, and were judged adequate samples for interpretation.

Results of the sputum cytology showed several (four) persons with moderate atypia. These cases correlated with smoking rather than oil shale exposure. Two other heavy smokers showed abnormal columnar cells, a common finding in heavy smokers. These findings also did not correlate with oil shale exposure.

Pulmonary function tests showed results similar to those already discussed. Decreased pulmonary functioning showed no correlation with oil shale exposure, but showed strong positive correlation with smoking.

LABORATORY RESULTS

Results on complete blood count, urinalysis, and chemistry profile showed no striking or significant trends. Results fit statistically expected patterns, with a small percentage of test results outside normal (two standard deviations) limits. This scatter occurred through all groups.

Results on special laboratory tests, including Ames testing on worker's urine, aryl hydrocarbon hydroxylase inducibility in peripheral lymphocytes and chromosome karyology in selected individuals, are still being evaluated.

INJURIES

Occupational lost-time injuries for the 12 months prior to August 1978 were recorded as part of the occupational history. Among the 87 employees evaluated, seven lost-time injuries occurred during this 12 month period. All were the result of accidental physical trauma.

It is not unexpected that activities which involve mining, rock handling, rock crushing and use of heavy mechanical equipment should present significant safety problems. This is particularly the case with current DEI operations at Anvil Points which are in a developmental stage and utilize a great deal of old and/or modified equipment. These operations certainly cannot be taken as representative of future commercial production. Nonetheless, the injury experience identified in this study clearly indicates the importance of occupational traumatic injury as a health factor in the oil shale industry.

SUMMARY

During August 1978, comprehensive health evaluations were conducted on 87 of the 92 employees of the DEI/Paraho oil shale

facility in western Colorado. This project was a joint undertaking of the American Petroleum Institute and the Los Alamos Scientific Laboratory of the Department of Energy.

The comprehensive health evaluations revealed DEI/Paraho employees to be in generally good health. No chronic or acute disease was found which could be attributed to exposure to oil shale or its products.

The occurrence of a significant number of lost-time job injuries during the 12 months prior to the study points out job-incurred trauma as a major health effect for workers in the developing oil shale industry. Future occupational health studies should continue to look at the sources and mechanisms of physical injuries to workers. Design of commercial scale equipment must include review of safety features and attention to development of safe operating procedures.

Health surveillance of employees in the developing oil shale industry should include periodic comprehensive medical evaluations. The aims of these examinations should be to aid employees in maintaining good health and to identify occupationally related problems, if any, at an early stage. Scope of the examinations should be such as to provide adequate information to meet these aims. Employee training programs should place particular emphasis on job safety and worker hygiene. Mechanisms must also be developed to correlate industrial hygiene data and health surveillance data.

The findings of this study should pose no impediment to development of oil shale, but rather should encourage the careful and appropriate development of this valuable resource.

PAPER NO. 7

THE NAVY'S TOXICITY AND SHIPBOARD HAZARD EVALUATION OF SELECTED OIL SHALE AND PETROLEUM DERIVED FUELS*

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INTRODUCTION

The Navy's principal interest in fuels derived from non-petroleum fossil fuel sources, or synthetic fuels, is to determine their compatibility with naval equipment and engines, and their suitability as naval fuels (U.S. Navy-ENRRDO, 1976a). Past Navy involvement with synthetic fuels has included the use of coal-derived fuels in the Navy ship, USS JOHNSTON, in 1972, and production and refining of 10,000 barrels of Paraho crude shale oil in 1974 (U.S. Navy-ENRRDO, 1976a; Applied Systems Corp., 1975a), and 100,000 barrels just completed. While product fuels obtained from the earlier refining of crude shale oil did not meet all military and Federal specification requirements due to fuel instability and the presence of wax, gum, and particulate matter, the project did demonstrate the feasibility of obtaining useful fuels from crude shale oil (U.S. Navy-ENRRDO, 1976a; Applied Systems Corp., 1975a).

*Naval Medical Research and Development Command, Research Project No. ZF57.572.001-4009. The opinions and assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large. The animals used in this study were handled in accordance with the provisions of Public Law 89-44, as amended by Public Law 91-579, the "Animal Welfare Act of 1970," and the principles outlined in the "Guide for the Care and Use of Laboratory Animals," [U.S. Department of Health, Education and Welfare Publication No. (NIH)73-23].

Current interest in synthetic fuels remains focused on crude shale oil from surface retorting, and the Navy is participating in an interagency effort to produce and refine larger quantities of crude shale oil into Military Specification fuels for subsequent test and evaluation (U.S. Navy-ENRRDO, 1976a). It is anticipated that the use of these larger quantities of crude and an improved refining process will produce acceptable fuel products in at least six fuel fractions: JET-A, JP-4, JP-5, JP-8, diesel fuel marine (DFM), and a residual fuel (U.S. Navy-ENRRDO, 1976a; Roberts, 1977).

In order to assess the suitability of shale JP-5 and diesel fuel marine (DFM) to be used by the Navy, it is important that both the toxicity of the fuels and typical fuel exposures be characterized. Comparative data on the toxicity and hazards of the synthetic and petroleum fuels are also desirable to assess engineering controls and work procedures. Unfortunately, little data has been available on the dermal, ocular, or inhalation toxicity of the distillate fuels used by the Navy, thus precluding a comparative evaluation of the fuels. The lack of such data has prompted many current research activities in this area, as well as recommendations for research by the National Institute of Occupational Safety and Health (NIOSH, 1977).

Fuels resulting from the current shale oil production and refining program may include full-scale, shipboard demonstration tests. The early availability of data on the comparative toxicity and shipboard hazards of naval fuels is desirable, and has led to the initial evaluation of selected fuel materials as a comparative baseline for future investigations with shale-derived product fuels. The following summary will provide an overview of current research in these areas, which is particularly responsive to questions related to biological effects following low-level, long-duration exposures.

MATERIALS

In order for fuels, both petroleum and shale oil-derived, to be acceptable as military fuels, they must meet certain specifications. Some of these specifications are depicted in Table 1. It is evident from this table that a lot of variation is allowed within a given specification.

TABLE 1. SOME SELECTED MILITARY SPECIFICATION REQUIREMENTS FOR JP-5 AND DFM*

	JP-5	DFM
Distillation temp °C		
Initial B.P.	---	---
10% recovery	205	---
End point, max temp	290	385
Aromatics, vol %, max	25	---
Olefins, vol %, max	5	---
Sulfur, total wt %, max	0.4	1.0
Sulfur, mercaptan, wt %	.001	---
Hydrogen content, wt %	13.5	---
Freezing point, °C, max	-46	---
Density, g/ml at 15 C, min	.788	---
max	.845	---
Flashpoint, °C, min	60	60

*Source: MIL-T-5624K, Turbine Fuel, Aviation, Grades JP-4 and JP-5, 1 April 1976.
MIL-T-16884G, Fuel Oil, Diesel, Marine, 7 March 1973.

JP-5

JP-5 is a high flashpoint aviation turbine fuel with a specified distillation temperature of 205 C (10% recovery) to 290 C (end point) (Military Specification, 1976). The fuel is a complex, kerosene-type mixture that, in some cases, has been found to contain higher boiling components than specified by Military Specification (Naval Biosciences Laboratory, 1978). Specifications permit up to 25 percent aromatic hydrocarbons and 5 percent olefins. Substituted phenols or amines are added to inhibit oxidation.

DIESEL FUEL MARINE

Diesel fuel marine (DFM) is also a distillate fuel, with a specified boiling range lower than 385 C (Military Specification, 1973). The content of aromatics and olefins is not specified but is limited only by the requirement that the fuel be clear and bright in appearance.

The following discussion will describe investigations employing these materials up to the present time.

METHODS

Personnel exposures to these fuels are being characterized by thorough industrial hygiene evaluation of ships expected to be characteristic of the Navy in the 1980's and beyond. A principal objective of the study is to quantify both atmospheric contaminants and fuel exposures associated with fuel use. Both personal (breathing zone) and general area samples for total and respirable particulate, combustion products, and hydrocarbon vapors are being collected and analyzed by a variety of methods. Jobs and occupations involving fuels are also being identified, along with exposure durations and an assessment of the potential for intermittent or prolonged skin contact with the fuels.

Until 1976, when Knave and coworkers (Knave et al., 1976) reported signs of polyneuropathy in Swedish aircraft workers chronically exposed to jet fuel vapors, the vapors of aliphatic hydrocarbons were generally reported to produce few harmful effects, even for prolonged exposures. Coincidentally, a phased, behavioral toxicological evaluation of these materials had already been initiated on naval fuels. The initial phase of this work was a general behavioral screen, in which spontaneous home-cage activity, food and water consumption, weight, and behavioral profiles of individual rats were observed after acute dosing. This phase of investigation serves to identify the range of doses that can produce gross toxic effects, as compared to those that produce no detectable behavioral signs of toxicity. In Phase 2, a battery of tests was used to define the specific type of behavior affected, and consisted of specific tests for changes in visual function, peripheral sensitivity, aggression, and motor integration. Subsequent iteration of the screening procedures involves more detailed evaluation of affected behaviors, and definition of dose and time effect relationships.

Before the behavioral experiments began, inhalation investigations were initiated to assess the inherent toxicity of military grade fuels. Table 2 is a summary of fuels inhalation toxicity studies that have been conducted since December 1974. Table 3 shows the experimental design of the first study initiated in December 1974. A high concentration of petroleum JP-5 vapor (643 mg/m^3) was selected to maximize the likelihood of observing effects due to the exposure. Fuel vapors were generated by bubbling air through the JP-5 and into a two cubic meter Rochester-type inhalation chamber. The JP-5 used for vaporization was changed every 12 hours, and the chamber concentration was sampled every 15 minutes and monitored as

total hydrocarbons. More recently, a pair of experiments (Table 2) designed to extend this earlier work on petroleum JP-5 and to provide data to serve as a basis for comparison of oil shale-derived JP-5 was initiated at the Naval Medical Research Institute (NMRI) Toxicology Detachment at Wright-Patterson Air Force Base, utilizing Thomas Domes at the U.S. Air Force Aerospace Medical Research Laboratory/Toxic Hazards Research Unit. Petroleum JP-5 vapors were generated for this study by passing the fuel through a column heated to 120 F; exposure concentrations were established for 750 mg/m^3 and 150 mg/m^3. Two-thirds of the male and female rats are presently being held for approximately their normal lifetimes. At that time, 42 tissues will be submitted for histopathologic evaluation of the 90-day continuous exposure to petroleum JP-5.

TABLE 2. SUMMARY OF FUELS INHALATION TOXICITY STUDIES

I. Petroleum Derived

A. JP-5
1. Continuous exposure
a. 643 mg/m^3
b. 750 mg/m^3 } paired exposures
c. 150 mg/m^3 }
2. Intermittent exposure
a. 500 mg/m^3 (80 6-hour exposures)

B. DFM
1. Continuous exposure
a. 14 mg/m^3
b. 167 mg/m^3
c. 50 mg/m^3 } paired exposures
d. 300 mg/m^3 }

II. Shale Derived

A. JP-5
1. Continuous exposure (planned 1979)
2. Intermittent exposure
a. 500 mg/m^3 (80 6-hour exposures)

B. DFM (planned 1979)

TABLE 3. PETROLEUM JP-5 CONTINUOUS EXPOSURE

Laboratory:	NMRI
Test Material:	Military Specification Petroleum JP-5
Concentration:	643 mg/m^3 total hydrocarbons (vapor)
Duration:	120 days (23 hours/day)
Animal Complement:	Rat 15 Guinea Pig 15
Clinical Tests:	Body Weight, Hematocrit, Hemoglobin, WBC
Histopathology:	Lung, Liver, Heart, Kidney, Spleen

In addition to the petroleum JP-5 study and its companion DFM study (Table 4), two other inhalation experiments with petroleum DFM have been conducted in the last five years, with essentially the same objectives and designs as the previously discussed JP-5 studies. The first experiment utilized a two cubic meter Rochester chamber with a total chamber hydrocarbon vapor concentration of 14 mg/m^3 chosen to assess inhalation exposures to low levels of petroleum DFM. Twenty-four of the exposed and control rats were held for approximately two years for histopathologic evaluation. A second inhalation experiment was conducted with a vapor and aerosol. The chamber concentration was maintained at 167 mg/m^3 total hydrocarbons for a duration of 120 days in an attempt to obtain an effect which could serve as a benchmark for further observations. In both of these experiments, 24 rats were held for two years in order to assess the long-term effects of 120-day continuous inhalation exposure to petroleum DFM.

The continuous inhalation toxicity studies with shale JP-5 and DFM that are listed as planned for 1979 on Table 4 have begun this month with the arrival of batches of shale fuel large enough to do 90-day continuous studies at two concentrations. This table outlines the protocol to be used in these studies. They will be conducted at the U.S. Air Force Aerospace Medical Research Laboratory/Toxic Hazards Research Unit. These experiments are companion studies to the petroleum JP-5 and DFM studies that will be reported tomorrow. They are designed to provide a basis of comparison between the petroleum and shale fuels.

TABLE 4. SHALE JP-5 AND DFM CONTINUOUS EXPOSURE

Laboratory:	NMRI/AMRL/THRU
Test Material:	Military Specification Shale JP-5 and DFM (Source: SOHIO refined, Paraho crude)
Concentrations:	JP-5: 750 mg/m^3 and 150 mg/m^3 total hydrocarbons
	DFM: 300 mg/m^3 and 50 mg/m^3
Duration:	90 days
Animal Complement:	(at each concentration) Rats: 75 male and 75 female (50 of each being held for 2 years) Mice: 150 female Dogs: 3 male and 3 female
Clinical Tests:	Body Weight, Hematocrit, Hemoglobin, RBC, WBC, RBC Indices, Sodium, Potassium, Calcium, Serum Proteins, Glucose, Alkaline Phosphatase, SGOT, SGPT, Bilirubin, Creatinine, BUN
Histopathology:	90 days - 42 tissues 2 years - 21 tissues

In the most recent set of experiments, groups of Fischer 344 rats, both male and female, were exposed to petroleum JP-5 and shale JP-5. An outline of the protocol used for each group is depicted in Table 5. The exposure was an intermittent inhalation exposure to a concentration of 500 mg/m^3 of vapor and aerosol in the case of the petroleum JP-5 (Table 5) and to a concentration of 500 mg/m^3 of vapor in the case of shale JP-5 (Table 6). In each case, there were 80 six-hour per day exposures over 16 weeks. Each fuel exposure group had a corresponding control group. At exposure end, 24 hour urine samples and blood samples were collected and the rats were necropsied for histopathologic evaluation.

TABLE 5. PETROLEUM JP-5 INTERMITTENT EXPOSURE

Laboratory:	NMRI/TD
Test Material:	Military Specification Petroleum JP-5 (Source: Wright-Patterson AFB)
Concentration:	500 mg/m^3 vapor and aerosol
Duration:	80 six-hour/day exposures
Animal Complement:	Rats (18 male and 18 female)
Clinical Tests:	Weekly Body Weight, HCT, HGB, RBC, WBC and Differential, Urine R&M, Bilirubin, SGOT, SGPT, SDH, LDH, BUN Clearance, Creatinine Clearance, Glucose
Histopathology:	Exposure End - Major Organs

TABLE 6. SHALE JP-5 INTERMITTENT EXPOSURE

Laboratory:	NMRI/TD
Test Material:	Military Specification Shale JP-5 (Source: Paraho Crude, Exxon Refined)
Concentration:	500 mg/m^3 vapor
Duration:	80 six-hour/day exposures
Animal Complement:	Rats (18 male and 18 female)
Clinical Tests:	Weekly Body Weight, HCT, HGB, RBC, WBC and Differential, Urine R&M, Bilirubin, SGOT, SGPT, SDH, LDH, BUN Clearance, Creatinine Clearance, Glucose
Histopathology:	Exposure End - Major Organs

At this point, I would like to spend a few moments discussing the two fuels used in these experiments since, as we have seen earlier, the Military Specification allows for quite a bit of variation from one batch of JP-5 to another.

The petroleum JP-5 used in this experiment met these specifications and was obtained from stock available at Wright-Patterson Air Force Base. The shale JP-5 used in this experiment was one of two Mil Spec fuels produced in 1977 by hydrotreating a 125 barrel batch of Paraho crude. Table 7 compares some selected Mil Specs with the sample inspection findings for this batch of shale JP-5. It was prepared by Exxon under an Air Force Aero Propulsion Laboratory contract and met all the Mil Specs with the exception of freezing point. Since tailoring the fuel to make a minor adjustment in this Spec would have consumed valuable product, no further distillations were attempted.

TABLE 7. COMPARISON OF SELECTED MILITARY SPECIFICATIONS, NMRI/TD 9-79 SHALE JP-5

	MIL SPEC	ANALYSIS
Boiling Range	*/554	330/490 F
S, Wt %, Max	.4	0.032
N, WPPM		12
FIA, Vol %, Max		
Aromatics	25	12.9
Olefins	5	1.1
Saturates		86.0
Flashpoint, °F	140	140
Freeze Point, °F	-46	-41.8
Smoke Point, Min	19	28
Viscosity, CS @ -30 F, Max	8.5	9.72
API Gravity	36 min/48 max	46.3

*Report only.

RESULTS

In the case of the intermittent exposures to petroleum JP-5 at 500 mg/m^3, review of the hematologic and blood chemistry data did not reveal any striking changes between the test and the control animals in either sex. There was, however, a small but statistically significant decrease in some of the hematologic parameters, namely the red blood cell count, white blood cell

count, and hematocrit and the hemoglobin for both sexes. Of the blood chemistries performed, slight statistically significant increases were seen in the alkaline phosphatase and blood urea nitrogen values in both sexes. Female rats also exhibited slightly elevated plasma creatinine and slightly decreased bilirubin values.

In the case of the intermittent exposures to shale JP-5 at 500 mg/m^3, there was a slight decrease in female red blood cell counts and an increase in plasma creatinine. The male animals exhibited only a slight increase in plasma creatinine. Comparison of test and control animal body weight data showed a normal growth curve for the test animals of both sexes and both fuels.

Since both of these are recent experiments, completion of the histopathologic evaluation is not yet available; however, review of the necropsy reports gave no indication of gross lesions.

Turning to earlier investigations with inhalation exposures of rats and guinea pigs to petroleum JP-5 at 643 mg/m^3, no deaths were attributable to the fuel and the results of hematologic and histopathologic examinations were unremarkable. In none of the earlier studies of petroleum JP-5 were animals held for a lifetime after exposure in order to examine for latent effects.

Earlier investigations of continuous inhalation exposures to petroleum DFM are represented in Tables 8 and 9. Table 8 represents results of an exposure to 14 mg/m^3, a concentration that could conceivably occur aboard a naval ship. Table 9 depicts results of hematologic examination and body weight data on rats exposed continuously for 120 days to approximately 10 times the amount of DFM. In both cases, the hematologic and body weight data are unremarkable. The results of histopathologic evaluation at exposure end were also unremarkable; however, half of the exposed rats were held for a lifetime. Examination of tissues taken at that time were, in general, consistent with incidental findings in aged rats. There was, however, an overall increase in tumors seen in the high dose group over the low dose and both control groups. Table 10 is a review of the findings. The addition of gross tumor-like masses to the total tumor chart is justified on the basis of the location and gross appearance of these masses that were inadvertently not sampled for histopathology. The presence of an overall increase in tumor incidence in high dose exposed animals, with no

individual tumor predominating, certainly does not constitute grounds for the identification of a carcinogen, it does stress the need for holding exposed animals for examination of latent effects.

TABLE 8. PETROLEUM DFM CONTINUOUS EXPOSURE

Laboratory: NMRI Concentration: 14 mg/m^3

Histopathology: 90 days - unremarkable
2 years - heart, liver, bile duct, kidney, lung, and spleen

90-DAY RAT RESULTS (AS PERCENT OF CONTROL)

	30 Day	60 Day	90 Day
Hematocrit	108	95	90
Hemoglobin	102	101	89
WBC	78	114	75
Body Weight	121	102	104

TABLE 9. PETROLEUM DFM CONTINUOUS EXPOSURE

Laboratory: NMRI Concentration: 167 mg/m^3

Histopathology: 120 days - unremarkable
2 years - heart, liver, bile duct, kidney, lung, and spleen

120-DAY RAT RESULTS (AS PERCENT OF CONTROL)

	30 Day	60 Day	90 Day	120 Day
Hematocrit	99	94	93	100
Hemoglobin	96	93	96	97
WBC	131	120	111	98
Body Weight	88	90	91	89

TABLE 10. PETROLEUM DFM CONTINUOUS EXPOSURE TUMORS

	167 mg/m^3	Control	14 mg/m^3	Control
Number of Animals Examined Microscopically	20	19	18	22
Tumors	8	5	5	7
Gross Tumor-Like Mass	4	0	1	2
Total Tumors	12	5	6	9
Tumor Incidence	60%	26%	33%	41%

DISCUSSION

Few data have been available to assess the inherent toxicity of product fuels derived from petroleum, with the exception of earlier investigations related to the chronic toxicity of the Air Force JP-4 jet fuel (U.S. Air Force, 1974). The inhalation toxicity studies described here are part of a continuing attempt to identify acute and subchronic effects of the two primary Navy fuels. In none of the Navy petroleum fuel inhalation studies done over the last five years or in the limited recent experiment with shale JP-5 has there been recognizable manifestations of specific organ dysfunction. In the 120-day continuous exposure of rats to petroleum DFM, there was an indication of increased tumor incident. In the just completed 90-day, two dose level, petroleum JP-5 and DFM studies, provisions have been made to hold animals for a lifetime for the purpose of examining for latent effects and specifically oncogenesis. The companion shale JP-5 and DFM experiments starting this month will also provide for lifetime holding of animals.

These previously described studies, along with other coordination activities, were initiated to assure the availability of adequate health criteria information on product fuels of interest to the Navy.

Before closing, I would like to point out the other research activities cooperating in the effort to develop a complete picture of petroleum and shale product fuel toxicity. Within the scope of the Navy/Air Force cooperative effort, mutagenesis assays are being conducted at the Toxic Hazards Research Unit of the Aerospace Medical Research Laboratory. Efforts are underway at the Naval Research Laboratory, Washington, D.C., to characterize representative samples of fuels from both petroleum and shale sources. The Navy Ship Research and Development Center, Annapolis, Maryland, in conjunction with the Navy Environmental Health Center, Cincinnati, is involved in characterization and quantitation of Naval shipboard atmospheric contaminants. Also, there are numerous other governmental and private organizations that have obtained samples of the fuels used in the studies described here. Incidentally, samples are available through the Chemical Repository program at the Department of Energy's Oak Ridge National Laboratory. We ask only that the Naval Medical Research and Development Command be informed of research resulting from their use.

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DR. HAZLETT (Naval Research Laboratory): I would like to ask Dr. Slomka if he has conducted studies on petroleum crude oil comparable to the acute exposure studies on crude shale oil samples.

DR. SLOMKA (Shell Oil Company): There have been acute toxicity studies done on petroleum crudes; and if I remember correctly, the results were within the same range of response. I don't think that you can expect to get greater precision than that using the materials that we are testing.

DR. HAZLETT: Would that statement include crudes such as those produced on the Alaskan north slope?

DR. SLOMKA: I haven't seen any acute toxicity information on crudes or refined products from that source.

DR. CROCKER (University of California, Irvine): Are there qualitative analyses of the raw shale oil and spent shales that would identify the common toxic components? It is noteworthy that all preparations seemed nearly alike in toxicity.

DR. SLOMKA: I didn't conduct an analysis of the oils but the API group who was responsible for the selection of the samples indicated that they were quite similar to each other qualitatively. There may be some quantitative differences but they are said not to be very large.

DR. CROCKER: One would have expected that extraction of oil would have changed the toxicity of the spent shale but it did not seem that spent shale was different in toxicity from the raw shale or the shale oil.

DR. SLOMKA: I'm not sure that we know what causes the toxicities of the raw and spent shales, particularly when they produce irritation without evidence of systemic toxicity. The response seen with spent shale may be related to the shape and the nature of the material that's left. That has not been changed. It's hard to understand, for example, why two of the spent shales produced eye irritation in contrast to the other materials tested.

MR. LEAHY (University of California, Irvine): Dr. MacFarland, how were the aerosols of the shale materials generated; and how were these aerosols controlled, monitored and sized?

DR. MAC FARLAND (Gulf Science and Technology Company): The two dusts that were used in the long-term inhalation studies were micronized to reduce the particle size and were disseminated from Wright Dust Feed mechanisms. The concentration in the chambers was determined daily by means of gravimetric samples taken on millipore filters. The particle sizing was done every two weeks using a cascade impactor system. The mean concentration for the low concentration of raw shale was 10.2 mg/m^3 and the standard deviation was 4.28. That was much the same for both concentrations of spent shale. The highest shale concentration tested was 29.55 mg/m^3 - the target was 30 - and the standard deviation was 8.97 mg/m^3.

DR. HARRIS (NIOSH): Can you give us the basis for the selection of the shale aerosol concentration tested?

DR. MAC FARLAND: It was not done with a table of random numbers. We selected the TLV concentration for nuisance dusts which currently is 10 mg/m^3 and we initially thought of running a second concentration at 20 mg/m^3. However, that difference was a little too small so we settled on 30 mg/m^3. We really did not anticipate that these dusts would prove to be very noxious. The selection of test concentration was based on the consideration that sometime in the future NIOSH will be interested in a standard for dust levels in shale operations, and the highest concentration they would ever be expected to consider would be 10 mg/m^3. We believed that if we could produce evidence that at three times that concentration, 30 mg/m^3, there was no effect, there would be some hope establishing a TLV of 10 mg/m^3 for this dust. If there's any evidence of fibrotic change or other significant injury, the TLV is obviously going to be less than 10 mg/m^3. We picked the highest possible concentration that would be allowed in any case and another one that was even higher for testing.

MR. VERNOT (University of California, Irvine): I'd like to ask Dr. Rudnick what tissue he used for the measurement of aryl hydrocarbon hydroxylase?

DR. RUDNICK (University of California, Los Alamos Scientific Laboratory): This enzyme determination was made on peripheral lymphocyte culture preparations.

DR. CROCKER: Dr. Coomes, I have a question about the various retorting processes and what might happen to the spent shale composition in the event that the retorting process involves burning of the shale. I would expect that the spent shale would then include ash as well as the residue after extraction of the oils from the rock that had not been burned in

the other retorting processes. In those retorting processes that do burn the shale, ash would also be generated which could produce environmental pollution problems. The small particulate size ash would have an added environmental impact. It also might be an added health risk if it produces the particle sizes that might be expected to be inhaled. I would like to hear your thoughts on this point. The second question I would like to ask is which of these extraction processes produces more in the way of the usual regulated effluent emissions, for example, SO_2, NO_2, hydrocarbons and the like? As one thinks about the local environmental impact on human populations, these issues could be of importance in the choice of retorting procedures. It would be interesting to know what the scale of environmental pollution is.

DR. COOMES (Tosco Corporation): That's a rather difficult set of questions to answer. I would say that in answering your question concerning the pollutants, the best available answers would probably come from the Oil Shale Office of the Department of the Interior in Grand Junction, Colorado. They are in charge of monitoring the activities that go on at the four federal shale oil lease sites. In detailed developmental plans, companies have to specify what emission levels they anticipate their extraction process will produce. I don't know the answer for each individual process.

The first question concerned burning the carbon residue off the processed shale and the impact of that action on the environment. The particulate ash produced may result in an increased hazard from the mineral content; but at the same time, most of the polycyclic hydrocarbon residues or oil residues from the processed shale are removed so that an organic hazard is eliminated. The hazard of the ash would then have to come either from specific minerals or trace metals. Tosco has published a paper on trace metal balance using a Fischer assay retort. We were trying to define the fate of the trace metals from raw shale in this laboratory retorting process. We've done the same thing but not quite as successfully around the Tosco II retort, but that information has not been published. We're using a laboratory retorting method where all of the products are totally contained. We find essentially that all of the nonvolatile trace elements, such as nickel and iron, stay in the processed shale and that since organic materials have been removed, the concentrations of metals are higher than they were in the raw shale. The voaltile trace elements such as arsenic and mercury are removed during the retorting process and end up either in the retort water, the oil, or the product gas, depending on exactly what

happens. The trace mineral content of the processed shale is essentially the same as that found in the soil in the Piceance Basin because the entire basin was laid down in one time period as sedimentary rock deposit. There is a remarkably uniform concentration of the various trace metals in the shale. Any leaching of soluble metals from the process shale enbankments at disposal areas would be an environmental problem. I'm not sure that that's any more of a problem than the leachate from materials that are already present on the basin surface. From my own experience with Colony development operations, our embankment would cover about 800 acres over a 30 year period. The shale embankment, as I recall, could reach a depth of 600 or 800 feet. The processed shale, when it is deposited for disposal, is impacted to about 95 to 100 foot pound density. Water really doesn't penetrate the surface after that treatment. Water leaching doesn't appear to be a problem. We have studied the Colony site embankments that are on the order of 80 feet deep to test for water migration and have found no movement of water after 8 years. There is a potential surface water problem. A severe thundershower will produce surface water runoff. This could carry spent shale particles with it. The Colony facility has been designed with an earthen dam below the disposal pile embankment to trap and confine any runoff water which can then be used in the process to moisturize the shale. In this way, the runoff water is utilized.

DR. JENKINS (University of California, Berkeley): These waste waters contain significant amounts of cyanide, thiocyanate, mono and polyhydric, and substituted phenols, significant concentrations of the more volatile heavy metals you mentioned, high and variable concentrations of chloride and lots of fatty acids. There are very high concentrations of ammonia. In many ways, they are more intractable to treat than the waste waters generated from the gasification of coal by processes that have been used in Europe. I would like to throw out a word of caution. Though you might have been lucky not getting any infiltration from embankments of spent oil shale and ash, I think that this is a problem that may be more extensive than you have indicated.

DR. COOMES: The disposal of the spent shale can definitely be a potential environmental problem. We have conducted some unpublished studies in which we added retort water to processed shale and then attempted to leach materials from the spent shale water. We found that the spent shale retains some of the organic materials irreversibly from the retort water. So you can add retort water with organic content to spent shale and you cannot leach all of those organics back from the spent shale. The surface of the spent shale is covered with essentially elemental

carbon. So you can have a large surface area of carbon black which absorbs organic materials. Some of the materials you mentioned, such as ammonia in the water, would be removed before moisturizing processed shale with a foul water or sour water stripper.

DR. LANDER (AFAPL/SFF, Wright-Patterson Air Force Base): What circumstances are needed for shale oil production to become commercially feasible either by private or government sources?

DR. COOMES: Essentially, the problem is a mixed political and economic one. The first factor is inflation. I'll refer to Colony because it's the only shale operation I'm really familiar with. Before the oil embargo of 1973-74 and the high inflation that took place, Colony had developed detailed engineering plans that were made by C. F. Braun. The detailed cost estimate at that time was just under $300,000. We had another cost estimate made near the end of 1975 and it was one billion dollars. That's a real inflation impact. The second factor is that the prices of the product are controlled by government regulations. Oil shale development would require being able to sell the finished product at comparable world prices for the petroleum product. The third factor, and it is really a problem, is meeting the environmental requirements. It's not that they can't be met. Since we've been trying to develop these plans, and in the case of Colony, that's been since 1970, the environmental regulations have been changing continually. By the time we have a completed engineering design, we have to start over. We have to emit less NO_x or SO_2 or something else. Again, it's not that we can't meet any set of regulations. It's that the regulations keep changing. There are other reasons but that probably summarizes the major factors.

DR. SUSKIND (University of Cincinnati): I know you have had an opportunity to examine crudes and spent and raw oil shales for their polycyclic aromatic hydrocarbons. I think we can learn a good deal by comparing the American experience with the Scottish experience, and I was just wondering whether or not you had any data which allowed us to compare the comparable material from the Pufferston retort or the Westwood which was the last retort used in the Westlothian operations and the Midlothian operations with the Colorado experiences whether they came out of Paraho, indirect, direct or Union or Colony?

DR. COOMES: We personally have not had that opportunity. The only person I know to investigate only the processed shales from the Scottish work is Dr. Schmitt Golaris from DRI. His samples of the Scottish processed shale were from the disposal piles. Of course, they've aged in the piles for some 25 years since the retorting took place. But I don't know the results of that analytical data.

DR. SUSKIND: There are analytical data supported, I believe, by the Medical Research Council prior to the closing of Pufferston works that would be prior to 1960. Analytical methods were different. Is there any usefulness in being able to compare what data was acquired during the 40's and the 50's in Scotland with the Colorado material?

DR. COOMES: I'm not really familiar with the analytical data. I know that during that period of time they were isolating the fluorescent fractions of the shale oil and then testing it biologically by skin painting. I think you can compare the biological results with what we obtain currently. Port and Port did a lot of work in the early 30's using a large number of animals. Their biological data from skin painting are very similar to what we obtain for the crude oil. I don't believe that you can infer from their skin painting studies that the crude shale oils would be chemically dissimilar. Neither oil is, I feel, very unique from the other. Retorting is a pyrolytic process which would produce similar types of organic material. You might find different proportions but they should be basically the same qualitatively.

DR. GIBSON (Gulf Science and Technology Company): I'd like to ask Dr. Rudnick how he intends to conduct follow up health studies on the Paraho worker group. How will you track them?

DR. RUDNICK: We really haven't approached that question. We built in a mechanism for trying to track them down, and we hope that follow-up studies for health surveillance will be undertaken periodically but we really haven't determined what should be done or what we can do. Practically, the immediate problem is that the place has closed down so our exposure is a nonexposure at the moment.

DR. GIBSON: Would you care to comment on any of the industrial hygiene results?

DR. RUDNICK: The few results that I have seen reported were done by some of the Los Alamos people who indicated that the only real problem areas are in the mine shortly after a shot.

They found fairly high hydrocarbon exposures around things like leaky blowers and top seals on the retort that were not sealing very well, problems of this sort which certainly would not be problems in a commercial operation because there would be much better equipment and attention would be concentrated on keeping everything enclosed properly. The exposures that they have reported don't look bad in general. There's a very hot debate going on right now as to what the silica content of the rock is. The values that people are talking about are all over the map, and I don't think anybody knows what the silica content of the rock is. But it doesn't appear to be very high. Dr. Holdsworth has some information on silica content on the samples we were using at API.

DR. HOLDSWORTH (American Petroleum Institute): Relatively high values of total silica in shale solids constitute something like 25% in raw shale and about 40% in spent shale. That's the basis for looking at pneumoconiosis as a potential hazard from exposure to shale dust.

DR. COOMES: That really gets my attention because the alpha quartz levels that we've determined on raw shale samples taken in Utah at Parachute Creek, the Colony property and Anvil Point seem to run about 10% alpha quartz. Of course, this amount of quartz forces you to control the dust more carefully than a nuisance dust. That's the same type of material that the western slope is built of.

MR. LEAHY: I get the impression from Dr. Rudnick that the workers get a clear bill of health basically. I wonder if there is a hazard or potential for latency problem which would show up after a number of years.

DR. RUDNICK: I think there certainly is a question of latency of effects on the workers. There are two parts in answer to that question. One is that we only took one slice in time. You can't really establish latency with a single point in time study. But we did have some people in the study population who had exposures in the industry for over 30 years and these people also came away with essentially a clean bill of health. So, if there are any latent problems, they certainly weren't apparent at this point in time.

The second part of the problem is that these people will continue to work in a variety of mining jobs that may result in other hazardous exposure and they may also continue their smoking habits so that latent effects of shale and shale oil fractions may be obscured due to subsequent occupational factors.

MR. LEAHY: Can we assume that the health facilities or the engineering for health protection used in shale oil production to date are adequate and there is essentially no exposure of the personnel?

DR. RUDNICK: I don't think anybody would characterize them in the developmental kind of make-do mode that all of these operations are in right now as being ideal. But clearly, they aren't producing any manifest problems right now either.

DR. GIBSON: It may be of interest that the Russians have been producing shale oil for quite a number of years and have reported pulmonary changes in a few of the workers with long-term exposures, varying from pneumoconiosis to interstitial fibrosis to bronchitis and almost anything else that happens in the respiratory tract.

DR. HODGE (University of California, San Francisco): Would the panel be willing to compare qualitatively and quantitavely the probable health hazards of the shale oil industry with the experience of the petroleum oil industry?

DR. SLOMKA: When we once get the oil out of the shale, we are dealing with a product similar to that the petroleum industry has been handling for years with reasonable safety. I think you may change the kinds of hazards or change the timing of the hazard in some way. For example, as Dr. Suskind showed this morning, there's a higher carcinogenic risk for crude shale oil as evidenced by the mouse skin painting studies. That could be handled in a variety of ways just as the petroleum industry is handling the problem. There are some extraction procedures to reduce the PNA content of the oil. Hydrotreating has been utilized for that purpose. There are some petroleum products in which the PNA content is concentrated even above that which shale oil contains. We've been able to remove some of the hazard by hydrotreating in a reasonable and safe way. I think that we can handle that kind of situation.

DR. HOLDSWORTH: I would like to try to answer Dr. Hodge's question a little more directly. I think qualitatively there is a very good comparison between finished shale and petroleum oils. Quantitatively, there are a lot of differences between crude shale and crude petroleum. We've done some extensive analytical studies on the chemistry of the products, and there's no clear cut way that you can extrapolate from chemical composition to bioassay results. We've looked at the various

contents of PNAs in different crude oils with orders of magnitude difference between some of them, and yet, as you saw, the latency periods in the skin painting studies demonstrate a very close reaction time. There's more than just benzo(a)pyrene to deal with. As several people mentioned, there's a whole series of polynuclear aromatic compounds included, some of which are biologically active, some of which are not active. You really can't predict how these things interact or what the end results will be without conducting actual bioassays on some of these things. As with petroleum crudes, you get a large variation in chemical composition of shale crudes from area to area; and yet, they fall into the usual spectrum of definitions of crude oil and their subsequent boiling fractions. There's just no clear cut way even with extensive analytical data in that kind of a broad specification for refined fuels that you can predict what the bioassay results are going to be. I think that by traditional, historical, methods in toxicology these materials are rather nontoxic. But if you test these raw shales, shale crudes and refined products from the mutagenesis, teratogenesis, carcinogenesis, and perhaps neurotoxic behavior, you've got a different story again. This is also true with petroleum crudes. So it's pretty much the same story.

I have been asked to tell you about the next step in our studies. Two crude shale oils are being refined so that we can do analytical and bioassay work on downstream products. Toxicology research that's been done to date has been done, of course, on the crude oils. This is a cooperative effort being sponsored by the Department of Energy, the Navy and API. Each one of us is studying a little bit different part of the problem. There will be, I guess, two crude oils and that group will actually be tested, too, in addition to the downstream products. We hope to know a little bit more about the impact of the product that's going to reach the consumer after some of these test results are in. As Dr. Slomka alluded, there are new techniques in petroleum refining, hydrotreating, etc. that reduce PNA levels and that are going to make things a little better.

We have an ongoing skin painting study now at the University of Cincinnati, Kettering Laboratories, that is part of an extensive analytical and bioassay research program. We are fractionating the shale crudes that produced skin tumors and painting animals with these fractions to try to determine the specific causative agent for tumorigenesis. We are also trying to find out what kind of promoters, cocarcinogens, and carcinogens are found in the various fractions that show biological activity. This project began almost 3 years ago, and there's no end in sight because it's such a large effort to fractionate, analyze and then go back and conduct bioassays on these fractions and then to try and isolate the very active, or less active ingredients in these materials.

DR. NEWELL (National Academy of Sciences): This question could probably be directed to anyone on the panel and it is somewhat related to the previous one. That is, if you are moving forward with the work, is there any analytical chemistry information or biological data about the types of combustion exhaust components that are coming out of regular gas fired engines or diesel engines burning shale oil products in relation to things that we're looking at from regular petroleum at the present time?

DR. HOLDSWORTH: There is an effort underway to coordinate the work and get some of the answers that you are referring to. The CRC APRAC program is actively coordinating with the automotive manufacturers to look at these kinds of answers. We're not doing this alone by any means. It's a cooperative effort by several groups. The automotive industry is taking an active part in looking at some of the combustion products from the emission standpoint while we'll be looking at things from the raw product end, not only of the fuels themselves but also the additives of various types of lubricants and oils associated with the internal combustion engine.

DR. GIBSON: The CRC APRAC group will also be conducting an epidemiology study of some type still to be determined on people who may be exposed to diesel exhaust.

DR. CHRISTIAN (University of Cincinnati, Kettering Laboratories): As Dr. Conaway knows, I'm quite interested in some of the discussions this morning concerning tumor promotion. I think there are a few points you can make about this. There are three people who have described in vitro tests which may have the same kind of advantages as the test that Dr. Conaway talked about this morning. Heidelberger talked about promotion of carcinogenesis with his 10T½ cells. Trosco, using UV radiation for the promotion of mutagenesis, and we have described several chemicals which can be promoted by classical promoters such as TPA and some linear alkanes as well. I think that this may give us an opportunity to separate some of the effects of carcinogenesis. Dr. Suskind pointed out this morning that there just didn't seem to be enough of the classical BAP type carcinogens in the shale oil products to account for their greater activity on mouse skin through petroleum oils. He suggested that there may be other compounds present, and I think it's imperative to look for some of these other active agents that we now suspect. I think that there is probably one more very practical point. Promoters are reversible. I think this may have a practical application if you can determine that some of your biological activity for carcinogenesis is due to

promoters, this emphasizes the need for restricting exposures of workers in a particular process. I think that whereas carcinogens apparently are not reversible, promoters may be. And if promoters are an essential or a very important part of carcinogenesis caused by petroleum type products, then perhaps they can still be worked on and used with safety if we can limit the exposure and if we can use proper hygiene. I guess that's a question for you, Dr. Conaway.

DR. CONAWAY (Beacon Research Laboratories): I agree that more work should be done in this area, and we need to determine which particular compounds are active promoters. Also, there are some which are thought to act as antipromoters. These things need to be studied to assess what carcinogenesis activity we do have.

DR. SUSKIND: Dr. Conaway, I'm very impressed with the work that you have done. It's clean and clear cut. It does presumably point to the fact that polycyclic aromatic hydrocarbons are involved and that conversion to electrophilic metabolites is necessary with your S-9 fraction. I'm not at all sure that explains the potential for carcinogenicity because accelerators, promoters which are not mutagenic, also play a role. The concentrations of benzpyrene are so low in the shale samples tested that they are not likely to be responsible for the level of carcinogenicity that we see in animals. There are probably other things that are biologically active in these oils and perhaps these materials can also be converted to electrophilic metabolites. Perhaps somebody in this audience knows more about this. But there's no doubt that the conversion that you pointed out indicates that PNAs or polycyclic aromatic hydrocarbons are involved.

DR. CONAWAY: We agree with that. This work points out the opportunity and the necessity of doing more work, studying the effects of promoters such as the linear alkanes and other promoting materials which are present in the shale oils. Perhaps this will be done at a later time.

DR. NEWELL (National Academy of Sciences): These results, in combination with the skin painting experiments at the University of Cincinnati, show that the in vitro type mutagenesis test procedure can serve as a useful tool if cleanup of the shale oil fractions is desired -- to remove potential carcinogenesis. Also, if such an approach is used, be sure to use several different mutagenesis tests for any one evaluation, not just the Ames procedure.

DR. CONAWAY: Yes, certainly.

DR. CROCKER: Dr. Suskind, you have given a very helpful and important presentation. I am interested to know how much skin irritation precedes the tumors. As we know from the experiences in Scotland, for example, skin irritation was a fairly useful warning but not uniformly available as an indication of likely hazard to that worker from excess exposure. What do you know of the risks that we may encounter? How much can we hope for in the way of certain signs that might be leading to carcinogenicity?

DR. SUSKIND: In this instance, all of the raw shale oils were irritating. As a matter of fact, we had planned to use a protocol which exposed the mice more frequently but we found that not only were the mice irritated, we found that they became toxic as well. I think it simply bears out what Dr. Slomka has indicated with respect to the percutaneous toxicity. I would assume that the irritation would enhance the absorption of the polycyclic aromatic hydrocarbons or the accelerating substances, the promoting materials; and as you know, in the paraffin wax experience in Scotland, the concentration of the carcinogen was extremely low, but the accelerator, the long chain hydrocarbon content, was very high. The induction period was comparable to coal tar where the polycyclic aromatic hydrocarbon content is high. I think that irritation does enhance increased absorption. I'm not altogether sure that what we call irritation or the induction of inflammation actually enhances carcinogenesis. I don't think that's ever been shown.

DR. CHRISTIAN: Dr. Conaway, in the mouse lymphoma results that you talked about this morning, was there any enzymatic activation?

DR. CONAWAY: Yes, there was activation from the mouse liver. We tested both a nonactivated and activated system.

MR. LEAHY: Dr. Conaway, I wonder if you could comment on how the aerosol in the teratological study was produced from that thick crude shale oil and also how this was monitored and sized?

DR. CONAWAY: The aerosol was produced by heating the material to approximately 100 degrees and then aerosolized with a Laskin generator. Monitoring was done by, I think, a standard method where they use an IR method for analysis.

DR. FRIESS (Naval Medical Research Institute): I should like to follow up on a question that Dr. Hodge asked relative to attempting to find some comparative hazard estimation for shale derived crude or fractions with petroleum derived crude or its fractions. To get a handle on relative hazard for a spectrum of biological effects, I'd like to focus on the total aromatic content and ask the members of the panel whether whole aromatic content ranging from small rings upwards can be looked upon as some rough factor increase of health effects from the shale derived versus petroleum fuels. Can you say anything from the analytical chemical background about the relative increase in total aromatics in the crude or its fractions in shale over petroleum in relation to probable health effects?

DR. GIBSON: Dr. Holdsworth has some information on shale.

DR. HOLDSWORTH: Yes, I do. But I don't have it with me. I can't tell you off the top of my head what the aromatic content is. I'd like to defer that question to Dr. Coomes. He may have that information at hand.

DR. COOMES: I thought I had the actual information with me so that I could quote it rather than relying on my memory but I don't have it. Dr. Peter Jones, who was with Batelle Columbus Laboratories, analyzed side by side Fischer assay shale oil, one of the synthetic coal liquefied oils, I don't remember which one, and Prudhoe Bay crude petroleum. He analyzed these oils for polycyclic aromatic hydrocarbons, saturated hydrocarbons, and olefins. He presented this work at the 1976 fall meeting of the American Chemical Society in San Francisco. The work has never been published. Essentially what the comparison said, as I recall, was the Prudhoe Bay crude and the shale oil derived from Fischer assay were very similar in aromatic character. The coal liquid had a higher aromatic content. In the paper, Dr. Jones identified up to 10 methyl substituted polycyclics and he compared the ratio of these methyl substituted polycyclics in the various liquids. Again, the shale oil and the Prudhoe Bay crude were more similar to each other than the coal liquid.

DR. FRIESS: The total aromatic content then is similar in this single comparison of petroleum based crude with the shale based?

DR. COOMES: In this single comparison, yes. There is wide variability in crude petroleum; but in this one instance, they were comparable.

DR. HAZLETT (Naval Research Laboratory): I would like to comment on that last question. I think crude shale has one other aspect which is distinctly different from petroleum crudes and that's the high nitrogen content. As a consequence, the aromatic content is considered to be very high when actually it includes many polar compounds. The 2% total nitrogen content can give you a nitrogen compound corresponding to around 40% if you assume a molecular weight of about 300. So the shale crude is really a very polar material with a large number of polar compounds and I think distinctly different from many petroleum crudes in that aspect.

DR. COOMES: One comment of the nitrogen content which is about 2%. It doesn't require very severe hydrotreating of the shale oil crude to knock the nitrogen content down to very low levels. Another comment along those lines, you can find some crude petroleums that have at least that high a nitrogen content.

CAPT. REED (Air Force Rocket Propulsion Laboratory, Edwards Air Force Base): Dr. Coomes, do you know whether or not there's any significant radioactive content in the shale deposits we have been talking about?

DR. COOMES: Laramie Energy Technical Center has analyzed oil shale for any radioactive materials. It's published in one of the older Bureau of Mines research reports. According to that report, they could find no significant amounts of radioactive material. This is probably quite reasonable when you examine the nature of the deposit.

DR. RUDNICK: I've asked this same question of several people, and the answer has been exactly the same. The radioactivity is below most normal background levels.

SESSION II

TOXICOLOGY OF HYDRAZINE COMPOUNDS

Chairman

Frank N. Dost, D.V.M.
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PAPER NO. 8

METABOLIC FATE OF HYDRAZINE

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INTRODUCTION

The extensive use of hydrazine in both military and industrial applications has necessitated a thorough toxicologic and hazard evaluation. In spite of a great deal of study over recent decades, there are still serious gaps in understanding the biological effects of hydrazine. Similarly, the metabolic fate of hydrazine is not well defined.

This report describes research which further defines the behavior of hydrazine once it enters the animal. The existing literature in this area of research is limited. One reason is that isolation and identification of simple nitrogen compounds is often difficult since no radioisotope of nitrogen is available, and use of tritium labeling is futile in view of extensive exchange reactions. There has also been frustration over the apparent "disappearance" of hydrazine after administration; no one has succeeded in establishing a complete general inventory.

The impetus to learn the details of reactions of hydrazine with biological entities has been intensified recently with the possibility that hydrazine may be a weak carcinogen. The presence of small amounts of hydrazine in tobacco smoke (derived from synthetic plant growth regulators) adds to the importance of the problem (Liu et al., 1974).

Metabolic study of hydrazine as a toxic substance began only in the early 1950s. McKennis et al. (1955) found that a variable but substantial amount of hydrazine emerged in urine of dogs within 48 hours after acute dosage with 15 mg/kg hydrazine, a treatment which is frequently lethal.

Dambrauskas and Cornish (1964) followed the time course of tissue levels and excretion of hydrazine as measured spectrophotometrically following reaction with p-dimethylaminobenzaldehyde. Hydrazine continued to appear in urine for 20 hours or more after a single injection, and tissue concentrations decreased steadily over the same period. About 50% of the administered hydrazine could not be found in either tissues or urine, and was assumed to have been metabolized. The amount "metabolized" was almost constant throughout the time course, indicating that it was either lost experimentally or was not subject to the processes mediating removal of presumably intact hydrazine from tissues.

Very few in vivo chemical reactions of hydrazine have been documented. McKennis et al. (1959) observed significant diacetylation of hydrazine in rabbits but found that such conversion in the dog was limited. Acetylation of many hydrazine derivatives, notably isoniazid, is known to be genetically determined within humans (Evans et al., 1960; Dufour et al., 1964) and possibly other species, and the significance to other hydrazine reactions of such differences in this pathway can only be speculated upon.

Hydrazine is a carbonyl reagent and is generally accepted as being reactive in vivo with pyridoxal phosphate and possibly otheraldehydes and ketones. Elevation of blood ammonia by hydrazine (McKennis and Weatherby, 1956) suggests that some hydrazine can cause alteration in ammonia metabolism or even may proceed to formation of ammonia. Hydrazine was used in studying the physiology and chemistry of hemoglobin around the turn of the century; the reaction was stated by Buckmaster (1913, 1914) to form gaseous nitrogen.

In the work reported here, we find that about 15% of a single dose of ^{15}N-hydrazine is converted to nitrogen-15 gas within 30 minutes after administration, and that an additional 10% emerges in the subsequent 24-48 hours. The extent of conversion is not significantly influenced by dosage. Measurable hydrazine remains in blood at least 24 hours after a single injection, as does a hydrolyzable hydrazine metabolite. Urinary excretion continues for at least 48 hours, to the extent of about 50% of dose. Hydrazine infusion at low dose rates results in a constant blood hydrazine concentration for the duration of infusion.

METHODS

MEASUREMENT OF $^{15}N_2$ IN RESPIRATORY GASES

The problem to be solved in measuring volatile nitrogen-15 in respiratory gases of rats can be summarized briefly: Very small amounts of a noncondensable gas must be quantitatively recovered from a closed atmosphere in a system that can support life in an intact animal for an indefinite period. Several requirements are obvious. The system should: (1) utilize a filling gas that is condensable or otherwise separable and nontoxic; (2) be gas-tight at ambient pressure and able to withstand substantial vacuum; (3) be designed to permit acute or continuous injection of intoxicants or other agents to experimental animals without opening the system; (4) have a sensitive, demand-driven oxygen supply; and (5) include CO_2 and water traps that do not otherwise compromise the experimental atmosphere. At the end of a metabolism period, there must be associated capability to remove oxygen, remove the bulk condensable gas, and trap $^{15}N_2$ or other noncondensable gases and move them to a mass spectrometer for analysis.

DETERMINATION OF ^{15}N IN URINARY AMMONIA

A 24-hour urine sample was collected at dry ice temperature in acid from a rat that had been treated with ^{15}N hydrazine sulfate (1.0 mmole/kg). Ammonia from the entire sample was collected into a 16 mm by 50 mm shell vial containing 1.0 ml of 2.0 N HCl, according to the method of Conway (1957). After evaporation to dryness at 95 C, the crystalline ammonium chloride salt was converted to nitrogen gas by the method of Ross and Martin (1970) and Porter and O'Deen (1977). This method involves oxidation of ammonium chloride and collection of the nitrogen gas in mass spectrometry tubes.

For standardization, known amounts of $^{15}NH_4Cl$ were converted directly to nitrogen gas or added to urine from untreated rats, then treated as described. The amount of nitrogen-15 in the standards or spiked sample was determined by mass spectrometry. Recovery from "spiked" control urine was consistently about 50% of the amount converted directly from $^{15}NH_4Cl$. Because samples from rats treated with ^{15}N-hydrazine contained no detectable ^{15}N in ammonia, further improvement of recoveries was not attempted.

MEASUREMENT OF HYDRAZINE AND HYDRAZINE DERIVATIVES IN BIOLOGICAL FLUIDS

Hydrazine was analyzed in samples of urine and blood by a micromodification of the method of Reynolds and Thomas (1965), employing p-dimethylaminobenzaldehyde (DMBA) as the

color reagent. Whole blood is diluted with enough water to rupture all erythrocytes, the lysate is treated with 10% trichloroacetic acid (TCA) to precipitate all protein, and then centrifuged. Samples are held on ice during each step prior to acidification. DMBA is added to an aliquot of the TCA extract which is incubated 20 minutes at room temperature. Absorbance is read at 470 nm. The color is stable for at least two hours.

For determination of the hydrolyzable derivative(s), an aliquot of the TCA extract is acidified to 1N with HCl and incubated 48 hours at 45 C. Analysis then proceeds as above. Standards of hydrazine and diacetylhydrazine are prepared in rat serum for the respective assays. (Absolute identity of the hydrolyzable derivative is not established but diacetylhydrazine is an appropriate model substrate.) The assay is linear between 0.006 and 0.09 μmoles in a final volume of 3 ml. Standards of each compound are generally used in the range between 0.01-0.06 μmoles.

Urine samples are diluted to volume with water, but since protein precipitation is not needed, HCl may be used alone to acidify samples. There are several interfering substances in urine which react with the color reagent, and which are variable from sample to sample. To correct for this interference, the hydrazine in the sample is oxidized with alkaline $CuSO_4$ (18 mM in 1N NaOH), and the sample is reanalyzed to determine the background color. Standards are prepared by spiking unknown duplicate samples with hydrazine and diacetylhydrazine.

ANIMAL PREPARATION, SAMPLING AND ADMINISTRATION OF CHEMICALS

Male Sprague-Dawley rats were used in all phases of this work. Animals of 250-280 grams were surgically prepared, where necessary, and were allowed at least four full days for recovery before use. Animals were surgically implanted with intraperitoneal or subcutaneous cannulas for administration of hydrazine. In some experiments, subcutaneous treatment was infused through a hypodermic needle taped in place. As needed, cannulas for infusion, feeding, or sampling were also placed in the posterior vena cava and/or the small intestine. All surgical procedures were performed under sodium pentobarbital anesthesia supplemented by ether. Bladder cannulas were installed under ether anesthesia the day before treatment.

Subcutaneous injection of hydrazine provides distribution that more nearly parallels the circulatory routes following either inhalation or transcutaneous absorption, but recoveries

of $^{15}N_2$ in initial experiments were comparatively low, and while use of direct intravenous injection resulted in greater conversion, the variability was unacceptable. Intraperitoneal administration was found to be adequately consistent and was selected for the major series of intact animal studies with ^{15}N-hydrazine.

In the $^{15}N_2$ recovery experiments, the animal was placed in a restraining device, which was then placed in the metabolism chamber, the necessary cannulas were connected to hubless needles inserted through the rubber stopper in the cannula port, and tubing was similarly attached on the outside. Hydrazine or $^{15}N_2$ was administered as a single dose or as a continuous infusion.

During observation of blood hydrazine concentration and excretion time courses, animals were held in acrylic restrainers for the duration of the experiment. Blood samples were drawn from the intravenous cannula in volumes of no more than 0.5 ml at one time, nor more than 2.25 mls in 24 hours. Urine was collected either directly on dry ice or into HCl at 4 C. Fluid intake in long duration experiments was maintained by infusion of water into the small intestine.

CHEMICALS

Sulfur hexafluoride, 99.8%, and carbon tetrafluoride (Freon-14), 99%, Matheson Gas Products, East Rutherford, New Jersey; hydrazine sulfate ^{15}N (99 atom %) and ^{15}N-nitrogen, 99 atom %, U.S. Service, Ic., Summit, New Jersey; oxygen (ultra-pure), Liquid Air, Inc., San Francisco, California; oxy-absorbent, Altech; Drierite, W. A. Hammond Drierite Co., Xenia, Ohio; Sodasorb (soda lime), Dewey & Almy, Cambridge, Massachusetts; activated carbon, type Cal 12 X 40, Calgon, Pittsburgh, Pennsylvania; pyrogallic acid, Mallinckrodt; p-dimethylaminobenzaldehyde (DMBA) and hydrazine, 99%, Eastman. Diacetylhydrazine was synthesized by the method of Turner (1947). All other chemicals were reagent grade.

RESULTS

PRODUCTION OF $^{15}N_2$ FROM ^{15}N-HYDRAZINE IN VIVO

When hydrazine was injected intraperitoneally in single 1 mmole/kg doses, approximately 15% was converted to nitrogen gas within the first 30 minutes, followed by a much slower conversion over at least the subsequent 24 hours (Figure 1 and

Table 1, lines 1-6). The possibility that saturation of some reaction system slowed the conversion was examined by comparing $^{15}N_2$ production following i.p. treatment with doses of 0.5, 1.0, and 1.5 mmoles ^{15}N-hydrazine/kg. Recoveries were 16.0 ± 3.26%, 16.8 ± 1.53%, and 17.9 ± 0.99%, respectively, indicating a slight, but nonsignificant increase associated with increased dose. An experiment in which two 1 mmole/kg doses were administered two hours apart provided recoveries slightly higher than should be expected for a single 2 mmole/kg dose, on the basis of the above dose-response information. Comparison of routes of administration indicates that over a 4-hour collection period, the highest conversion arose from intravenous hydrazine, and the lowest following subcutaneous administration (Table 1, lines 4, 10, 12).

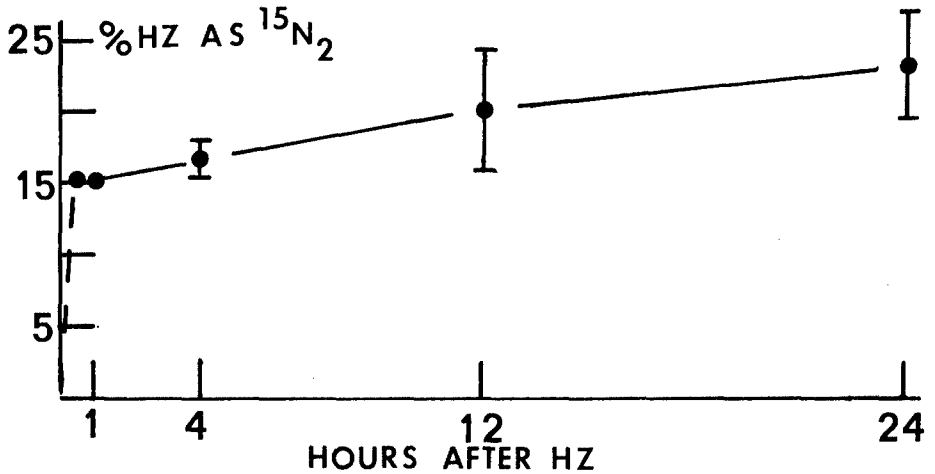

Figure 1. Conversion of ^{15}N-hydrazine to respiratory $^{15}N_2$ following a single 1 mmole/kg dose, i.p. (N = 3 at each time point.)

TABLE 1. FORMATION OF $^{15}N_2$ FROM ^{15}N-HYDRAZINE

Summary of Effects of Collection Time Following Administration, Route of Administration, Dose and Oxygen Atmosphere.

Line	Compare with Line #	Dose mmole/kg	Admin. Route	Collection Time, Hr.	N	$\bar{\mu} \pm s$	Comments
			TIME COURSE OF $^{15}N_2$ PRODUCTION AFTER SINGLE DOSE OF ^{15}N-HYDRAZINE				
1		1	IP	0	2	0.615 ± .304	A
2		1	IP	0.5	3	15.4 ± 3.18	
3		1	IP	1	3	15.3 ± 2.02	
4		1	IP	4	3	16.8 ± 1.53	
5		1	IP	12	2	20.2 ± 4.24	
6		1	IP	24	3	23.3 ± 3.76	
			EFFECT OF INTRAVENOUS ADMINISTRATION				
7	1	1	IV	0	2	0.84 ± 0.45	A
8		1	IV	5 Min.	2	6.0 ± 3.44	
9	3	1	IV	1	3	14.4 ± 2.57	
10	4	1	IV	4	3	18.9 ± 5.30	
			EFFECT OF SUBCUTANEOUS ADMINISTRATION				
11	3	1	SC	1	3	12.6 ± 1.64	
12	4	1	SC	4	3	14.0 ± 1.5	
			EFFECT OF DOSAGE				
13		0.5	IP	4	4	16.0 ± 3.26	
14		1.0	IP	4	3	16.8 ± 1.53	Line 4 Repeated
15		1.5	IP	4	3	17.9 ± 0.99	
16		2.0	IP	4	2	20.8 ± 1.98	B
			EFFECT OF 100% O_2 ATMOSPHERE				
17	4	1.0	IP	4	2	12.0 ± 0.5	
			COLLECTION UNDER 30 MINUTE VACUUM TO ASSURE RELEASE OF ENTRAINED $^{15}N_2$				
18	4	1.0	IP	4	2	17.4 ± 0.28	

A - Animal euthanized at time of hydrazine injection.
B - Two 1 mmole/kg doses two hours apart.

An attempt was made to observe the first few minutes of the course of the reaction by using intravenous administration of hydrazine in spite of the inherent variability associated with that route. Two samples obtained five minutes after injection were quite different (Table 1, line 8), but showed that significant reaction had taken place.

In view of the known reaction of hydrazine in oxygenated hemoglobin, two animals were maintained in 100% oxygen, resulting in somewhat decreased conversion (Table 1, line 17). To assure that all free nitrogen produced was adequately mobilized at termination, two animals were subjected to vacuum for 30 minutes after euthanasia but no significant increase in collection resulted (Table 1, line 18).

FORMATION OF AMMONIA

No ^{15}N has been detected in urinary ammonia of rats administered a single, 1 mmole/kg dose of ^{15}N-hydrazine.

REACTION OF HYDRAZINE WITH HEMOGLOBIN AND OTHER BLOOD CONSTITUENTS

We are seeking an in vitro model that may be useful in explaining the mechanism of N_2 formation from hydrazine, and the nature of any other factors that may influence the reaction rate. Early studies with whole blood and blood constituents suggest that such a system may be a model for the sequence observed in vivo.

Whole blood converted ^{15}N-hydrazine to $^{15}N_2$ in vivo to a greater extent than did intact animals. Conversion under an atmosphere of 100% O_2 was not appreciably different from that in 20% oxygen, and both were quite variable. The time course of the reaction under 100% oxygen, based on the data so far available, shows a relatively constant rate of conversion for three hours, reaching a total of about 35% (Figure 2). Whole blood incubated under 100% SF_6 converted 9.9 ± 2.32% (N = 5) of ^{15}N-hydrazine to $^{15}N_2$ in three hours. The oxygenated system contained roughly 0.16 meq hemoglobin subunit and 0.115 mmole (15 mg) hydrazine sulfate in 22 ml; 0.230 mmole hydrazine was added to the deoxygenated series.

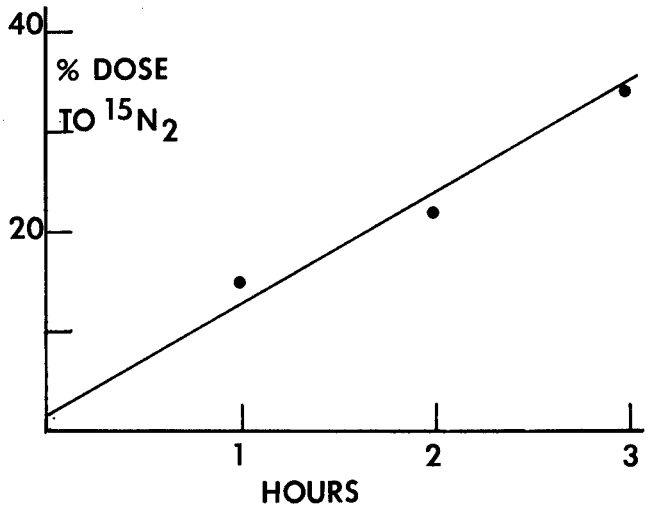

Figure 2. Conversion of ^{15}N-hydrazine to $^{15}N_2$ by whole blood, in vitro (0.16 meq hemoglobin subunit, 0.115 mmole hydrazine/22 ml blood, 100% O_2).

Solutions of hemoglobin under 100% oxygen and in the absence of other protein or cell membranes react with unlabeled hydrazine readily. The stoichiometry suggests that hemoglobin is either catalytic or serves as a replenishing oxygen carrier in the system. The kinetics of the reaction have not yet been fully studied, however.

Early experiments also show that bovine serum albumin protects hydrazine from reaction with hemoglobin, and that the extent of reaction is related to the amount of albumin added. Surprisingly, rat plasma, without modification, did not confer such protection, and the reaction proceeded at the same rate as in buffer with no protein. Such factors as previously occupied albumin binding sites, influence of anticoagulants, and the role of the red cell membrane, either as a barrier or as a binding surface, are being examined.

CONCENTRATION OF HYDRAZINE AND ITS METABOLITE(S) IN BLOOD FOLLOWING HYDRAZINE ADMINISTRATION

During the first hour after a single i.p. injection of 1.0 mmole hydrazine/kg, blood hydrazine concentration declined rapidly, probably reflecting distribution and early metabolic activity. There appeared to be two separate subsequent phases between hours 1 and 6, and 6 and 24, associated with movement from tissues, excretion, and slower metabolism. The half times of disappearance increased substantially for each successive phase. Data presently available are not sufficient to construct an adequate mathematical representation. At 24 hours, concentration of hydrazine and its metabolite was about 10% of the hydrazine concentration 15 minutes after administration (Figure 3).

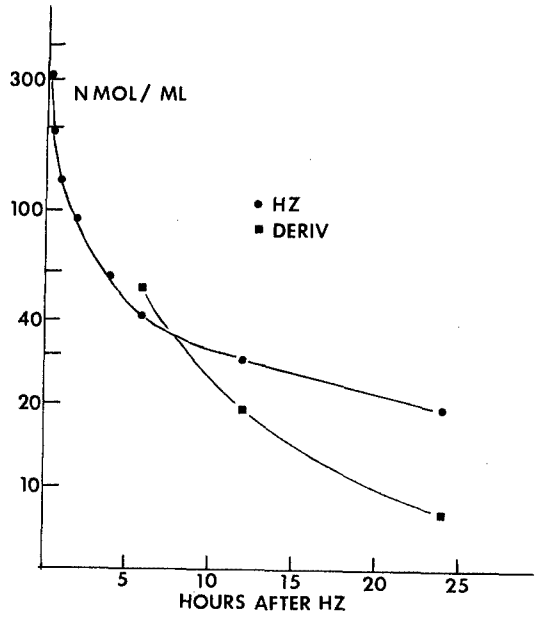

Figure 3. Concentration of hydrazine and a derivative(s) in blood after 1 mmole hydrazine/kg, single dose, i.p. (N = 6 at 6 hours, 3 at all other points).

Continuous infusion of hydrazine over 6-12 hours has shown that at lower dose rates, a steady state blood hydrazine concentration usually is established, proportional to input rate. Figure 4 shows data collected between hours 5 and 12 of infusion. Such regulation occasionally occurs at dose rates in excess of 0.167 mmole/kg/hr.

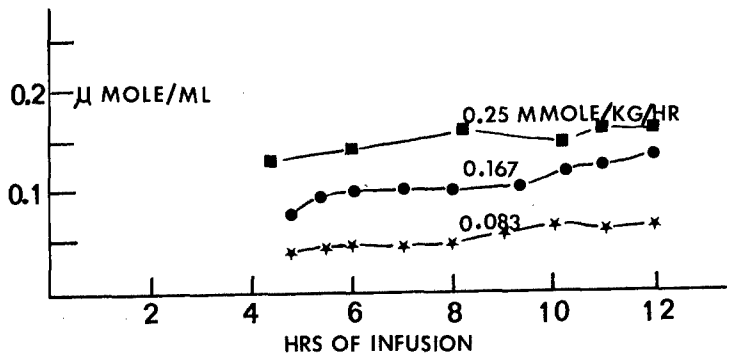

Figure 4. Concentration of hydrazine in blood during continuous sc infusion at indicated dose rates.

An attempt was made to identify a range of blood hydrazine concentrations that might be associated with lethal effect, but no clearcut relationship was found. Death has occurred at blood concentrations ranging from 0.3-1.05 μmole hydrazine/ml whole blood. A number of continuously infused animals have been sampled over the time sequence immediately prior to death, in experiments intended to obtain other information. In most such cases, the blood hydrazine levels have tended toward a constant concentration but after 8-10 hours the hydrazine level rose abruptly just prior to death.

URINARY EXCRETION OF HYDRAZINE

The persistence of hydrazine in blood is paralleled by an extended period of excretion in urine. Rate of excretion of hydrazine and its metabolite(s) is in excess of 5% of the dose per hour immediately after administration, about 3% at 6 hours, 1% at 24 hours and 0.25% at 48 hours (Figure 5). The excretion rate of the hydrazine metabolite was relatively constant through the first 6 hours after administration, and was not characterized by an initial high output rate. Cumulative excretion over 48 hours totaled about 52% (Figure 6), of which slightly more than half was hydrazine.

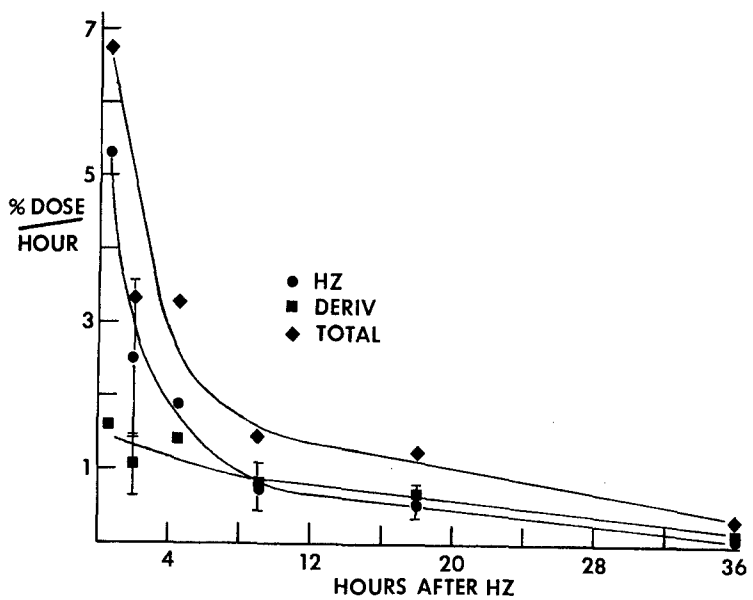

Figure 5. Urinary excretion rate of hydrazine and a hydrolyzable derivative(s) following a single 1 mmole/kg dose, i.p. (N = 4-10 animals/point. Each point located in middle of collection period).

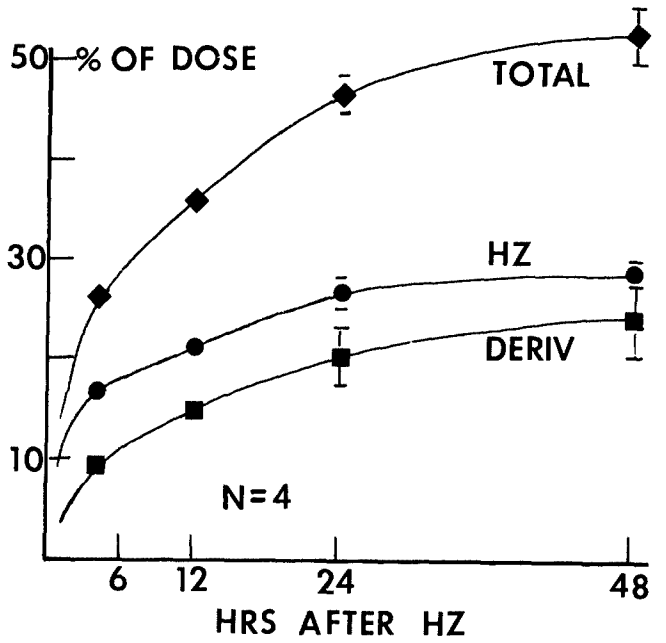

Figure 6. Cumulative excretion of hydrazine and a hydrolyzable derivative over 48 hours following a single 1 mmole/kg dose i.p.

DISCUSSION

The time course of conversion of ^{15}N-hydrazine to $^{15}N_2$ presents an interesting pattern. The reaction is initially very rapid and is quite slow thereafter. There is some correspondence between the decline of nitrogen production and the decline in blood hydrazine. However, during the period from 1 to 3 hours, when nitrogen production has slowed sharply, blood hydrazine seems higher than would be expected if a direct relation with nitrogen production exists. As more data are assembled, this relationship should become clearer. The kinetics of the conversion are clearly not a function of saturation of either a reaction system or of a sink for hydrazine, because the change in output relative to dose is negligible.

We speculate that the reaction is catalyzed by hemoglobin. We find that 50% of added ^{15}N-hydrazine is converted to $^{15}N_2$ in whole blood in three hours, and in hemoglobin solution conversion can exceed 80%. In preliminary experiments, addition of albumin to hemoglobin solutions decreases loss of hydrazine from solution presumably through binding of hydrazine. Fresh serum had no effect.

We are extremely interested in the nature of this system, because it seems reasonable that the very rapid metabolic activity might be exploited in some way for reversal of acute hydrazine intoxication. It appears that the general problem is to find a way of increasing the fraction of circulating hydrazine that is accessible to the reaction.

It is evident that a somewhat more complete inventory of administered hydrazine has been achieved than past studies have accomplished. About 23% of administered hydrazine emerged as nitrogen gas at 24 hours, and a recovery of about 25% can be assumed if an extrapolation is made to 48 hours. Cumulative recovery of hydrazine and its derivatives in urine was slightly in excess of 50%, totalling, with $^{15}N_2$, more than 75%. Both $^{15}N_2$ production and urinary excretion are very nearly ended by 48 hours, suggesting that either an unusually large fraction of hydrazine is tissue bound, or that we have a metabolite that has not been detected.

There is an interest in the expected behavior of hydrazine at steady, low level intake, as a simulation of possible work place exposure. Animals continually infused with hydrazine at relatively low rates are able to maintain a constant blood hydrazine concentration for at least 12 hours of infusion. The steady state may be established as early as two hours after beginning of infusion but occasionally requires 4-5 hours.

The absence of ^{15}N in urinary ammonia is interesting, in view of the potential for ammonia formation from hydrazine in nonbiological reactions, and in view of the suggestion by Procellati and Preziosi (1954) that intact rabbits and isolated rat tissues could enzymatically make that conversion.

We have begun work on emission spectroscopy techniques which ought to be considerably more sensitive for measuring ^{15}N in tissues, whether as hydrazine or as a product, and we are hopeful that these questions can be resolved in the near future.

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PAPER NO. 9

RATIONAL APPROACH TO THE PREVENTION OF ISONIAZID HEPATITIS

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Text of this presentation is not available for publication.

PAPER NO. 10

ALTERATIONS IN DNA IN HYDRAZINE-TREATED RATS AND MICE

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A few years ago the Toxic Hazards Research Unit at Wright-Patterson Air Force Base (Dayton, Ohio) initiated a series of carcinogenicity bioassays on the propellant hydrazines; hydrazine, monomethylhydrazine (MMH), and 1,1-dimethylhydrazine (UDMH). This was a multiple species inhalation assay, using the Fischer 344 rat, the C57Bl/6 mouse, the Golden Syrian hamster, and the beagle dog. Cognizant of the species variations in the acute toxicities of these compounds, it was decided to embark on comparative metabolism studies on these compounds in the same rodent species and in human tissues obtained at surgery. If the metabolic pathway relevant to the carcinogenicity of these compounds could be elucidated in the animal models, it was hoped that the extent of such metabolism in human tissues could be measured to assist in determining which animal species most closely approximates man in susceptibility to the carcinogenicity of these hydrazines.

Several metabolic pathways for the propellant hydrazines in rodents are known and the work of Dr. Dost and Dr. Mitchell, from whom we have just heard, has added greatly to our knowledge of the metabolic fates of these compounds. We have chosen a slightly different approach in considering the hydrazines as carcinogens. There now is considerable evidence supporting the proposal that chemical carcinogens are relatively inactive as they exist in the environment but undergo transformation, either metabolically or spontaneously, in target cells to form highly reactive electrophiles. It is thought that these electrophiles react immediately with nucleophilic sites in the cell, and that when these sites are critical moieties of information molecules, alterations arise in the cell's information transfer processes; such alteration could conceivably result in cancerous cells (Miller and Miller, 1977). We postulated, therefore, that if the propellant hydrazines are carcinogenic, their administration

to sensitive animals should result in the formation of electrophilic reactants which would bind covalently to critical sites in DNA and other information molecules; thus we set out to measure the formation of DNA adducts in animals poisoned with hydrazine, MMH, and UDMH. We intend to extend our studies to include human tissues, once the animal studies are completed. Our preliminary results on alteration of DNA in hydrazine-treated rats and mice are reported here.

DNA METHYLATION IN HYDRAZINE-TREATED RATS

In the first studies, we decided to look for methylation of DNA in hydrazine-treated rats. In 1967, while one of us (R. C. Shank) was working with Dr. Saul Villa-Trevino and Professor Peter N. Magee in the MRC Toxicology Research Unit in England, it had been observed that liver DNA in rats poisoned with hydrazine was labeled by ^{14}C-methyl-methionine to a greater extent than in saline-treated animals.

In the current studies, young adult fasted male Fischer 344 rats were given by intubation 60 mg hydrazine per kg body weight in 0.1 N HCl (0.1 ml); this dose approximates an LD 0.01 by extrapolation from log dose-probit response data analyzed by the Litchfield-Wilcoxon (1949) method. At the same time each animal was given 20-25 μCi ^{14}C-methyl-methionine ip in 0.1 ml 0.9% NaCl; the radioactive methionine injections were repeated hourly for a total of five injections. Five hours after hydrazine administration, and one hour following the last methionine injection, the animals were decapitated, and DNA was isolated from various tissues by the phenolic extraction method of Swann and Magee (1968). The DNA underwent mild hydrolysis in 0.1 N NCl at 70 C for 30 minutes and was then fractionated into pyrimidine oligonucleotides and purine bases by a high pressure liquid chromatography method developed in our laboratory. Up to 1 ml of hydrolysate (5 mg DNA/ml) was applied to a Whatman Magnum 9 preparative strong cation exchange Partisil 10 column, 50 cm long with an internal diameter of 9.4 mm; the mobile phase was 0.1 M ammonium phosphate pH 2.0 at 4 ml/minute. Elution of ultraviolet absorbing bases was monitored at 275 nm. In most cases carrier 7-methylguanine and 0^6-methylguanine were added to the hydrolysates to serve as markers in the chromatography.

A typical elution profile for liver DNA fragments from control animals is shown in Figure 1. Some label from methionine is incorporated normally into thymine (in the pyrimidine oligonucleotide fraction), guanine and adenine; there is no indication of the presence of either 7-methylguanine or 0^6-methylguanine in DNA from unpoisoned animals.

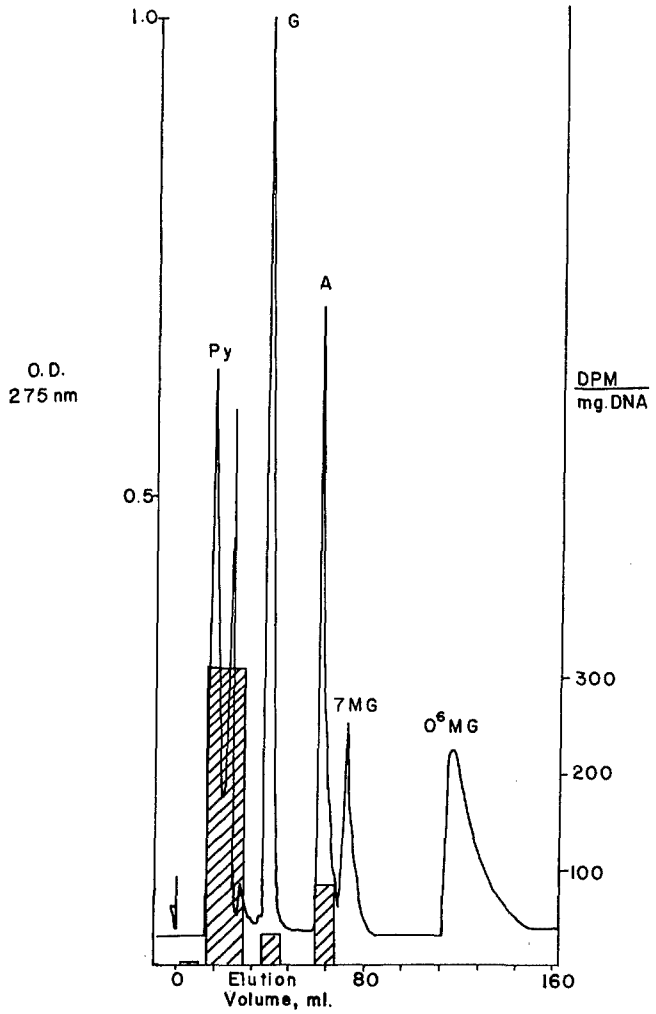


Figure 1. Elution profile of liver DNA hydrolysate prepared from control rats.

A similar elution profile for liver DNA from hydrazine-poisoned rats is shown in Figure 2. Here it is apparent that there is a readily detectable amount of 7-methylguanine-^{14}C in this DNA hydrolysate. This chromatographic method has been in use for two years in the laboratory and much experience has been gained with the elution of several methylated purines and pyrimidines and ethylated purines; the only base found to co-chromatograph with 7-methylguanine in this system is 5-methyl-cytosine, the only normally methylated base in mammalian DNA. The hydrolysis technique used in these experiments does not release free pyrimidines and thus 5-methylcytosine would be expected to elute with the pyrimidine oligonucleotide peaks and not with 7-methylguanine. Analysis of 7-methylguanine-^{14}C

material by paper chromatography indicated that all radioactivity migrated with the 7-methylguanine and none with added carrier 5-methylcytosine. To confirm the identity of 7-methylguanine, a separate experiment was conducted in which 10 rats were given 60 mg hydrazine/kg body weight and killed 5 hours later; methionine was not given to these animals. Pooled livers yielded 96 mg purified DNA which was hydrolyzed and fractionated as above; no carrier 7-methylguanine was added to the hydrolysate. Separation and purification of the DNA fractions by high pressure liquid chromatography and descending paper chromatography yielded suspect 7-methylguanine, which, in acid and alkaline solutions, gave ultraviolet absorption spectra nearly identical to those of authentic 7-methylguanine.

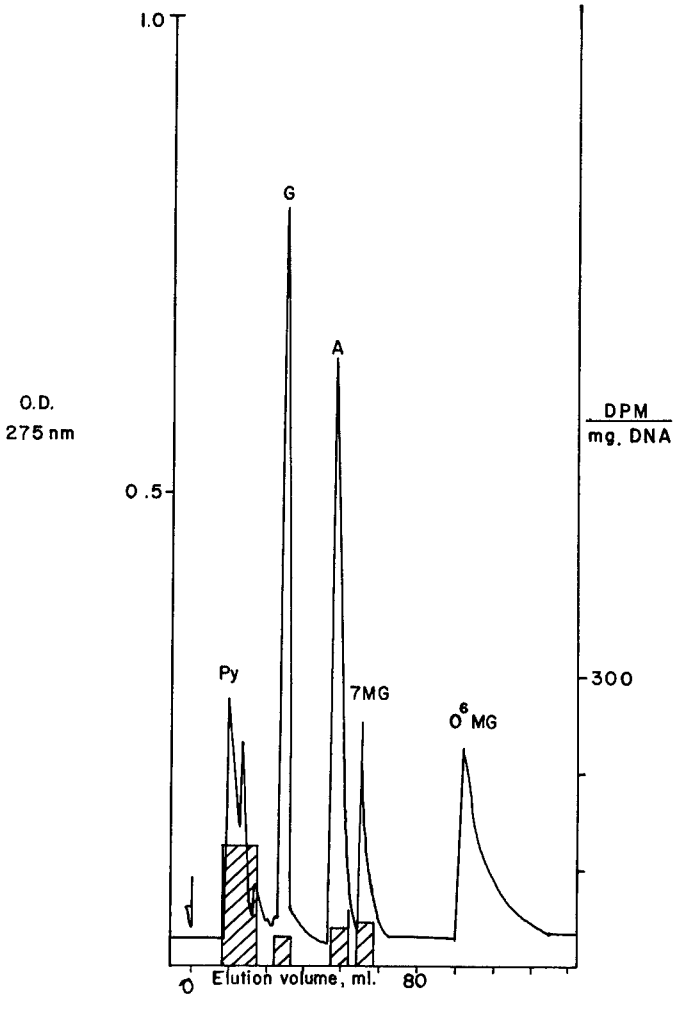
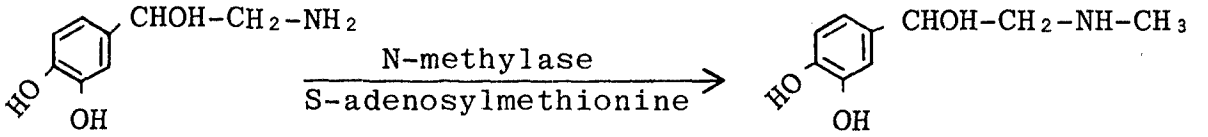
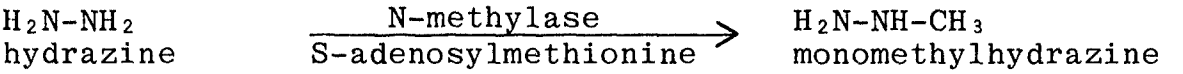


Figure 2. Elution profile of liver DNA hydrolysate prepared from control rats.

At least two mechanisms can be proposed for the methylation of DNA in hydrazine-poisoned animals involving methionine as the methyl donor. There are a few examples of N-methylation of amines by endogenous N-methylases, for example, the methylation of norepinephrine to epinephrine,



It is possible that some of the administered hydrazine might be methylated by a similar mechanism,



The methylation product, monomethylhydrazine, then might be metabolized to the strong electrophile, methonium, CH_3^+, in much the same manner as dimethylnitrosamine has been proposed to form this reactive ion. Because of the relatively low level of activity of the N-methylase system in most cells, one would predict that only a small fraction of administered hydrazine would be expected to be methylated to MMH.

A second mechanism that might be proposed to explain DNA methylation in hydrazine-poisoned rats is that a rapid response to administration of the liver toxin is an increase in the activity of native DNA methylase, the endogenous enzyme that normally uses S-adenosylmethionine in the methylation of the 5-position in DNA-incorporated cytosine; in order to form 7-methylguanine, it would be necessary to postulate an aberrant action of the enzyme in response to the hydrazine insult.

Both these proposed mechanisms are illustrated in Figure 3. Some experiments have been carried out to determine if either of these two mechanisms might have merit.

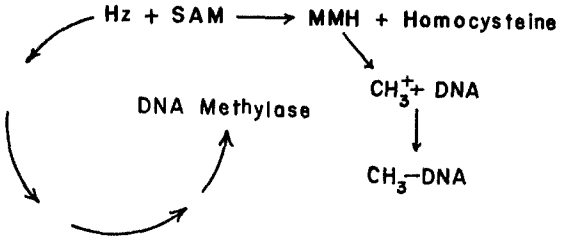


Figure 3. Possible mechanisms for DNA methylation in hydrazine-treated rats.

DNA METHYLATION IN MMH-TREATED RATS

If DNA-methylation in hydrazine-poisoned rats occurs via the formation of MMH as an intermediate to the generation of methonium ion, then one would expect a greater extent of DNA methylation in MMH-poisoned animals, for MMH is closer structurally and metabolically to the actual alkylating agent. To test whether it is likely that MMH is an intermediate in this mechanism, young adult rats were given 15 mg ^{3}H-MMH/kg body weight orally in 0.1 ml 0.1 N HCl; this dose approximates an LD 0.01. The animals were decapitated 5 hours later and liver DNA was isolated, hydrolyzed, and fractionated, as described above. Table 1 gives the distribution of radioactivity in the elution fractions for this DNA; the presence of ^{3}H-7-methylguanine is evident.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN ELUTION FRACTIONS FROM LIVER DNA HYDROLYSATES PREPARED FROM RATS TREATED WITH HYDRAZINE AND ^{14}C-METHYL-METHIONINE

	DPM/MG DNA				
	Control		Hydrazine		
Fraction	I	II	I	II	III
Prepyrimidine	3	0	0	0	0
Pyrimidine oligonuc.	312	106	95	64	105
Guanine	31	35	6	5	8
Adenine	81	11	3	0	33
7-Methylguanine	0	0	40	26	23
Trough	0	0	5	0	0
O^{6}-Methylguanine	0	0	0	0	0
Trough	0	0	0	0	0

Hydrazine dose, LD 0.01, 60 mg/kg BW.

A comparison was made of the methylation of rat liver DNA with hydrazine and MMH treatments. To quantitate the 7-methylguanine formation in the hydrazine-treated animals, it was assumed that the specific activity of the 7-methylguanine was the same as that of the S-adenosylmethionine that served as the methylating agent. The specific activity of S-adenosylmethionine was measured according to the method of Craddock (1974). Due to the dynamic state of the S-adenosylmethionine pool, the specific activity of this compound could only be estimated and was taken as an average value over the period of an hour. The quantitative results of DNA methylation in hydrazine- and MMH-treated rats are given in Table 2. The doses of each toxin are equitoxic (LD 0.01)

and methylation of DNA in hydrazine treated rats was obviously greater. Equimolar doses could not be studied in the rat, for doses of MMH higher than 15 mg/kg killed the animals in less than 5 hours and 30 mg hydrazine/kg resulted in no detectable DNA methylation.

TABLE 2. COMPARISON OF LIVER DNA METHYLATION IN RATS TREATED WITH EQUITOXIC DOSES OF HYDRAZINE OR ^{3}H-METHYL-MONOMETHYLHYDRAZINE

Toxin	Dose Mg(Mmoles)/Kg	Methylated Purines, µMoles/Mole Guanine 7-Methylguanine	O^6-Methylguanine
Hydrazine	60(1.88)	150-196	ND
MMH	15(0.33)	5-21	ND

The study was repeated, this time using the C57Bl/6 mouse. As shown in Table 3, equimolar doses of the two toxins produced different levels of 7-methylguanine in mouse liver DNA, MMH administration resulting in approximately 50% more methylation. Although such results do not refute the possibility that DNA methylation in hydrazine-treated animals may be a consequence of the in vivo methylation of hydrazine first to MMH, it is felt that such a small increase in DNA methylation resulting from MMH rather than hydrazine administration does not support strongly the mechanism relying on the intermediary methylation of hydrazine.

TABLE 3. LIVER DNA METHYLATION IN MICE TREATED WITH HYDRAZINE AND ^{14}C-METHYL-METHIONINE OR ^{3}H-METHYL-MMH

Toxin	Dose	µMoles 7-Methylguanine/Mole Guanine
Hydrazine	10 mg/kg	220
MMH	14.4 mg/kg	338

Kudryaskova and Vanyushin (1976) have shown that normal mammalian DNA methylase activity is increased in animals given a pharmacological dose of hydrocortisone. It is possible then that administration of an acutely toxic dose of hydrazine might result in a release of endogenous corticosteroid hormones, which in turn could alter native DNA methylase activity and specificity; the formation of 7-methylguanine then would reflect a response to toxic injury to the liver and could be nonspecific to hydrazine insult. Indeed, we have been able to demonstrate in the laboratory that stressful intubation techniques involving no toxins can result in the formation of trace amounts of 7-methylguanine in liver DNA in control rats. It has also been shown that hepatotoxins other than hydrazine (aflatoxin B_1, yellow phosphorus,

dimethylnitrosamine) can cause the methylation of liver DNA in rats given near LD_{50} doses concurrently with ^{14}C-methyl-methionine (Shank, unpublished results).

In the current studies, young adult male rats were given ip 20 mg hydrocortisone/kg in 0.9% NaCl 5 hours before decapitation and isolation of liver DNA. As shown in Table 4, such treatment resulted in 15 μmoles 7-methylguanine/mole guanine in the DNA, a level about 10 times less than that which results from administration of hydrazine (LD 0.01). Such results make it difficult to rule out the proposed mechanism that DNA methylation in hydrazine-treated rats results in an increase in DNA methylase activity but a decrease in the specificity of the enzyme.

TABLE 4. LIVER DNA METHYLATION IN RATS TREATED WITH HYDROCORTISONE AND ^{14}C-METHYL-METHIONINE: COMPARISON TO METHYLATION IN HYDRAZINE- AND MMH-TREATED RATS

Treatment	7-Methylguanine Content (μMole/Mole Guanine)
Saline	0 (None detected)
Hydrocortisone, 20 mg/kg	15
Hydrazine, 60 mg/kg	150-196
MMH, 15 mg/kg	5-21

Table 5 compares the levels of DNA methylation in rats treated with hydrazine or MMH to those treated with a known strong carcinogen, 1,2-dimethylhydrazine (SDMH). In the rat, SDMH is carcinogenic to the colon with a few malignant tumors appearing in the liver and kidney. It is clear that neither hydrazine nor MMH poisoning results in the level of DNA methylation as that seen in SDMH poisoning.

TABLE 5. DNA METHYLATION IN RATS TREATED WITH HYDRAZINE, MMH, OR SDMH

Toxin	Dose (mg/kg)	Tissue	Methylated Purines, µMoles/Mole Guanine 7-MG	O^6-MG	Others
Hydrazine	60	Liver	150-196	ND	ND
MMH	15	Liver	5-21	ND	ND
SDMH	10	Liver	1716	332	ND
SDMH	10	Colon	103	36	ND
SDMH	10	Kidney	Trace	12	ND
SDMH	160	Liver	2450	264	7-MA, 3-MA
SDMH	160	Colon	548	55	3-MA
SDMH	160	Kidney	101	11	3-MA
SDMH	160	Brain	148	ND	ND
SDMH	160	Lung	57	?	3-MA
SDMH	160	Pancreas	50	?	ND

Several studies are now in progress to pursue further whether DNA methylation in hydrazine-poisoned animals is more likely the result of MMH formation or stimulation in aberrant activity in DNA methylase. For the time being, there are few data to associate this form of DNA methylation with the putative carcinogenicity of hydrazine and MMH. Several laboratories (Swann and Magee, 1968; Loveless, 1969; Gerchman and Ludlum, 1973; Singer, 1975) have provided evidence that sites of DNA alkylation other than the 7-position of guanine may be more relevant to carcinogenesis; however, Lawley (1976) cautions that not enough is known yet about the consequences of alkylating the 7-position of guanine in replication fidelity of mammalian DNA to discount the importance of this site in chemical carcinogenesis.

SUMMARY

Preliminary evidence has been obtained to show that in rats and mice given large but not fatal doses of hydrazine, liver DNA becomes methylated at the 7-position of guanine. The amount of methylation occurring is much less than occurs when a strong carcinogen, such as 1,2-dimethylhydrazine, is given. Two mechanisms by which hydrazine administration could result in DNA methylation are being explored: (1) hydrazine may be methylated in vivo by an N-methylase and S-adenosylmethionine to form MMH which then is metabolized to methonium ion, or (2) the toxic insult to the liver resulting from hydrazine administration stimulates, probably through corticosteroid hormone release, aberrant DNA methylase activity. In any case, hydrazine administration does result in chemical alteration of liver DNA.

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PAPER NO. 11

HYDRAZINE AND UDMH INDUCED NEOPLASTIC TRANSFORMATION AND FELINE SARCOMA VIRUS INDUCED COCARCINOGENIC EFFECT ON HUMAN DIPLOID CELLS IN VITRO

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INTRODUCTION

Hydrazine and related analogues (1,2-DMH) have been implicated as suspect inducers of the carcinogenic process in colon carcinogenesis in mice (Cooper et al., 1978). The induction of the process was reported to be via a specific alkylation of the colon DNA. Apparently in mammalian systems the DMH is activated to its alkylating carcinogen (Free et al., 1978) prior to the initial stages of the carcinogenic process. This induction stage of carcinogenesis was reported to be followed by a promotion stage and then expression of the carcinoma. Phenobarbital administered parenterally prior to the administration of DMH enhanced the incidence of tumor formation (Eidlen, 1978). As the mixed-function aryl hydrocarbon hydroxylase activity was increased, an increase in the incidence of DMH induced colon carcinogenesis was observed. Following an activation stage, Matheson et al. (1978) showed that unscheduled DNA synthesis (UDS) was stimulated in WI-38 cells in vitro by UDMH and monomethylhydrazine. Recently, Barrett and T'so (1978) observed a progressive nature of carcinogen induced neoplastic transformation in vitro with Syrian hamster embryo cell cultures. We (Milo and DiPaolo, 1978) have observed that human cells can be transformed in vitro and that there are progressive stages to the carcinogenesis process in this sytem. Each of the stages of the carcinogenesis process can be investigated with feline sarcoma virus to unravel the details of each stage. Moreover, the virus directed transformation can be quantitated in a predictable manner (Blakeslee and Milo, 1978; Milo et al., 1978; Casto et al., 1973). We, therefore, implemented studies with either UDMH, 1,2 DMH, MMH and/or HZ to study the interrelationships between the suspect chemical carcinogen induced carcinogenic process and the virus induced carcinogenic process and their interrelationships during the different stages of the process.

METHODS

Randomly proliferating cell populations were obtained routinely from different foreskin tissue dispersed with collagenase (Reigner et al., 1976). These cell populations at low population doublings were serially subpassaged in complete medium (CM) composed of Minimum Essential Growth medium - 25 mM Hepes at pH 7.2 supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM glutamine, 0.2% sodium bicarbonate, 50 µg/ml gentomycin, in an atmosphere of 4.0% CO_2 enriched air at 37 C. Preconfluent (70-80%) cultures were transferred to Dulbecco's modified Eagle's medium lacking arginine and glutamine, at pH 7.2, supplemented with 10% dialzyed FBS (Dulbecco's modified Eagle's medium lacking arginine and glutamine) to decrease cell multiplication. After overnight exposure, this medium was replaced with CM medium containing 0.5 IU of insulin. Ten hours later, the suspect chemical carcinogen(s), monomethylhydrazine (MMH), unsymmetrical dimethylhydrazine (UDMH), 1,2 dimethylhydrazine, and hydrazine (HZ) were added to the CM containing added insulin. This time coincided with the time when the maximum number of cells were entering S-phase of the cell cycle. The carcinogen stock solutions had been prepared by dissolving the chemical in spectra grade acetone and diluting with CM immediately before use. All dilution procedures were carried out under red light and an atmosphere of argon. After discontinuing the treatment, the cell cultures were washed 3 times with CM and immediately subpassaged 1:2 into CM containing 8 X nonessential amino acids and 2 X vitamins. The length of treatment with the carcinogens depends on the stability of the compound and time necessary to transport the compound into the nucleus of the cell. When the cell populations were 75% confluent, they were serially passaged 1:10. This ratio of subpassaging was continued for 20 population doublings (PDL). The saturation density of the transformed cell populations always exceeded the density of the normal untreated cell populations.

After 20 PDL, 50,000 cells from a rapidly proliferating nonconfluent culture were seeded into 2 ml of a 0.35% agar or 1.0% methylcellulose (6,000 cps) supplemented with modified Dulbecco's Lo-Cal growth medium over a 5 ml 2% agar base supplemented with RPMI 1629 growth medium - 20% FBS. Agar-containing cultures were refed every 7 days with 0.5-2.0 ml of Low-Cal medium-supplemented soft agar. The colonies were isolated from the semisolid medium, seeded into CM containing nonessential amino acids and grown until confluent. Colonies

were isolated and repopulated. Cell populations from treated populations were mixed with wheat germ agglutinin from 2500 µg/ml to 9 µm/ml. After 10 minutes, they were read. Twenty-thousand cells from the cultures that contained colonies that grew in soft-agar again were seeded into soft agar, removed after 11 days, and repopulated to $10^{6.3}$. These cells were inoculated into the subcapular region of the nu/nu mouse. After 4-6 weeks, the tumors were excised and examined histopathologically.

VIRUS INDUCED CARCINOGENESIS

Human foreskin fibroblast cells (HSF) were grown by conventional methods. Just prior to use, HZ, MMH or UDMH were added to prewarmed (37 C) CM and further diluted in CM to the desired concentrations (Blakeslee and Milo, 1978). Cells pretreated with nontoxic concentrations of chemicals prior to virus infection were intubated with designated concentrations of chemical for 3 hours then washed and infected with the Snyder-Theilen strain of FeSV or virus infected cells were treated 3 hours postinfection for 1 hour, washed and refed with growth medium. The foci were counted 9-13 days later. RDDP enzyme assays for determining the presence of endogenous virus following long-term (40-60 days) exposure to the chemicals were performed with the exogenous template-primer technique (Blakeslee and Milo, 1978).

RESULTS

Cytotoxicity data for each carcinogen was accumulated prior to transforming the human cells. It was found that the optimum transformation dose for each carcinogen was a cytotoxicity value from 25-50% (Effective Dosage 50 = ED_{50}). Hydrazine and 1,2 DMH were the most cytotoxic followed by MMH then UDMH (Table 1). The only cell populations to grow in soft agar were UDMH and HZ treated cells (Table 2). From 6 different treated cell populations, 100% of the populations grew in soft agar at a frequency of $1:10^{3.5}$ for UDMH and $1:10^{4.7}$ for HZ treated cells. However, for three different MMH or three different 1,2 DMH treated cell populations, none grew in soft agar.

TABLE 1. CYTOTOXICITY DATA (ED_{50})

Suspect Chemical Carcinogen	Concentration (µg/ml)
Monomethylhydrazine	25
Unsymmetrical Dimethylhydrazine	30
1,2 Dimethylhydrazine	10
Hydrazine	10

One thousand cells were seeded per 25 cm^2 and cloned for 9-11 days (Milo et al., 1976). The suspect carcinogen was dissolved in a suitable solvent and applied as described by Milo and DiPaolo (1978). The effective dosage (ED) values were relative plating efficiencies (cloning efficiencies), i.e. the cloning efficiency of the untreated controls, 18%, compared with the cloning efficiencies of cultures over a range of concentrations of the suspect chemical carcinogen expressed as µg/ml.

TABLE 2. FREQUENCY OF COLONY GROWTH IN SOFT AGAR*

Chemical	No. of Treated Populations	No. that grew in soft agar	Incidence
Monomethylhydrazine	3	0	--
Unsymmetrical Dimethylhydrazine	6	6	$1:10^{3.5}$
1,2 Dimethylhydrazine	3	0	--
Hydrazine	6	6	$1.10^{4.7}$

*Fifty thousand cells were seeded into 2 ml of 0.35% agar over a 5 ml 2% base in 25 cm^2 wells. These cultures were incubated at 37 C in a 4% CO_2 - enriched air atmosphere. The base medium was supplemented with RPMI 1629 (G.I.B. Co., Grand Island, N.Y) and the soft agar was supplemented by Lo-Cal (Biolabs, Worthbrook, Ill.). Colonies containing >100 cells per colony were scored after 11 days.

Agglutination endpoints for the transformed cell populations were 9 µg/ml for UDMH transformed cells and 19 µg/ml for HZ (Table 3). In all of the treated cell populations that did not grow in soft agar, we observed no altered agglutination profile when compared to the untreated population (Table 3). Moreover, the MMH and 1,2 DMH treated cell populations when injected into nude mice did not form tumors. The blebs regressed in 24-72 hours.

TABLE 3. AGGLUTINATION PROFILES OF CARCINOGEN TREATED CELL POPULATIONS

Cell Population	Lectin	Conc. of Agglutinin, µg/ml
Monomethylhydrazine	Wheat Germ	2500
Unsymmetrical Dimethylhydrazine	Wheat Germ	9
1,2-Dimethylhydrazine	Wheat Germ	2500
Hydrazine	Wheat Germ	19
No Treatment	Wheat Germ	2500

The cell sheet was washed 3X with Hank's salts minus calcium and magnesium containing 0.05 mM EDTA. The cell sheet was then lightly trypsinized with 0.01% trypsin (CLS Worthington Biochem). A stock solution of wheat germ agglutinin (Worthington Biochem) was made up in phosphate buffered saline (PBS). After centrifugation of the cells, the pellet was reconstituted twice in PBS. A stock solution of agglutinin was 2500 µg/ml and 0.025 ml was added to a microliter well along with 0.18 µl of the cell suspension at a stock concentration of 10^6 cells/ml. The mixture was agitated to completely mix the ingredients for a few minutes. These titrations were run in sets of triplicates (San et al., 1979).

Colonies were removed from the semisolid medium and propagated to a cell density of $10^{6.3}$ and injected into nude mice in the subscapular region. Eighteen to 25 days later, tumors appeared at the site of injection. After 4-6 weeks, the tumors were 0.8-1.5 cm in length. They were surgically removed and parts examined histopathologically. The incidence of tumor formation was 4/8 for UDMH and 4/8 for hydrazine treated cells. The tumors were composed of multiple vacuolated

cell types predominantly a syncytial network with eosinophilic cytoplasm and ovoid basophilic nuclei. There were also foci of more centrally, very vacuolated basophilic staining cells with central of peripheral nucleoli. The tumors were classified as myxofibroma-like.

COCARCINOGENS

Cells treated before virus infections with either 8 or 80 µg/ml MMH resulted in significant inhibition of ST FeSV focus-forming units by 62% and 56%, respectively, whereas HZ at 5 µg/ml inhibited focus formation by 29% when virus infected cells were treated 3 hours post infection (Table 4). However, pretreatment of cells with 100 µg/ml of UDMH resulted in a 1.8 fold enhancement of focus forming units while a 3 hour post treatment resulted in a 1.4 fold enhancement.

TABLE 4. HYDRAZINE, MONOMETHYLHYDRAZINE, AND UNSYMMETRICAL DIMETHYLHYDRAZINE EFFECTS ON ST FeSV VIRUS TRANSFORMATION

Chemical	Treatment Time (Hours)	Conc. (µg/ml)	FFU/0.2 ml X 10^{-2} Treated	FFU/0.2 ml X 10^{-2} Untreated	Effect on Transformation ENH.	INH.	(P)*
Hydrazine	-3	50	74.3±14.4	75.5± 9.9	-	2%	N.S.
		5	64.2±13.0	75.5± 9.9	-	16%	N.S.
	+3	50	87.4±22.4	75.5± 9.9	1.2 fold	-	N.S.
		5	53.6± 6.6	75.5± 9.9	-	29%	0.001
Monomethyl-hydrazine	-3	80	34.8±10.9	78.5±26.5	-	56%	0.001
		8	30.7±16.1	78.5±26.5	-	62%	0.05
	+3	80	-**	-	-	-	-
		8	-**	-	-	-	-
Unsym-metrical dimethyl-hydrazine	-3	100	112.6±28.7	63.5± 8.9	1.8 fold	-	0.001
		10	60.3±14.5	63.5± 8.9	-	5%	N.S.
	+3	100	89.0±16.1	63.5± 8.9	1.4 fold	-	0.05
		10	53.7±11.7	63.5± 8.9	-	16%	N.S.

* Significance determined by student's t-test.
** Not determined.

High speed pellets were assayed at various days of treatment with HZ, MMH and UDMH for RDDP activity as an indicator of endogenous virus expression. No increased activity was detected in samples beginning 12 days post treatment through 40 days (Table 5).

TABLE 5. RDDP ACTIVITY FROM HYDRAZINE(S) TREATED CELL SUPERNATANTS

Day Post Treatment	Control	60 µg/ml HZ	80 µg/ml MMH	100 µg/ml UDMH
		(CPM X 10^{-3})		
12	2.1	2.1	2.3	2.3
18	1.6	2.0	1.8	1.3
21	2.1	1.9	2.0	2.1
25	1.9	2.6	1.6	1.6
27	1.3	1.8	1.3	1.3
29	1.6	1.9	1.5	1.2
32	2.7	2.2	3.5	2.2
36	N.D.*	3.0	2.6	2.2
40	1.5	2.2	1.9	1.7

FeLV Standard - 96.6
Background - 1.2
*N.D. - Not determined.

DISCUSSION

We previously reported that select chemical carcinogens inhibited ST FeSV induced transformation in human cells at non-toxic concentrations (Blakeslee and Milo, 1978). Further studies by Milo et al. (1978) showed that these same carcinogens transformed human cells in vitro and the transformed cells grew as tumors in nude mice (Milo and DiPaolo, 1978). Thus, there appeared to be a good correlation between chemically induced in vitro transformation and inhibition of oncogenic RNA virus induced transformation.

When applying these criteria to the hydrazine carcinogens, only hydrazine demonstrated these properties. MMH, a non-carcinogen toxic chemical, inhibited virus transformation, but did not transform cells alone (Table 6). UDMH neoplastically transformed human cells, but did not inhibit virus transformation. The results of this study suggest that HZ and UDMH should be considered as chemical carcinogens and cocarcinogens. However, UDMH does not appear to inhibit the FeSV induced transformation events.

TABLE 6. CORRELATION BETWEEN INHIBITION OF VIRUS TRANSFORMATION, IN VITRO CHEMICAL TRANSFORMATION, AND NEOPLASTIC TRANSFORMATION

Chemical	In Vitro Chemical Transformation	Neoplastic Transformation	Inhibition of Virus Transformation
Hydrazine	Yes	Yes	Yes
Monomethyl-hydrazine	No	No	Yes
Unsym-metrical dimethyl-hydrazine	Yes	Yes	No

In that carcinogen treatment of randomly proliferating cells has resulted in only a rare transformation event and that we have observed with SV 40-carcinogen treated human cells that all the foci formed were virus induced (Milo and Blakeslee, 1978), we therefore conclude that chemical carcinogen induced transformation initiates the carcinogenic events at a different stage of the cell cycle than the virus. Moreover, in every case where a chemical carcinogen has been administered to human cell population, when the cells are predominantly in S phase of the cell cycle, the cells can routinely be transformed by chemical carcinogens (Milo and DiPaolo, 1978), either MMNG, β-propiolactone, 4NQO, benzo(a)pyrene, or propane sultone. At this time, the list includes many other carcinogenic substances presently under investigation.

It is our contention that DNA repair synthesis of the non-proliferating cell population repairs the DNA too accurately and rapidly (Milo and Hart, 1976) and upon the administration of the carcinogens during S, the error prone DNA repair synthesis system is functional and a transformation event occurs after initiation of the microinsult to the genetic machinery. These cell populations grow in soft agar and produce tumors in mice. There appears to be a high correlation between growth in soft agar and tumor growth in nude mice (San et al., 1979).

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PAPER NO. 12

ENVIRONMENTAL CHEMISTRY OF HYDRAZINE FUELS

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INTRODUCTION

The widespread use of hydrazine compounds and their documented toxicity to biological organisms makes it imperative that data be available to predict their persistence and fate in the environment. The purpose of this paper is to summarize some of the physical and chemical aspects of the environmental chemistry of the three hydrazine fuels, hydrazine (HZ), unsymmetrical dimethylhydrazine (UDMH), and monomethylhydrazine (MMH). This information will allow prediction of the environmental impact of accidental releases and design of protocols reducing the hazards to personnel and the environment. Table 1 summarizes chemical and physical characteristics which are important in understanding the fate and transport of the three compounds in the environment.

TABLE 1. CHEMICAL AND PHYSICAL PROPERTIES OF HYDRAZINE FUELS

Fuel	Density	Boiling Point	Vapor Pressure	Evaporation Rate	Half-Life Air	Half-Life Water
Hydrazine (N_2H_4)	1.0 g/cc	114.2 C	1892.9 kPa	0.49 mg/cm^2 min	1-10 hr	7 days
Monomethylhydrazine ($CH_3N_2H_3$)	0.87 g/cc	87.7 C	6598.4 kPa	1.7 mg/cm^2 min	2-7 hr	10 days
Unsymmetrical Dimethylhydrazine [$(CH_3)_2N_2H_2$]	0.78 g/cc	62.3 C	22274.4 kPa	13 mg/cm^2 min	100 hr	10 days

MATERIALS AND METHODS

REAGENTS

Reagents were ACS reagent grade or better except for the hydrazine fuels. Fuels obtained from the Rocky Mountain Arsenal were used in all evaporation, degradation, and spill treatment studies.

ANALYTICAL METHODS

Numerous different analytical techniques were adapted for use in this study: (1) potassium iodate titration was used as a general method for nonspecific reducing characteristics; (2) reaction with para-dimethylaminobenzaldehyde (PDAB) to form p-dimethylaminobenzolazine, a colored azo complex, was used for trace analysis in pure systems; (3) hydrazine specific analyses were conducted by pyrazole-derivative gas chromatography; (4) long path infrared spectroscopy was utilized for atmospheric degradation studies; and (5) gas chromatography interfaced to a mass spectrometer was used for component identification in some of the atmospheric degradation studies. Detailed information on the above techniques is available in MacNaughton et al. (1978), Stauffer and Eyl, (1978), Stauffer et al. (1979), Stone (1978), and Zirrolli, 1979).

EXPERIMENTAL PROCEDURES

EVAPORATION STUDIES

Evaporation studies were conducted on both the neat fuel obtained from Rocky Mountain Arsenal and aqueous solutions. Studies of the neat fuel included work on hydrazine samples placed outdoors under prevailing environmental conditions and work on all of the fuels under more controlled conditions in the laboratory. Only laboratory studies were conducted on the aqueous solutions.

A known volume of fuel was placed in round glass Petri dishes and weighed prior to introduction to the prevailing environmental conditions. At different time intervals, the samples were weighed and the density and N_2H_4 concentration determined. The laboratory studies duplicated the outdoor studies except that temperature and air velocity were controlled. The temperature was 21 ± 1 C and the air velocity at the evaporating surface, measured with a Velometer®, was 63.5 cm/sec.

DEGRADATION STUDIES

Aqueous degradation experiments were performed in a closed pyrex double-jacketed reaction vessel. Temperature was controlled by a Lauda Model K2R temperature recirculator and pH was measured, adjusted, and maintained with a Radiometer, Model TT2 titrator and Model ABU12 autoburette. Dissolved oxygen was monitored with a YSI Model 51A dissolved oxygen meter. Atmospheric oxidation studies were performed in various all glass and paraffin coated reaction vessels. These ranged from a 44 cm x 2 cm cell to a 305 cm x 15 cm cell with internal multiple reflection optics capable of path lengths up to 200 meters. Fuel concentration was monitored with a Digilab Model FTS-20 Fourier transform infrared spectrophotometer. The infrared source was a Nernst glower operated from a Variac transformer in series with a 100-watt incandescent lamp at 96 volts AC.

At various times during the oxidation, 0.1 ml gas samples were withdrawn and injected onto the GC/MS system. At the completion of the reaction (24-36 hours), the bulb was rinsed with 2 ml of deionized distilled water of which 2-4 µl aliquots were analyzed on a Finnigan 3200 GC/MS coupled to a System 150 data system.

RESULTS

EVAPORATION

Evaporation rates can be calculated using chemical rate equations or measured under simulated spill conditions. The evaporation rate of a liquid is a complex function of the ambient air temperature, ground temperature, wind speed, solar radiation, size and dimensions of the spill, vapor pressure, and diffusion coefficients. Both calculated and measured evaporation rate data are, at best, estimates of the real situation since rarely would the necessary model input parameters be available for expeditious use of a model or the circumstances be the same as those used in the evaporation studies.

The measured evaporation rates are shown in Table 1 for the three fuels under controlled laboratory conditions. There is more than one order of magnitude difference in the evaporation rate between hydrazine and UDMH. This reflects the large difference in vapor pressure of the fuels. Figure 1 illustrates the effect of solar insulation and the corresponding increase in pool temperature on hydrazine evaporation. CO_2 reduces the evaporation rate by forming a weak salt with hydrazine, thereby lowering the vapor pressure of the fuel.

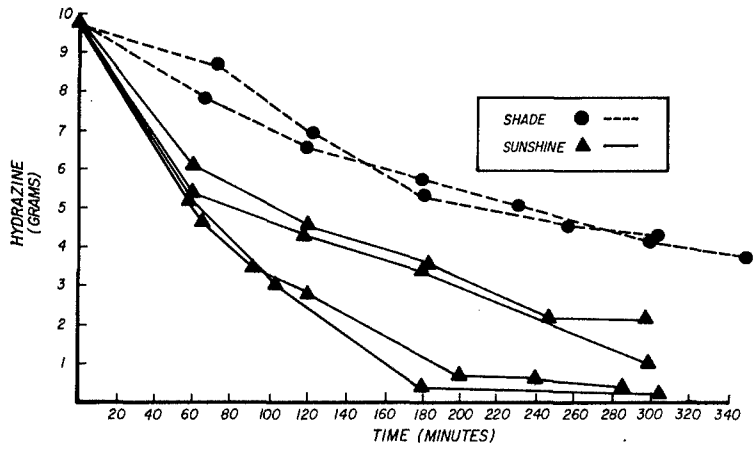

Figure 1. Evaporation of hydrazine as a function of time under uncontrolled ambient conditions.

Figure 2 illustrates that increased dilution of the fuel causes a significant decrease in the hydrazine vapor concentration. These data, generated by passing nitrogen over and through the various dilutions of hydrazine, show that hydrazine must be diluted with almost 1000 volumes of water to achieve the Threshold Limit Value (TLV) accepted by the Air Force (0.13 mg/m^3).

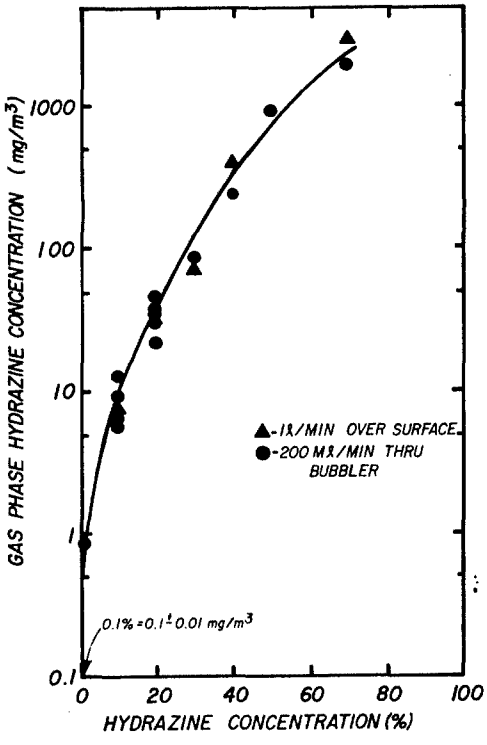

Figure 2. Gas phase hydrazine concentration as a function of percentage hydrazine concentration. Temperature 25 C.

DEGRADATION

Hydrazine and MMH are much more stable than UDMH in the systems used for these studies. The data presented in Table 1 demonstrate that, for an accidental spill, decay by oxidation can take several hours in air atmospheres and up to several days for fresh and marine waters.

Although the hydrazines are energetic reducing compounds, they are remarkably stable in the absence of appropriate catalysts. The degradation of hydrazine in various aquatic environments is shown in Figure 3. In distilled water, losses over the 6 days of the study were too low to be measured. The different pond and seawater samples exhibit increased degradation rates over that found in distilled water; however, this decreased resistance to oxidation could not be correlated with chloride content, ionic strength, or suspended material. This increased breakdown may be due to low concentrations of metals, such as copper, which were shown to act as catalysts for the oxidation of hydrazine. It is apparent from Figure 3 that degradation will not be a significant sink for hydrazine in aqueous environments, and downstream hydrazine concentrations will be controlled by dilution and dispersion.

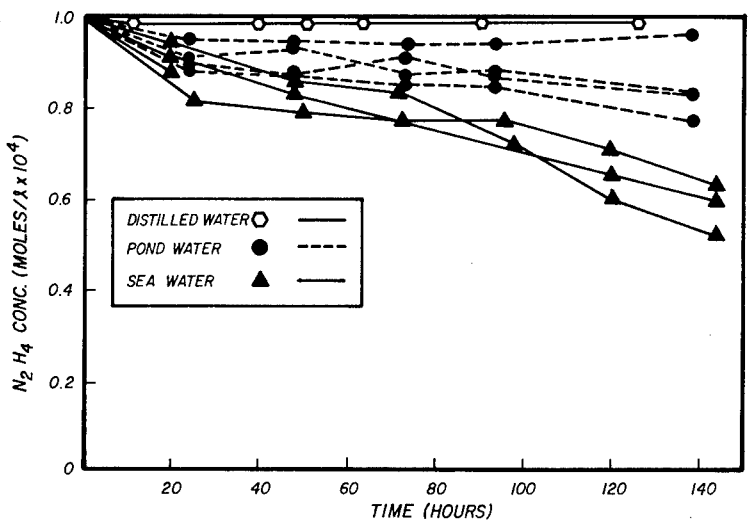

Figure 3. Aqueous degradation of hydrazine as a function of time and source of aqueous solution. Temperature - 25 C, pH - 8.0 ± 0.3, atmosphere - air.

Atmospheric studies in our laboratory have shown that hydrazine oxidation in air is highly dependent on the available surface area and the composition of the surface material. Because the decay rate is greatly influenced by these heterogenous reactions, extrapolation of reaction cell data to real ambient conditions should be made cautiously. Figure 4 displays the oxidative decay of hydrazine in a 5-liter paraffin coated all glass reaction flask. The paraffin coating gave the least reactive surface and produced a half life of approximately 5 hours. Duplicating the same experiment in the 60-liter long path infrared cell produced a half life of 139 minutes. The main reaction products under the conditions studied here were N_2 and water with some ammonia also observed.

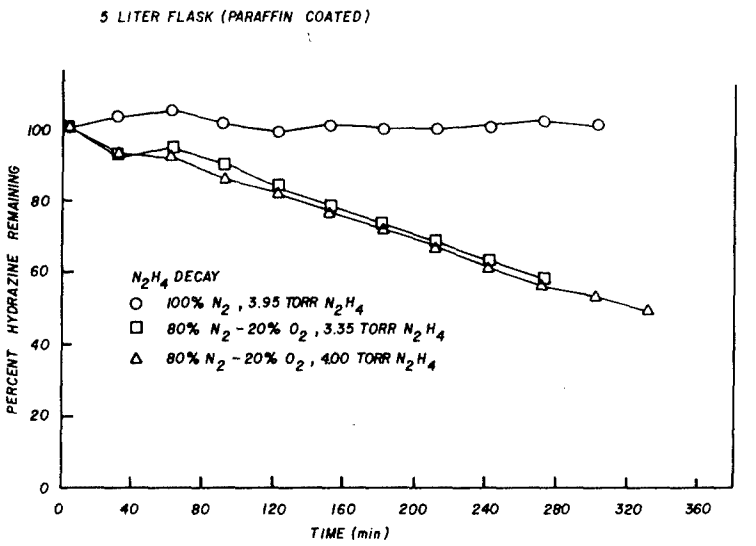

Figure 4. Atmospheric degradation of hydrazine as a function of time and oxygen concentration in a 5-liter paraffin coated reaction flask.

DEGRADATION PRODUCTS

The atmospheric degradation of hydrazine is simple compared to the more methylated compounds. Table 2 summarizes the reaction products found after gas phase oxidation of the three fuels. The low vapor pressure of many of these products results in their condensation on the vessel walls and makes identification in the gas phase difficult.

TABLE 2. IDENTIFIED DEGRADATION PRODUCTS OF HYDRAZINE FUEL

HZ	MMH	UDMH
Ammonia	Formaldehyde Dimethyl-hydrazine (FDH)	Tetramethyltetrazine
Nitrogen	Nitrogen	Methyl Triazole
Water	Water	Nitrosodimethylamine
	Hydrogen	Dimethylformamide
	Methane	Acetaldehyde Dimethyl-hydrazone
	Azomethane	Formaldehyde Dimethyl-hydrazone
	Formaldehyde Monomethylhydrazone (FMH)	Trimethylhydrazine
	Methyl Pyrazole	Trimethylamine
	Polymers of FMH	Dimethylamine
	Formaldazine	Triazole

It is impossible to determine the environmental impact of these oxidation products because little if any research has been performed on their toxicity to higher or lower organisms.

SUMMARY

The use and transport of the hydrazine fuels and their toxicity to both humans and lower organisms makes it important to understand their fate in the environment. It is apparent that in contrast to their energetic decomposition as fuels, in dilute concentrations, they are relatively stable in natural environments. The evaporation rates under the conditions studied varied from less than 0.5 mg/cm^2min for hydrazine to 13 mg/cm^2min for UDMH with the stability of the fuels increasing in the same order. The complex nature of the oxidation products of MMH and UDMH makes postulation of their environmental consequences difficult. Hydrazine, although the most toxic, degrades mostly to nitrogen and water with the possibility of some ammonia.

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DR. MITCHELL (Baylor College of Medicine): Major MacNaughton, you showed that only methane and nitrogen were being produced in your chamber but you didn't show anything about the stoichiometry of that reaction although you were showing the hydrazine to disappear.

MAJOR MAC NAUGHTON (Air Force Engineering and Services Center, Tyndall Air Force Base): Looking at oxygen, methane, and nitrogen bases it about on a molar basis. We do find, though, that the reaction is tremendously surface catalyzed. A number of experiments that we've done using glass vessels that have been silanized, glass vessels that have been paraffin coated, or glass vessels that have been heated up to 400 C and totally deoxygenated, gave us extremely different rates. The stoichiometries are different as well. In the studies that we have done with our largest system which is 55 liters, we're getting about 1 mole of oxygen per 1 mole methane nitrogen produced. Those studies only showed things that can be measured by thermal conductivity after separation of the main gas phase component on a molecular sieve.

MR. VERNOT (University of California, Irvine): You mentioned that you simulated sunlight in your system. Did you find that the oxidation of the hydrazine materials required UV or light activation?

MAJOR MAC NAUGHTON: Just the opposite. We found that sunlight made no difference in the oxidation rate at all. There are a number of reports put out by our laboratory. If anybody is interested in more specific data and actual numbers, I'll be glad to send you copies of both the gas phase decomposition and the aqueous decomposition studies that we've done in the past with some of the identification work that we are doing.

DR. DOST (Oregon State University): Major MacNaughton, I was interested in your data on the effect of concentration on evaporation rate. There's beginning to be some information, at least with organic materials, that the interface has significant effects on the rate of evaporation and that if you agitate or stir, you can increase the rate of volatilization from a solution. Is that the case with hydrazine?

MAJOR MAC NAUGHTON: Evaporation rate data, as simple as it seems, is very difficult to get. We have looked at a number of parameters and one that I didn't discuss is the actual surface area utilized to do the experiments. We have found that we have to go up almost to a 20 centimeter diameter size beaker to get very little surface effect. If you establish a ripple effect, or if you stir it or any other thing, you are going to increase the rate of evaporation. In the data that I showed, we were looking at the gas phase concentration over different solution concentrations and we actually bubbled the gas through at a very slow rate to achieve saturation at that concentration.

DR. DOST: I think we're beginning to learn that the data that's in the handbook doesn't necessarily apply to the real situation.

DR. JENKINS (University of California, Berkeley): Do you notice any difference either in the nature of the products or just in the overall rate of decomposition between the vapor phase results that you got and the aqueous phase studies? Could you just summarize?

MAJOR MAC NAUGHTON: In aqueous phase studies with monomethylhydrazine, we found less of the formaldehyde dimethylhydrazone. We believe that the formaldehyde dimethylhydrazone is also oxidized, and we find more of the MMH coming off as nitrogen gas and as methane. We don't find much formaldehyde dimethylhydrazone. We also see many intermediates that have not been identified, and they appear to be kinetic intermediates. If you take, for instance, unsymmetrical dimethylhydrazine and oxidize it, you get some colored intermediates that are very difficult to identify. As you conduct the experiments, the color comes and goes. You don't know really whether you're just catching a slower phase of the degradation or not. We haven't identified nearly as many of the oxidation products in the aqueous phase but the primary reason is when you do it in the gas phase and you get this condensate, it's much more concentrated than what you would find in the aqueous phase. It's formed in concentrations that we can analyze. As anybody who has done GC-MS with these compounds can attest, they are not very stable. As was shown in some of the other presentations, we have to go to derivatization and identify these breakdown products as benzaldehyde, ketone, and acetone derivatives. Many of these compounds do not form derivatives so we are unable to identify some of the components.

MR. HAUN (University of California, Irvine): Dr. Milo, could you comment on the purity of your UDMH samples used in your study? Specifically, did your UDMH contain dimethylnitrosamine?

DR. MILO (Ohio State University): I would defer that question to Miss Marilyn George. She analyzed it after we redistilled it and sent it to her.

MISS GEORGE (6570 Aerospace Medical Research Laboratory): There was less than .001% DMNA in the UDMH Dr. Milo used in his study.

DR. CROCKER (University of California, Irvine): Dr. Milo, I'd just like to clarify what I think you are telling us. You were using feline sarcoma virus to transform human foreskin fibroblasts, and you were getting a certain number of focus forming units in your standard focus forming unit assay. When you added the hydrazine compounds, you increased or decreased the number of forming units; but in the main, it was UDMH and hydrazine that did increase the number of focus forming units. These increased by a factor of some percentage, 29% in the case of UDMH, as I recall. Are you suggesting that the increase in the number of focus forming units by the hydrazines on the already virus transformed cells is an indication that this was chemical transformation of those cells?

DR. MILO: No, I'm not implying that at all. What I am saying is that many of these compounds that we consider to be carcinogens are complete carcinogens and incomplete carcinogens. We were looking at the virus as a transforming agent and we talk about the cocarcinogenic principle or property. In this particular case, UDMH is classified as a carcinogen in this system, but it has also cocarcinogenic properties. In other words, it can act with the virus in a randomly proliferating population where the carcinogen itself, without the virus, induces unscheduled DNA repair synthesis. But it does not affect transformation in that particular aspect.

DR. CROCKER: Just let me repeat what I've heard. UDMH on human foreskin fibroblasts does not transform by itself.

DR. MILO: Only in the blocked cells treated during scheduled DNA synthesis, not in the randomly proliferating population when they are treated when the cells are in the G1 part of the cell cycle.

DR. CROCKER: Did you show us any evidence of that cell transformation in the slides? By this I mean the transformation by UDMH in blocked cells?

DR. MILO: Yes.

DR. CROCKER: And are tumors formed by the cells that are treated this way?

DR. MILO: Yes. When Dr. Weisbrode evaluated these histopathologically, the interpretation was a myxofibroma or a mesenchymal tumor which essentially is then described and reported in Nature.

MR. JONMAIRE (Uniroyal, Inc.): I would like to ask Dr. Dost if he has searched for the other 25% of the hydrazine in his animals.

DR. DOST (Oregon State University): I've searched for it but I haven't successfully gotten a material balance. We're looking for other metabolites and we are preparing to look for residuals in tissue. We have conducted some wet chemistry analyses of tissue; and clearly, there is some hydrazine measurable as hydrazine in tissues. We are in the process of developing what we hope will be a more sensitive method of following ^{15}N by emission spectroscopy. If our efforts are successful, we will improve the analytical sensitivity by at least 1 and possibly 2 orders of magnitude and then I think we'll have better identification of what we are finding in the tissues.

MR. JONMAIRE: Is there any specific tissue or tissues where you're finding the ^{15}N being stored?

DR. DOST: I think we have two factors to consider. It seems evident that we are going to find substantial nonspecific binding, probably to proteins of whatever character you want to imagine and quite possibly binding to nucleic acids. That's one consideration. The other consideration is the amount of hydrazine nitrogen that we may find in association with acylations at that intermediate point where nitrogen has yet to come back off but the carbon is left on the molecule. That's going to demand sensitivity that I suspect we won't be able to achieve because sensitivity with ^{15}N is not very high. We're looking at mass in the one case and emission spectra on the other. There are clear limits. The techniques for analysis of ^{15}N are not as sensitive, for example, as when using radioisotope tracers. We also would like to see if we can label substrates with ^{14}C somewhat in a system like Dr. Shank has been describing and follow the fate of the ^{14}C. I'm not terribly optimistic, and I'm not going to make any promises about it.

MR. VERNOT: Dr. Milo, I'm not at all certain that I understand the technique that you're using. If I understood the beginning test that you used, you treated cell strains with some kind of an agglutinin. Is my interpretation correct that this was a sort of a screening test that would tell you whether the rest of your procedure would work on the chemicals?

DR. MILO: I agree. I guess you are somewhat confused. I guess it's my fault because I didn't clarify it in the beginning. The process of carcinogenesis can be identified as a multi-stage process. You can look at the different stages as they occur. Now this staging process is associated with the cell. And the cell has a cell cycle. It has a nonproliferating phase (G1). It has a phase which synthesizes DNA. It has a G2 phase which immediately follows the S phase and then the mitotic phase. The virus was used to probe the nonproliferating phase or the G1 part of the cell cycle. The chemical carcinogen would not induce carcinogenesis when the cell population was treated during the nonproliferating phase, in other words, G1. But if the cell was treated during scheduled DNA synthesis, it would transform the cell. What we wanted to do was to study the interaction of the chemical with the virus during G1 to see what role it played because it also induced unscheduled DNA synthesis or DNA repair synthesis which occurs during the G1 part of the cell cycle when the DNA is not replicating. It repairs the DNA error free. Otherwise, we'd all walk around with mutations and squamous cell carcinomas of the skin. The feline sarcoma virus system is identified in the chemical biological interactions, and CBI published this about three months ago. The chemical carcinogen system is published in Nature, Volume 275, pages 130-132, 1978. What we've done in the human cell is separate the carcinogenesis process and the cell cycles and used the virus to study the nonproliferating phase and the chemical carcinogen to study it during scheduled DNA synthesis.

MR. VERNOT: My question had to do with the treatment of the four systems, the four cell systems which had been treated with the four chemicals with this lectin. On the basis of whether there was agglutination or not, you rejected further work on two of the chemicals.

DR. MILO: That's to identify whether the cells, once they have gone into the early stages of the carcinogenesis process, during expression, would be considered as candidates to go on in the system to be passed through to the intermediate phase of carcinogenesis.

MR. VERNOT: What confuses me in this system is that you rejected symmetrical dimethylhydrazine as a candidate, and it is the one member of the hydrazine family which everyone accepts as a very strong carcinogen.

DR. DOST: SDMH is negative on that test but positive on others. It's accepted as a very strong carcinogen.

DR. MILO: That's right. But I told you in the beginning that this is a foreskin. All I can tell you is that in this system, it doesn't act as a cocarcinogen or a carcinogen. Let me give you examples of two other compounds. Ethyl methane sulfonate and methyl methane sulfonate. Methyl methane sulfonate in the mammalian systems is a very strong carcinogen. In our system, it has no carcinogenic property at all. 7,12-Dimethylbenzanthracine is a very good polynuclear hydrocarbon carcinogen in mammalian cells requiring an activation system and all this. And 7,12-DMBA has no carcinogenic activity or no cocarcinogenic activity in our system. I understand your point, but all I can say is that in this cell with these compounds, we find no carcinogenic activity and no cocarcinogenic activity associated with that compound which is considered to be a carcinogen in mammalian cells.

DR. CROCKER: Dr. Shank, I think it would be interesting if you would review the relationship between DNA adduct formation or especially N-7 guanine formation in animals where the liver is the target for carcinogenesis versus a target for toxicity. Would you care to do that with the studies you just described?

DR. SHANK (University of California, Irvine): The results on the carcinogenicity bioassay for hydrazine in the rat, I believe, is complete. And the only tumor associated with the hydrazine has been nasal pharyngeal carcinoma. No liver tumors have been associated with hydrazine exposure. So, in this case, we are looking at a nontarget organ. We have not looked at nasal pharyngeal epithelial DNA yet because of the problem of isolating enough DNA from that tissue. There are a variety of carcinogens which do not produce cancer in the liver but still are metabolized to agents which alkylate liver DNA at the 7 position. Just because you form an electrophile doesn't necessarily mean you get a tumor. That's another problem there. But the alkylation we've seen in liver DNA in the rat with hydrazine would indicate that you can get a small amount of electrophile generation and whether this is specific to the liver or not, we cannot say. However, the liver is not a target organ in this case.

DR. MC KENNA (Dow Chemical, USA): Dr. Shank, you just piqued my interest very much, and this raises a question. We've had some recent studies in our laboratory where we found

many reactive chemicals in rodents which are obligatory nasal breathers. These are inhalation studies, obviously. We come up with early detectable histopathological changes in both the olfactory and the respiratory epithelial lining of the nasal pharynx. I was wondering if you know which cell type was affected in this instance.

DR. SHANK: I will redirect that question to Mr. Vernot or Dr. MacEwen.

MR. VERNOT: These tumors were in the nasal turbonates. I believe they were epithelial but I'm not certain of that.

DR. MC KENNA: But you don't know which epithelial site. The epithelial lining is olfactory type epithelium and then a respiratory epithelium. The point I was going to make was that we were talking this over the other day, and we have identified a number of compounds in rodent bioassays where the primary target tissue is at the site of entry and that's the nasal turbonates. It's kind of interesting because it makes you wonder as to how much significance this is going to have in predicting hazard to man. There is a problem in setting a maximum tolerated dose in a bioassay system with an obligatory nasal breather, an animal with an exudative rhinitis that turns to mouth breathing and dies because he's swallowing air. Then you're left with a study with no target organ. So I think this is an area that's going to warrant a lot of further research.

I'd like to make another comment in regard to the previous question about target organ and nontarget organ toxicity and DNA alkylation. We've been doing some studies on vinylidine chloride and also on dimethyl nitrosamine, and we found that with VDC, in particular, you get tumors in the kidneys of mice following chronic inhalation exposure. You can't find anything in rats no matter how hard you try if you want them to live for two years. We've recently finished some studies in comparing VDC with a material like DMN where the alkylation is with DNA. It seems to be a direct acting nuclear type whereas with vinylidine chloride, we don't see a great deal of alkylation of DNA; and we don't see a great deal of DNA repair in the kidney of mice or in rat liver. But we do see a great deal of alkylation out in the cytosol, and this seems to result in a hyperplastic type of response and an increased level of DNA turnover. We think now that possibly this enhanced DNA turnover for a chronic period of time is something that results in a secondary carcinogenesis. You have a repeated insult and a lot of toxicity in an organ that eventually turns into something analogous to a 2 or 3 year old liver in a 1 year old rat or mouse, and these animals then develop tumors.

DR. SHANK: Do you see any alkylation in the DNA at all?

DR. MC KENNA: Very, very little. Almost none detectable.

DR. SHANK: What kind of alkylation is it in the cytosol?

DR. MC KENNA: It's primarily protein with a little bit of RNA but mostly it's all protein.

DR. SHANK: Is it methylation?

DR. MC KENNA: We don't know. Specific activities are so low that it's very difficult to get counts, and we are unable to pass it through a mass spectrometer and get any results.

DR. DOST: I want to mention that my colleagues, Dave Springer and Don Reid, and I have looked for ^{15}N in ammonia and cannot find it which bewilders us a little bit. We haven't found any nitrogen derived from hydrazine in ammonia.

I have a question for Dr. Mitchell. If I understand correctly, you found no change in the effect of hydrazine on hepatic necrosis after treatment with phenobarbital? Is that correct?

DR. MITCHELL (Baylor College of Medicine): Yes.

DR. DOST: And you do after treatment with acetylhydrazine?

DR. MITCHELL: Yes.

DR. DOST: Can we interpret that as meaning that hydrazine does not form acetylhydrazine as a metabolite or derivative in vivo?

DR. MITCHELL: Those doses were selected on a millimolar basis. The hydrazine would have been about twice the concentration of the acetylhydrazine, and the doses were in the milligrams per kilogram range. If you give a higher concentration of hydrazine at the LD_{25} dose or so, you can indeed produce interlobular necrosis in the phenobarbital treated animals. It's difficult to be certain without simultaneous metabolic experiments going on, but I would guess that necrosis is being caused by the acetylhydrazine. You see a similar phenomenon with diacetylhydrazine. At comparable molar doses, it's less necrotizing than is acetylhydrazine; but you can, indeed, produce liver necrosis with diacetylhydrazine.

DR. DOST: We have assumed that the metabolite that we see along with hydrazine is diacetylhydrazine, but we are losing a little of our certainty about that because we haven't been able to prove it. It would almost certainly have to be secondary to the formation of the monoacetyl compound.

DR. MITCHELL: We would be glad to assay a sample for you if you would like us to look in terms of it being diacetylhydrazine or not. I would guess that it's very unlikely to be diacetylhydrazine because most rats are relatively slow acetylators relative to humans. If you use a small dose, you can get up to maybe 75% of the dose being acetylated, at least for isoniazid and acetylhydrazine. If you look after administering acetylhydrazine to the rat, you find almost no diacetylhydrazine. And the same thing is true for isoniazid. So, I'm not sure what you found either. Are you sure it's not acetylhydrazine?

DR. DOST: It could very well be.

DR. MITCHELL: The half-life is about three hours for acetylhydrazine when you administer it directly to a rat.

DR. NEWELL (National Academy of Sciences): I have a question for Dr. Mitchell. At the end of your talk, you touched briefly on the changing practices in the use of isoniazid in treatment of patients in relation to age. I wonder if you would explain that a little more.

DR. MITCHELL: Well, I threw in those last three slides though it's not germane to the use of isoniazid in practice, I'll be glad to answer your question. The point of putting those slides in was a plea for more mechanistic study because I find routine data collection most of the time being nothing more than that because you don't know what to look for until you understand mechanism for most toxic chemicals. If you want to study the risk for man with drugs where there's considerable potential for benefit, you need to have some estimate of what this risk is and hence the need for developing some estimate of the formation of these reactive metabolites in terms of the exposure whether it be in the CO_2 and all of these various metabolites for isoniazid or whether it be something like mercapturic acids formed by additional reactions of two electrophiles. In answer to your question, I think once we understand some of the mechanisms we can begin to then look for certain types of populations. I think this is one of the biggest difficulties with epidemiological surveillance for carcinogenicity or for drug toxicity in that all population studies in general show subgroups having a much higher incidence of the effect. Frequently, for example, you'll hear that there's no dose response curve. But if, in fact, there's only a small subsegment, let's say people that are over the age of 50 that have a 25 to 50-fold greater

incidence of effect than for the population subsegment under 35, you don't know whether that population has a dose response curve or not for the toxicity. And when you look at population epidemiology, it's lost. To answer your question more directly in regard to the use of isoniazid, it's generally recommended now and since 1975 that isoniazid not be used routinely for treatment of all people with positive tuberculin skin tests that had previously been the recommendation of the American Thoracic Society and the recommendation of the Tuberculosis Research Division of the CDC since about 1968 or 1969. The use of isoniazid on all positive tuberculin skin test reactors is what literally triggered off this epidemic of isoniazid hepatitis. So the current recommendations are to use the drug only in people who are tuberculin converters, certain selected subgroups clinically at greater risk of developing tuberculosis and with much greater care in the older age group.

I'd like to ask Dr. Shank a question. If I understood your data correctly in the labelled methionine experiment, rather than calling that an alkylation from hydrazine, it was my interpretation of that experiment that you were producing injury which was stimulating the methylase to produce 7-methyl guanine. Is that the correct interpretation?

DR. SHANK: We feel we don't have enough data yet to say which mechanism is active. You're absolutely right that we cannot say that hydrazine is methylating the DNA. We observed DNA methylation in hydrazine poisoned animals. We think the DNA methylase may play a role but we have not yet eliminated the possibility that hydrazine might be methylated to form monomethylhydrazine.

DR. MITCHELL: Has anyone ever shown methylation of hydrazine by SAM or S-adenosyl methionine?

DR. SHANK: No, not that I know of. And we have tried. The measurement of monomethylhydrazine in tissue is extremely difficult, and we have not yet been able to do it.

DR. MITCHELL: Another approach one might consider is the use of alternative controls such as the treatment of your animals with a growth hormone and then give them radio labelled methionine or look at animals after a partial hepatectomy in terms of the liver that's left and see if these species are now developing 7-methyl guanine.

DR. SHANK: We have tried other hepato toxicants in very brief experiments with unlabelled dimethylnitrosamine and radioactive methionine. We get about 100th of 1% of the alkylation

from dimethylnitrosamine poisoned animals that we get from methionine. If you give cold alphatoxin which is a liver toxin and radioactive methionine, you get a small amount of 7-methyl guanine in liver DNA labelled from the methionine.

DR. MITCHELL: Small relative to the hydrazine treated animals?

DR. SHANK: About the same as in the hydrazine treated animals but small relative to the kind of adduct formation you get from a strong carcinogen. The other compound we tried was yellow phosphorus which is also a hepatotoxin, and again, we found small amounts. This week we are trying to measure the effect of carbon tetrachloride on this system. We feel that what will come of this will probably be some description of a nonspecific response to toxic insult to the liver which has nothing to do with the specific toxicity of hydrazine itself.

COLONEL CARTER (6570 Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base): Dr. Dost, would you care to give a semi-educated guess at this point on the half-life of hydrazine in the mammalian system in a single dose?

DR. DOST: Several hours. The strange thing is that what little we have looked at suggests that there are good solid slopes but that those half-times increase with increasing dose until we get up to a point where we're approaching a lethal dose and then the whole system degrades again, and there is no real order to the decrease. I would say 2 to 5 hours in the rat.

SESSION III

INHALATION TOXICOLOGY

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PAPER NO. 13

A SIX-MONTH CHRONIC INHALATION EXPOSURE OF ANIMALS TO UDMH TO DETERMINE ITS ONCOGENIC POTENTIAL

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In the past several years in our laboratory, a large amount of time and money has been spent conducting chronic inhalation studies to determine the toxicity of three important chemicals used by the military. The earliest studies reported at this conference in the last few years defined rather well the chronic toxicity of methylhydrazine and hydrazine (Haun, 1970; MacEwen and Haun, 1971; Haun and Kinkead, 1973). These studies were not designed for carcinogenic assessment. However, this concern prompted us to hold hydrazine exposed mice for one year postexposure.

At necropsy, a larger incidence of tumors was seen in the mice exposed to 5 ppm hydrazine than was seen in their controls. This occurred in 1974.

This information, together with the knowledge from the literature that other investigators have produced tumors in animals by various routes of administration, set forth the plans for and the conduct of inhalation studies which would be definitive of a suitable carcinogenic risk or no-effect level for MMH, hydrazine, and UDMH.

This work has been in progress since 1974. Sufficient information and results from the UDMH study permits, at this time, report of the tumorigenic and toxic effects of UDMH exposure.

Four animal species were used in this study. The concentration levels used at or near the current TLV of 0.5 ppm were 5, 0.5, and 0.05 ppm. Exposures conducted were of the industrial type, 6 hours/day, 5 days/week, for six months. This was followed by a prolonged observation period for potential tumor induction in each species. Animals and the numbers used in each exposed and control group were 400 female C57Bl/6 mice, 200 male Fischer 344 rats, 200 male Golden Syrian hamsters, 4 male and 4 female beagles. A concurrent set of control animals was provided for the 0.05 ppm test as it was not started at the same time as the 5 and 0.5 ppm experiments. Dogs and rats were housed in one dome and mice and hamsters in a companion chamber. All control animals were maintained in animal housing facilities.

The inhalation chambers were operated with nominal air flows of 35 cfm at a slightly reduced pressure of 725 mm Hg to prevent leakage of UDMH into the laboratory. Nearly continuous monitoring of chamber concentrations was performed with an AutoAnalyzer, the same instrumentation and techniques used for chronic hydrazine and MMH studies (Haun, 1970; MacEwen and Haun, 1971; Haun and Kinkead, 1973).

Dimethylnitrosamine (DMNA) is used in the commercial production of UDMH and remains in the final product. The supply that we used in this study contained approximately 0.12% DMNA. DMNA is a known potent carcinogen. This is a significant consideration in the assessment of the oncogenic and nononcogenic effects of UDMH inhalation.

All animals were observed hourly during exposure and nonexposure periods for signs of UDMH intoxication and mortality. Gross and histopathologic examinations were made on all dead animals. Rats, hamsters, and dogs were weighed individually at biweekly intervals during exposure and monthly

during the postexposure period. Mice were weighed in groups, and group mean weights were followed on a monthly basis throughout the experimental period.

Blood samples were drawn from dogs at biweekly intervals, and clinical determinations were made for the battery of tests shown in Table 1.

TABLE 1. CLINICAL BLOOD TESTS PERFORMED ON DOGS EXPOSED TO 5, 0.5, AND 0.05 PPM UDMH AND CONTROLS

RBC	Sodium	Albumin
WBC	Potassium	Globulin
HCT	Calcium	SGPT
HGB	Glucose	Alkaline Phosphatase
Differential Cell Count	Total Protein	

Blood measurements not included in the regular biweekly schedule during the exposure phase of the study but made at the conclusion of the 5 ppm and 0.5 ppm exposures are shown in Table 2.

TABLE 2. CLINICAL BLOOD TESTS PERFORMED ON DOGS AT THE END OF EXPOSURE TO 5 AND 0.5 PPM UDMH AND CONTROLS

Blood Urea Nitrogen	SGOT
Chloride	Prothrombin Time
Cholesterol	Cephalin Flocculation
Creatinine	Bromsulfalein

Of these, tests giving abnormal values were repeated post-exposure at regular intervals until recovery.

To examine for possible hemolytic effects in rodents, blood samples for hematocrit and red blood cell counts were taken from 5 rats and 5 hamsters from each group at the conclusion of the 5 ppm and 0.5 ppm exposures.

Significant exposure effects of UDMH were limited to slight to moderate but transitory hepatotoxicity in dogs exposed to the 5 ppm concentration as shown in Table 3. Dogs exposed to the 5 ppm level developed significantly elevated serum glutamic pyruvic transaminase (SGPT) levels by the fourth week of exposure. At 6 weeks, the mean SGPT value

for the exposed dogs was 3 times the control level. Throughout the remaining 20 weeks of exposure, values for the exposed dogs were stable at levels 3-4 times those of the control group.

TABLE 3. DURING EXPOSURE EFFECT OF 6-MONTH INHALATION EXPOSURE TO 5 PPM UDMH ON SERUM GLUTAMIC PYRUVIC TRANSAMINASE LEVELS IN DOGS

[Group Mean Values (N = 8)]

Weeks of Exposure	Control Group	5 ppm Group
2	26*	32
4	27	79**
6	27	102**
8	25	118**
10	26	118**
12	31	116**
14	--	---
16	22	88**
18	23	107**
20	23	99**
22	20	97**
24	22	100**
26	25	86**

*International Units
**Significant at the 0.01 level.

As shown in Table 4, there is a trend to recovery, approximately a 50% reduction, in measurements made at 2 weeks postexposure. Subsequent values at 4, 8, and 11 weeks postexposure showed no further reductions. However, when the dogs were sampled again (at Brooks Air Force Base where they were being maintained) at 27 and 47 weeks postexposure, values were completely normal when compared with control animal values. Periodic measurements have been made since that time and are continuing. They show no abnormalities.

Liver function tests were performed on dogs at exposure termination and at 4, 8, 11, and 38 weeks postexposure. This information is shown in Table 5. Bromsulfalein (BSP) measured in the blood of the 5 ppm exposed dogs 10 minutes following a 10 mg/kg injection showed significant retention at exposure termination, 4, and 8 weeks postexposure. Recovery occurred at 11 weeks postexposure. BSP measurements made at Brooks Air Force Base 38 weeks postexposure show no abnormal values for the exposed dogs. Subsequent scheduled measurements have been normal.

TABLE 4. POSTEXPOSURE EFFECT OF 6-MONTH INHALATION EXPOSURE TO 5 PPM UDMH ON SERUM GLUTAMIC PYRUVIC TRANSAMINASE LEVELS IN DOGS

[Group Mean Values (N = 8)]

Weeks Postexposure	Control Group	5 ppm Group
2	22*	37**
4	23	42**
8	22	36**
11	23	35**
27[1]	33	30
47[1]	40	37

*International Units
**Significant at the 0.01 level
[1]Measurements made at Brooks Air Force Base.

TABLE 5. MEAN BROMSULFALEIN RETENTION VALUES* IN CONTROL AND 5 PPM UDMH EXPOSED DOGS

Time	Control	5.0 ppm
Exposure Termination		
26 Weeks	18.1	30.3**
Weeks Postexposure		
4 Weeks	20.7	29.5**
8 Weeks	12.8	30.0**
11 Weeks	18.0	21.8
38[1] Weeks	11.4	12.3

*Percent retention.
**Significantly higher than controls at the 0.05 level.
[1]Measurements taken at Brooks Air Force Base.

Hematocrit and RBC determinations done immediately postexposure on rats and hamsters showed no abnormalities. Likewise, clinical results on dogs other than those mentioned showed no effects of exposure to UDMH.

Mean body weights for all groups of exposed rats and hamsters were depressed during the exposure phase of the study, but they were not dose related. The weights of dogs and mice were unaffected by UDMH exposure.

Mortality during exposure was limited. No dogs died, a few rats and mice were found dead, but in no case was death attributed to UDMH exposure. A caging problem with hamsters early in the 5 and 0.5 ppm experiments caused a number of accidental deaths. The problem was successfully corrected.

Table 6 shows the time of postexposure sacrifices and the numbers of surviving animals. Longevity of the four species is shown in order. That is, the life span of hamsters is the shortest while that of dogs is the longest. This is reflected in the number of survivors relative to the original number used. It can also be seen that 2 dogs are missing, one in the high dose group and one in the low dose group. The reasons will be discussed.

TABLE 6. EXPERIMENTAL DESIGN USED FOR UDMH INHALATION EXPOSURE CONCENTRATIONS, TIME OF POSTEXPOSURE SACRIFICES AND NUMBER OF SURVIVORS

UDMH Conc., (ppm)	Animal Numbers, Sex and Species	Sacrifice (Months) Postexposure	Number of Survivors
5.0	200 male hamsters	12.5	17
	400 female mice	19.5	75
	200 male rats	18-19*	142
	4 male, 4 female dogs	Not sacrificed	7
0.5	200 male hamsters	12.5	19
	400 female mice	19.5	103
	200 male rats	18-19*	145
	4 male, 4 female dogs	Not sacrificed	8
Control	200 male hamsters	12.5	10
	400 female mice	19.5	56
	200 male rats	18-19*	74
	4 male, 4 female dogs	Not sacrificed	8
0.05	200 male hamsters	17-18*	46
	400 female mice	19-20*	142
	200 male rats	19-20*	145
	4 male, 4 female dogs	One sacrificed	7
Control	200 male hamsters	17-18*	37
	400 female mice	19-20*	88
	200 male rats	19-20*	139
	4 male, 4 female dogs	Not sacrificed	8

*Serial sacrificed over a one-month period.

The protocol for microscopic examination of tissue was that used by the National Cancer Institute biocollaborative carcinogenesis research program.

Table 7 shows the tissues routinely examined from each animal. Both nontumor and tumor histopathologic findings were recorded.

TABLE 7. TISSUES TAKEN ROUTINELY FOR HISTOPATHOLOGIC EXAMINATION, UDMH ONCOGENIC STUDY

Gross lesions	Esophagus	Pancreas
Tissue masses or suspect tumors and regional lymph nodes	Stomach	Spleen
	Duodenum	Kidneys
	Ileum	Adrenals
Skin	Colon	Bladder
Mandibular lymph node	Anus	Seminal vesicles
Mammary gland	Mesenteric lymph node	Prostate
Salivary gland		Testes
Larynx	Liver	Ovaries
Trachea	Thigh muscle	Uterus
Lungs and bronchi	Sciatic nerve	Nasal cavity
Heart	Sternebrae, vertebrae, or femur (plus marrow)	Brain
Thyroids		Pituitary
Parathyroids		
	Thymus	

The nontumor pathology results have not been completely analyzed. However, there were no striking changes in exposed animals when compared with their controls.

One male dog in the high dose group died one year and nine months followng the start of the study. The cause of death, and the only tumor seen in this dog, was a metastatic reticulum cell sarcoma that most likely arose in the mediastinum. The other dog in the low dose group was euthanized 4 years and 2 months following the start of the study. The sacrifice was made because of posterior paresis. The cause of the paralysis was a ruptured intervertebral disc. No neoplasms were seen in this dog. The dogs are between 5-6 years of age at this time and are all in good health.

Microscopic examination of hamster tissues was done by four USAF veterinary pathologists at Brooks Air Force Base working in unison. Tumor nomenclature was developed for an automated data processing capability to compile results from hamsters. Incidence tables and statistical analysis of results was done by the THRU. There were no tumor types seen in these hamsters that were significantly increased over that seen in controls. Table 8 compares, by exposure group, incidence of tumors seen in hamsters.

TABLE 8. TUMOR INCIDENCE IN CONTROL AND UDMH EXPOSED GOLDEN SYRIAN HAMSTERS

Tumor Type	Unexposed Control	0.05 ppm Exposed	0.5 ppm Exposed	5.0 ppm Exposed	Unexposed Controls
Lung					
Pulmonary Sarcoma	0/190	0/178	3/166	1/158	0/167
Pleural Mesothelioma	1/190	1/178	0/166	0/158	0/167
Bronchogenic Adenoma	1/190	0/178	0/166	0/158	0/167
Tracheobronchial Adenoma	1/190	0/178	0/166	0/158	0/167
Adrenal					
Cortical Adenoma	29/185	33/167	27/146	25/138	25/149
Medullary Adenoma	2/185	2/167	7/146	1/138	3/149
Bone Marrow Tumors	2/168	1/148	0/128	3/121	2/129
Reticulo-endothelial System					
Reticulo-Endotheliomas	20/171	13/144	16/171	10/164	9/177
Gastro-Intestinal Systems					
Esophageal Papilloma	0/179	0/172	1/197	0/140	0/154
Stomach Papilloma	1/182	1/169	1/148	0/148	0/160
Stomach Adenocarcinoma	0/182	1/169	0/148	1/148	0/160
Colon Papilloma	1/149	0/143	0/84	0/99	0/82
Colon Adenocarcinoma	1/149	0/143	0/84	0/99	0/82
Thyroid					
Thyroid Adenoma	0/147	1/131	0/83	0/75	1/85
"C" Cell Adenoma	0/147	3/131	0/83	2/75	0/85
Parathyroid Adenoma	1/87	0/73	1/28	1/27	1/29
Skin					
Trichoepithelioma	0/193	0/184	1/171	0/164	0/177
Melanoma	1/193	0/184	0/171	0/164	0/177
Undifferentiated Tumor, Malignant	1/193	0/184	0/171	0/164	0/177
Kidney					
Adenoma	1/191	0/177	0/168	0/155	2/170
Other Tissues					
Salivary Gland Adenoma	0/193	0/184	0/171	0/164	1/177
Gingiva Epithelioma	0/193	0/184	1/171	0/164	0/177

The microscopic examination of rat and mouse tissues was done by two groups of three USAF veterinary pathologists working in unison in our laboratory. The NIH biocollaborative carcinogenesis program methodology was used for mice. Nomenclature of tumors conformed to the miniaturized systematized nomenclature of pathology (minisnop). The NIH automated data processing contractor (Mason EG&G) was employed for compilation of incidence tables and statistics. The synopsis of rat tumor nomenclature was developed during conduct of the histologic examination of rat tissues. Incidence tables and statistical analysis of the data were prepared by the Toxic Hazards Research Unit (THRU).

Table 9 shows tumor incidence in the various groups of exposed and control mice. The outstanding finding in mice was the increase in hemangiosarcomas and Kupffer cell sarcomas. In the 5 ppm exposure group, there are 19 hemangiosarcomas versus 3 in controls. Also eight Kupffer cell sarcomas are seen compared with none in the control group. The numbers are modest, but significant in the 5 ppm group. There is a fairly large incidence of malignant lymphomas in each group of exposed and control mice, but note that incidence in the 5 ppm group is statistically different, higher than the incidence in the controls.

Tumor types seen in rats are shown in Table 10. Two types of lung tumors are listed. Bronchiolar adenomas are somewhat more prevalent. There is a statistically significant increase in the high dose group. Although there is no statistical validity, squamous cell carcinomas are conspicuous in that 4 such tumors were found in the high dose group but none in any of the other control or exposed groups. The incidence of these tumors occurring spontaneously in rats is quite low. Hepatocellular carcinomas are seen for six rats in the 5 ppm exposed group. This tumor type was not seen in the controls. Many studies have shown the liver to be a target organ for DMNA carcinogenicity. A significant increase in islet cell adenomas is seen in the 0.5 ppm dose group. The high dose group does show a higher incidence than controls, but this is not statistically significant. Chromophobe adenomas are prevalent in aged F-344 rats. Both the high and the intermediate dose groups show a significantly higher incidence than their controls. Brain tumors of the central nervous system are rare in rats. Seven were seen in exposed animals, none in controls. There was no statistically significant increase in any one type seen. Four of the seven tumors were astrocytomas, which is in keeping with reports that astrocytomas are the most prevalent type of brain tumor seen in rats. Fibrous histiocytomas are a fairly common tumor seen in the skin and soft tissue of rats. There were increases of this type seen in both the high and intermediate dose groups, statistically significant at the high dose level only.

TABLE 9. TUMOR INCIDENCE IN CONTROL AND UDMH EXPOSED C57B1/6 MICE

Tumor Type	Unexposed Controls	0.05 ppm Exposed	0.5 ppm Exposed	5.0 ppm Exposed	Unexposed Controls
Lung					
Alveolar/Bronchiolar Adenomas	3/340	4/355	7/331	11/337	6/320
Alveolar/Bronchiolar Carcinomas	1/340	1/355	3/331	0/337	0/320
Liver					
Hepatocellular Carcinoma	2/349	1/363	7/344	4/342	2/332
Pituitary					
Carcinoma	3/270	3/320	1/257	1/251	2/255
Adenoma	13/270	10/320	3/257	3/251	1/255
Chromophobe Adenoma	48/270	34/320	2/257	24/286	29/255
Thyroid					
Follicular Cell Adenomas	20/293	17/311	20/278	13/286	25/258
Follicular Cell Carcinomas	0/293	1/311	8/278	5/286	2/258
Uterus					
Leimyosarcoma	1/328	3/348	3/311	3/312	0/304
Ovary					
Tubular Adenoma	2/234	4/336	4/287	1/308	3/279
Circulatory System					
Hemangioma	1/355	2/374	2/368	5/360	6/346
Hemangiosarcoma	1/355	8/374*	3/368	19/360**	3/346
Hematopoietic System					
Malignant Lymphomas	94/355	102/374	98/368	112/360*	87/346
Kupffer Cell Sarcoma	1/355	4/374	0/368	8/360*	0/346
Plasma Cell Tumor	1/355	4/374	1/368	2/360	1/346

*Significant at the 0.05 level as determined using Fischer's Exact Test.
**Significant at the 0.01 level as determined using Fischer's Exact Test.

TABLE 10. TUMOR INCIDENCE IN CONTROL AND UDMH EXPOSED FISCHER 344 RATS

Tumor Type	Unexposed Controls	0.05 ppm Exposed	0.5 ppm Exposed	5.0 ppm Exposed	Unexposed Controls
Lung					
Bronchiolar Adenoma	5/189	0/192	2/182	8/191*	1/196
Squamous Cell Carcinoma	0/189	0/192	0/182	4/191	0/196
Liver					
Hepatocellular Carcinoma	0/197	0/193	2/189	6/188*	0/193
Pancreas					
Islet Cell Adenoma	0/170	3/174	12/169*	8/158	3/132
Pituitary					
Chromophobe Adenoma	69/171	76/182	72/169**	89/174**	39/166
Kidney					
Carcinoma	0/195	0/196	0/196	1/190	0/197
Adrenal					
Adenoma	0/194	1/188	1/177	1/188	1/189
Brain					
Astrocytoma	0/199	2/182	1/189	1/178	0/182
Glioblastoma	0/199	0/182	0/189	1/178	0/182
Malignant Neural Neoplasm	0/199	0/182	1/189	0/178	0/182
Pinealcytoma	0/199	0/182	0/189	1/178	0/182
Hematopoietic System					
Malignant Lymphoma and Leukemia	51/200	39/197	20/193	19/196	17/199
Skin and Miscellaneous Fibrous Histiocytomas	1/200	0/197	6/193	9/196*	1/199

*Significant at the 0.05 level as determined using Fischer's Exact Test.
**Significant at the 0.01 level as determined using Fischer's Exact Test.

In summary, a dose related tumor response was not seen in the data for mice. It was seen in most of the tumor types for rats, but without statistical support.

Overall total tumor incidence was greater in all exposed rats and mice than in controls. Statistical evidence for significant tumor production was seen in rats and mice exposed to the highest dose used. Of particular importance are the hepatocellular carcinomas in rats and the hemangiosarcomas and Kupffer cell sarcomas in mice.

In previous studies, we showed that the small amount of DMNA impurity in UDMH caused hepatotoxicity in dogs (Haun, 1976) and increased liver cell changes in orally dosed mice (MacEwen and Vernot, 1976).

As a follow-up experiment, we are currently conducting a 12-month exposure of mice to purified UDMH at the 5 ppm level to determine if tumors can be produced without the DMNA contamination.

Certainly we must await the results of this mouse study, but at this point in time, it is reasonable to theorize that the very small amount of DMNA present in UDMH as a 0.12% impurity contributed significantly, if not entirely, to the tumor production observed in mice and rats exposed to 5 ppm UDMH.

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PAPER NO. 14

METHYLCYCLOHEXANE ONE-HOUR EMERGENCY EXPOSURE LIMIT

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Methylcyclohexane (MCH) is a solvent found in the aircraft fuel designated JP-9. This fuel is a mixture of three primary ingredients, JP-10, RJ-5, and MCH. JP-10 and RJ-5 are high density hydrocarbons yielding a greater BTU output than conventional aircraft fuels. They also have a higher viscosity causing flow problems at low temperatures which is the reason for the addition of MCH to the mixture.

Chronic inhalation exposures to RJ-5 have been conducted in our laboratory and are reported in the annual report (MacEwen and Vernot, 1975). Acute and chronic toxicity studies on MCH have been reported by Treon et al. (1943). Six-hour, acute exposures of rabbits to inhaled concentrations of MCH above 10,000 ppm caused convulsions, light narcosis, labored breathing, salivation and conjunctival congestion. Between 5500 ppm and 7300 ppm, lethargy and impaired coordination were the only signs.

Lazarew (1929) reported that 7500 to 10,000 ppm for two hours produced narcosis in mice while 10,000 to 12,500 ppm caused death. Lehman and Flury (1943) indicated that the acute toxicity of MCH was greater than that of heptane, but less than that of octane.

Similar high level narcotic effects were reported when mice were exposed to heptane vapors between 10,000 and 15,000 ppm (Fuhner, 1921). In addition, Patty and Yant (1929) reported slight dizziness in man after exposure to 1000 ppm for six minutes. Concentrations of 2000 to 5000 ppm resulted in marked vertigo, nausea, incoordination, and hilarity which persisted for several hours after exposure. No human toxicity information exists for MCH.

The American Conference of Governmental Industrial Hygienists (ACGIH) lowered the threshold limit value (TLV) for MCH in 1976 from 500 ppm to 400 ppm or 1600 mg/m^3. These values are based on analogy of the toxicity of heptane and are identical to the TLV and STEL of heptane.

It was felt that even though the narcotic effects of MCH and heptane on mice were similar at high concentrations, the low level CNS effects might not be the same. If this were the case, the STEL set on analogy with heptane might be somewhat lower than necessary. It was the purpose of this study to find a concentration of MCH that would not cause chronic or irreversible tissue damage or produce CNS effects which could impair coordination or prevent a man from self-rescue.

MATERIALS AND METHODS

The MCH used in this study is a colorless liquid produced by Eastman Organic Chemicals, Rochester, New York. The pertinent physical properties of the reagent grade MCH are as follows:

Molecular weight:	98.18
Boiling point @ 760 mm:	100.93 C
Density 25°/4°:	0.76501
Lower explosive limit (% by vol. in air):	1.15
Flashpoint (closed cup):	25 F
Vapor pressure:	43 mm

Air saturated with methylcyclohexane at 20 C and 760 mm contains 56,500 ppm vapor. At these same conditions, 1 ppm vapor = 0.00401 mg/ℓ and 1 mg/ℓ = 249.5 ppm.

Groups consisting of twenty male Sprague-Dawley rats and 20 female ICR mice were exposed to MCH vapors for one hour periods. A control group of each species was maintained for comparison to the test group. Ten animals of each species, including controls, were sacrificed immediately following exposure while the remaining animals were observed for 28 days postexposure.

Once the concentration causing no narcotic effect in rodents was achieved, four dogs were exposed at that level. A group of four control dogs was also maintained for comparative purposes.

Rodent exposures were made in a Rochester chamber while dogs were exposed in a Longley chamber. The rodents were housed in Longley cages to allow for greater freedom of movement and provide more opportunity for visual observations. Dogs had no confinement within the chamber.

The animals used in this experiment were male Sprague-Dawley rats and female ICR mice, both supplied by Harlan Industries of Indianapolis, Indiana. The purebred beagle dogs were provided by the Air Force. The rodents were fed ad libitum and were cage changed twice per week. The dogs were fed once per day and had their cages cleaned daily.

GENERATION AND ANALYSIS

MCH vapors were produced by generating a known liquid flow through a spiraled glass evaporator. A measured air flow through the evaporator carried the vapors into a Rochester chamber where an air flow of a minimum of 30 cfm was maintained.

The chamber concentration was measured using a Miran I infrared analyzer. Calibration of the Miran I was done using 50 liter Mylar® bags containing known concentrations of the contaminant.

OBSERVATIONS AND CLINICAL EVALUATIONS

All animals were carefully observed for signs of toxic stress during and after the exposure period. The rats were weighed immediately prior to exposure and at 1, 2, 3, 7, 14, 21, and 28 days postexposure.

Prior to exposure, the dogs were trained by animal technicians to perform four basic tasks. The dogs were trained to fetch, come, stay, and lead. It was necessary to spend considerable time acclimating the dogs to the laboratory environment. Approximately six weeks of training was done including weekly tests prior to the experimental exposure and twice immediately after exposure.

Besides the field trial evaluation, the dogs were neurologically examined after exposure using the following tests:

flexor reflex, extensor thrust reflex, tonic neck reflex, tonic eye reflex, righting reflex, and placing reflex. Each reflex was tested by the method described by Hoerlein (1971).

The dogs were weighed before exposure and at 2 and 4 weeks postexposure. On the same schedule, blood samples were taken from each dog for the following determinations:

HCT	SGPT	Sodium
HGB	SGOT	Potassium
RBC	Alkaline Phosphatase	Glucose
WBC	Bilirubin	BUN.

Gross examinations were done on all experimental and control animals while histopathology was done on half the rodents and all dogs. All major organs were sampled and examined with special emphasis on the liver and kidney.

EXPERIMENTAL RESULTS

The first concentration tested, 6564 ppm to rats and mice, caused immediate hyperactivity in both species. A slight loss of coordination was first seen in mice at 12 minutes followed by rats at 29 minutes. Prostration was noted in both mice and rats at 42 and 54 minutes, respectively. At 55 minutes, one mouse experienced tonoclonic spasms which lasted for 15 to 20 seconds. No deaths resulted from this one-hour exposure.

No signs of stress were noted during the subsequent 28-day observation period in either species, and mean body weight gains of the test animals compared favorably with their respective controls. Gross pathology examination of the animals that were sacrificed immediately following exposure and those sacrificed after a 28-day observation period revealed no lesions which could be attributed to the MCH exposure.

The second concentration tested was to rats alone. This exposure, with a mean concentration of 4172 ppm, caused increased activity in the animals for the duration of the experiment. The activity demonstrated in this exposure was somewhat less than that of the first exposure; however, the rats maintained normal coordination throughout and demonstrated no observable CNS effects.

The mean body weight gain of the exposed rat group was slightly less than the control group but well within the normal weight gain range which would be expected for this time period. Gross pathology examination of these rat groups failed to show any contaminant-related lesions.

An equivalent mouse exposure was run at a mean concentration of 4758 ppm. The only observable sign noted was hyperactivity which lasted during the entire exposure period. Following exposure, the mice returned to their normal activity pattern. The effects of this contaminant level on the mice appeared to be very similar to the effect reported for rats at 4172 ppm. No loss of coordination or CNS effects were noted during or after the one-hour exposure.

Mean body weights of the test mice at 28 days did not differ significantly from the controls. Gross pathology examination of the animals, both immediately after exposure and at 28-days postexposure, failed to reveal any exposure related lesions.

From the rodent data, a concentration of approximately 4200 ppm appeared to be a safe one-hour exposure limit for rats and mice. The dog exposure was then designed for a nominal concentration of 4200 ppm wherein the dogs would be observed carefully during exposure and tested postexposure for neurological effects.

The four test dogs were exposed to a measured mean concentration of 4071 ppm for one hour. The dogs all acted normally throughout the exposure showing no signs of eye or nose irritation or effects upon coordination.

Immediately following the exposure, each of the dogs performed its trained tasks with assigned animal technician. All dogs performed this exercise adequately to the standard established during the training program. The subsequent neurological testing of each dog revealed no exposure-related effects. During the 28-day postexposure observation period, all dogs appeared normal.

Blood samples examined at 14 and 28 days postexposure showed all normal values. Gross pathology at necropsy revealed no exposure-related lesions.

Histopathologic examinations of the test and control dogs showed no significant lesions. Examinations were also done on one-half the control and test rodents from the low level exposures. This examination revealed that two test rats held for 28 days postexposure had convoluted tubule adenomas of the kidneys. It is unusual to find lesions of this kind in rats of this age (73 days) and it is unlikely that this lesion would develop in this short time span after a single exposure to the compound; however, this lesion was not seen in the rats sacrificed immediately after exposure or in either of the two control rat groups.

To determine whether this lesion was real or an anomaly, the kidneys from the 6700 ppm group were examined. Three sections were examined from the kidneys of each of the ten test rats. No adenomas or abnormal lesions were found in the kidneys of these rats. Therefore, it is assumed that the two adenomas seen in the low level exposed rats were anomalies and had nothing to do with exposure effects.

The only signs noted on low level exposed mice were minimal to mild cytoplasmic changes in the liver which are considered by our pathologist to be of a reversible nature. This lesion was seen in five of the test mice and in one control mouse.

The MCH concentrations selected for the dog and rodent exposures (4071 to 4758 ppm) produced no adverse signs of toxic stress during or after exposure and the animals gained weight normally during the subsequent 28 days. Dog blood parameters remained normal throughout the 28-day observation period. Pathologic findings in the exposed animals were essentially the same as seen in the respective control animals.

The MCH concentrations are in the same range as heptane concentrations which caused considerable toxic effects to man as reported by Patty and Yant (1929). Although there are no human MCH data for comparison, it appears from our experiments that the effects of these two compounds, while reported to be similar in animals at high levels, are not necessarily similar at the 4000 to 4700 ppm range.

While man is reported to lose coordination and experience marked vertigo and nausea after six minutes of exposure to this concentration, the dogs remained alert and coordinated throughout a one-hour exposure period. At no time during the post-exposure trials did the dogs show any signs of CNS effects. During the retrieving portion of the dog trials, the dogs ran rapidly while chasing a ball and showed normal coordination in the process.

Until human MCH exposure data are available which would show that man is more susceptible to the vapors of MCH than the animals tested, one could assume that the responses would be similar. Based on the effects seen in the mice, rats, and dogs, there is no reason to believe that a concentration of 4000 ppm of MCH would hamper self-rescue or cause any irreversible damage to a man within a 60-minute period. This recommendation of 4000 ppm for a one-hour emergency exposure limit is based solely on animal data and any human exposure information to the contrary would preclude this recommendation.

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PAPER NO. 15

SUBCHRONIC INHALATION TOXICITY OF TWO PETROLEUM FUELS, JP-5 AND DFM

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INTRODUCTION

Petroleum distillate fuels have been used as an energy source for over a century and during that time have become materials essential in daily life. The advent of the internal combustion engine has resulted in large quantities of these distillate fractions being introduced into the working environment.

Despite long industrial and environmental experience with petroleum distillates, little investigative work had been done on the toxicological characteristics of fuels until Drinker et al. (1943) exposed groups of human volunteers to known concentrations of gasoline vapors. They found that for a concentration up to 0.03% (1060 mg/m^3) the major complaint was eye irritation. When the concentration reached 0.26% (9150 mg/m^3), symptoms of mild exhilaration and muscular incoordination characteristic of moderate ethanol ingestion appeared. At a concentration of 1.1% or 38,000 mg/m^3, the subjects were described as decidedly drunk, most within a period of 5 minutes. Symptoms of chronic gasoline poisoning include dizziness, headache, mental confusion, nausea, and tightness in the chest (Machle, 1941). Tissue damage including fatty livers, kidney damage in the convoluted tubules, polyneuropathy, and bronchitis have been among the reported effects of chronic gasoline exposure (Machle, 1941; Wilson, 1949).

In 1963, Elkins et al. pointed out that the concentration of benzene in air after total or partial evaporation of gasoline could exceed the TLV of benzene (25 ppm at that time) even though the TLV of gasoline (500 ppm, 1760 mg/m^3) was not exceeded. In response, the American Conference of Governmental Industrial Hygienists in 1967 changed its approach in favor of determining the TLV of gasoline on the basis of benzene, other aromatics and additives in gasoline or petroleum distillates. The reported effects of benzene exposure in humans are blood disorders. Reductions in the number of erythrocytes, leucocytes, and platelets were found after long-term exposure to benzene at high concentrations (Greenberg, 1939; Hardy and Elkins, 1948; Aksoy et al., 1972).

The studies reported here involved two petroleum distillate fuels which primarily differed from gasoline in that they were less volatile. The studies were designed to determine the toxic effects, including oncogenesis, of 90 days of continuous exposure of test animals to the jet fuel JP-5 or diesel fuel marine (DFM). The studies were conducted at the request of the U.S. Navy. A 90-day continuous exposure was chosen to simulate conditions onboard Navy vessels where the fuels would be carried or transferred in enclosed areas and where crewmen might be exposed continually for the length of a cruise.

MATERIALS AND METHODS

Groups of purebred beagle dogs (3 male, 3 female), 147 Fischer 344 rats (75 male, 72 female), and 108 C57Bl/6 female mice were exposed to 0.15 mg/liter or 0.75 mg/liter JP-5 in Thomas Dome inhalation chambers (Thomas, 1968). A control group consisting of an equal number of animals was housed in laminar air flow rooms in a separate facility. Groups of 6 purebred beagle dogs (3 male, 3 female), 150 Fischer 344 rats (75 male, 75 female), and 140 C57Bl/6 female mice were exposed to 0.05 mg/liter or 0.30 mg/liter DFM in the Thomas Domes. Equal numbers of control animals for this study were also held in laminar air flow rooms. All animals had access to food and water ad libitum.

Upon termination of the 90-day exposure period, all of the dogs and one-third of the rodents were killed for detection of any pathologic lesions caused by exposure. The remaining rodents are presently being held for observation for a period of 19 months. At that time, one-half of the rodents will be killed. These animals should have attained

a normal lifetime age and should provide a statistically satisfactory sample of animal tissues for histopathologic examination which will not be compromised by cannibalism or postmortem degeneration seen in animals that die spontaneously. The rest of the rodents will be held until mortality in any group reaches 90% of the original number, when all representatives of that species will be killed and examined.

All animals were carefully observed throughout the exposure and postexposure periods for signs of altered physical condition. Rats and dogs were weighed individually at biweekly intervals during exposure and rats monthly during the postexposure period. Mice were weighed monthly throughout the study, and the group mean weights were monitored. All animals that died or were killed were necropsied and the tissues taken for histopathologic examination. The liver, spleen and kidneys of individual dogs and rats were weighed during necropsy and the ratios of organ to body weight of exposed animals were compared to unexposed controls. Blood samples were drawn from dogs biweekly and from killed rats for clinical determinations as shown in Table 1. Most of the tests were included to detect the onset and presence of blood dyscrasias since these have been the major effects of exposure to benzene. Other tests could reveal toxicity to specific organs such as liver and kidney. Red blood cell osmotic fragility tests were conducted on dog blood using a modification of the method described by Davidsohn and Henry (1969).

TABLE 1. HEMATOLOGY AND CLINICAL CHEMISTRY TESTS ON DOGS AND RATS EXPOSED TO JP-5 OR DFM VAPORS

Hematology	Chemistry
Hematocrit	Sodium
Hemoglobin	Potassium
Total RBC	Calcium
Total WBC	Albumin/Globulin
Differentials	Total Protein
Mean Corpuscular Volume (MCV)	Glucose
Mean Corpuscular Hemoglobin (MCH)	Alkaline Phosphatase
Mean Corpuscular Hemoglobin Concentration (MCHC)	SGPT
	Bilirubin
	Creatinine
	BUN

The JP-5 and DFM used in the studies consisted of mixtures of aliphatic hydrocarbon compounds along with a small portion of aromatic hydrocarbons. The distillation endpoint temperature of JP-5 is 290 C, and the majority of the straight chain hydrocarbons are between C_{10} and C_{15}. DFM contains a greater number of higher boiling components with the endpoint distillation temperature of 385 C. The majority of straight chain hydrocarbons in DFM are between C_{12} and C_{18}. Each fuel typically contains a small amount of benzene.

The basic design of the contaminant generation system is shown in Figure 1. Since the fuels were multicomponent mixtures with a wide boiling range, both animal exposure chambers were operated from a single generation system to assure similar exposure environments. Vapors were generated by passing the liquid fuel down through two heated evaporator towers. Air passing up through the towers created a fuel vapor/air mixture. This mixture was split into the approximate volume ratio of the chamber concentration before entering the respective chamber air streams. The air stream flow was then used for maintaining finer control of the chamber contaminant concentration. Spent fuel which did not vaporize in the towers drained through the bottom of the tower and was collected for disposal.

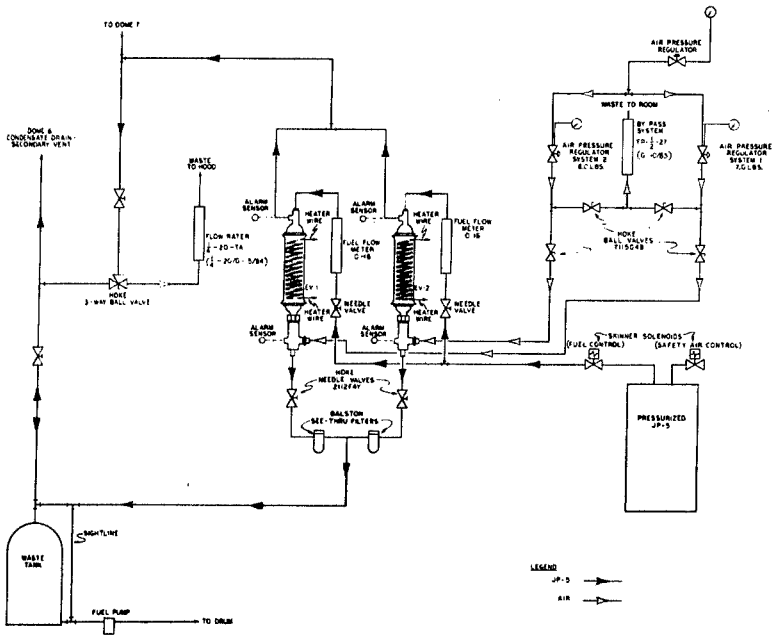

Figure 1. Contaminant introduction system.

A Beckman Model 400 hydrocarbon analyzer was used for continuous mass analysis of the chamber atmosphere. In addition, a Varian 1200 gas chromatograph equipped with a flame ionization detector was used for quality control checks of each drum of fuel prior to use, as well as for routine fingerprint analysis of the chamber atmosphere. The column was 12 ft. x 1/8 in. stainless steel tubing packed with 10% SE30 on Chromosorb W. The system was maintained isothermally at 40 C.

Statistical analyses of body weights, hematology, clinical chemistry, and organ weights were carried out using the Student's T method.

RESULTS

ANALYTICAL MEASUREMENTS

Initially, the high level JP-5 exposure concentration was set at 1.5 mg/liter. With 6 exposure days, 50% of the mice were dead and the dogs were lethargic. There were no rats in the exposure chamber at this time. Oily deposits were noticed on the fur of the mice and dogs and on the windows of the chamber. No significant increase in hydrocarbon concentration was evident. A condensate aerosol appeared to be formed when the heated JP-5 vapors cooled in the chamber air stream, and this aerosol was lethal to mice after continued exposure.

> [Histopathologic examination of the lungs of 10 randomly chosen dead mice and 10 surviving mice revealed lesions which were probably the result of irritation. All of the lungs had minimal to mild diffuse congestion. Seven of 10 survivors and 2 of 10 mice that died had minimal to mild patchy areas of edema. All of the survivors and 3 of 10 mice that died had mild degeneration of the bronchiolar epithelium. One survivor and 2 mice that died had small foci of hemorrhage in alveolar spaces.]

A Royco 225 particle counter was used to estimate the aerosol. Resulting data indicated that there was a considerable amount of aerosol formed at the 1.5 mg/liter JP-5 level. The study was restarted with new groups of animals and the high level concentration was lowered to 0.75 mg/liter. This reduction in concentration greatly reduced aerosol formation. The DFM exposure concentrations were based on the highest total hydrocarbon concentration possible without significant aerosol formation.

Exposure concentrations were well controlled throughout both studies. The JP-5 vapor concentrations (mean ± s.d.) over the 90-day exposure period were 0.750 ± 0.013 mg/liter and 0.151 ± 0.003 mg/liter for the high and low exposure levels, respectively.

Figure 2 shows the first 20 minutes of a typical gas chromatogram of chamber air containing JP-5 jet fuel. The hydrocarbon peaks are representative of the lower boiling fractions of JP-5. The benzene peak was eluted from the column at 10.5 minutes. Benzene concentration in the 0.75 mg/liter JP-5 exposure was approximately 0.5 ppm with a range of 0.33 to 0.57 ppm. In the 0.15 mg/liter JP-5 exposure, the benzene concentration was approximately 0.1 ppm, ranging from 0.06 to 0.12 ppm. DFM vapor concentrations (means ± s.d.) were 0.299 ± 0.005 mg/liter and 0.50 ± 0.001 mg/liter for the high and low exposure levels, respectively. The first 20 minutes of a typical gas chromatogram of DFM vapor in the 0.30 mg/liter exposure chamber are shown in Figure 3. The benzene concentrations were 0.26 ± 0.04 ppm in the 0.30 mg/liter dome and 0.04 ± 0.02 ppm in the 0.05 mg/liter dome.

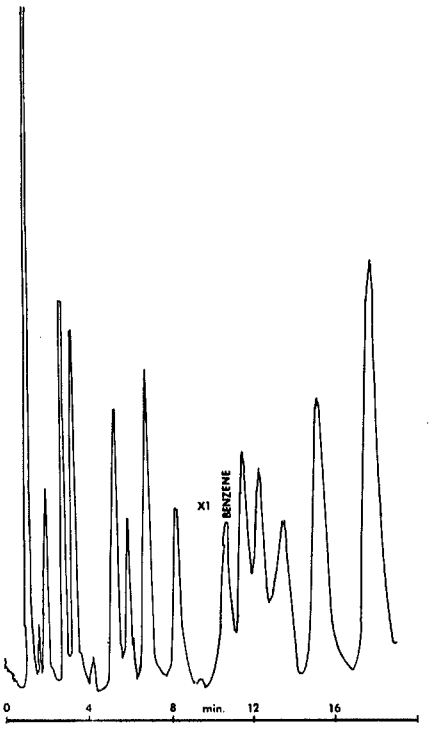

Figure 2. Typical chromatogram of chamber air containing JP-5 vapors.

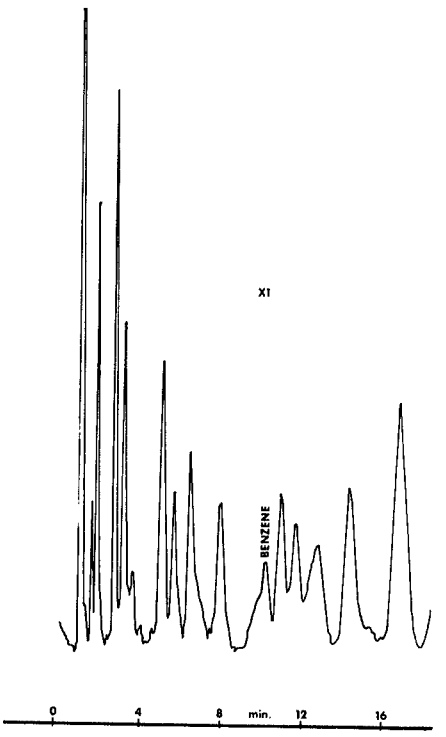

Figure 3. Typical chromatogram of chamber air containing DFM vapors.

EFFECT ON ANIMALS: JP-5

The growth of beagle dogs exposed to JP-5 vapors was unaffected after 90 days of continuous exposure. Statistically significant decreases in serum albumin levels of exposed dogs were noted when compared to control dogs. This resulted in decreased A/G ratios in both exposed groups. The decreased albumin levels and A/G ratios were within normal biological limits, except for one dog which had an inverted A/G ratio for the last two sampling periods. The blood urea nitrogen levels of the exposed dogs were also increased sporadically through the exposure. All other clinical chemistry values of blood samples from the dogs were within normal biological limits. Red blood cell osmotic fragility was significantly increased in the female dogs exposed to the 0.75 mg/liter JP-5 concentration and, although not statistically significant, was also increased in the lower exposure group (Figure 4). Increased RBC fragility was evident in both groups of exposed male dogs (Figure 5).

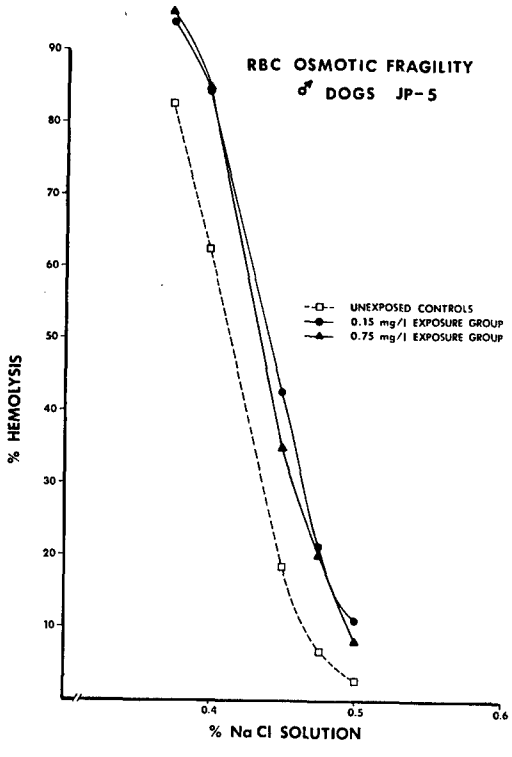

Figure 4. Effect of 90-day continuous exposure to JP-5 vapors on red blood cell osmotic fragility in male beagle dogs.

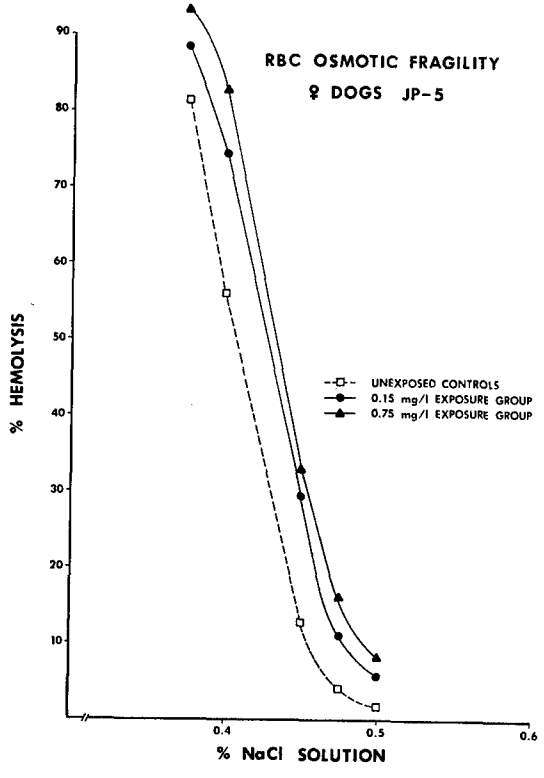

Figure 5. Effect of 90-day continuous exposure to JP-5 vapors on red blood cell osmotic fragility in female beagle dogs.

In dogs, exposure related lesions were noted only in the liver. Diffuse, mild, cloudy swelling of hepatocytes occurred in all of the high level exposure group, in two (33.3%) of the low dose group, and in none of the controls. Affected hepatocytes were moderately swollen, pale, and had a foamy cytoplasm which was negative for both fat and glycogen with special stains. The lesions were considered reversible.

The growth of male Fischer 344 rats was retarded by exposure to JP-5 vapors (Figure 6). The mean body weights of both JP-5 exposure groups were significantly different from the unexposed control group at the 0.01 confidence level throughout the exposure phase and until the eighth month of the study when the difference dropped to the 0.05 significance level. No dose response was apparent. The mean body weights of females exposed to JP-5 (Figure 7) were generally not as great as those seen in the male rats. A period of weight loss in the control female rats decreased the apparent difference between these control and exposed female rats.

Figure 6. Effect of 90 day continuous exposure to JP-5 vapors on the growth of male rats.

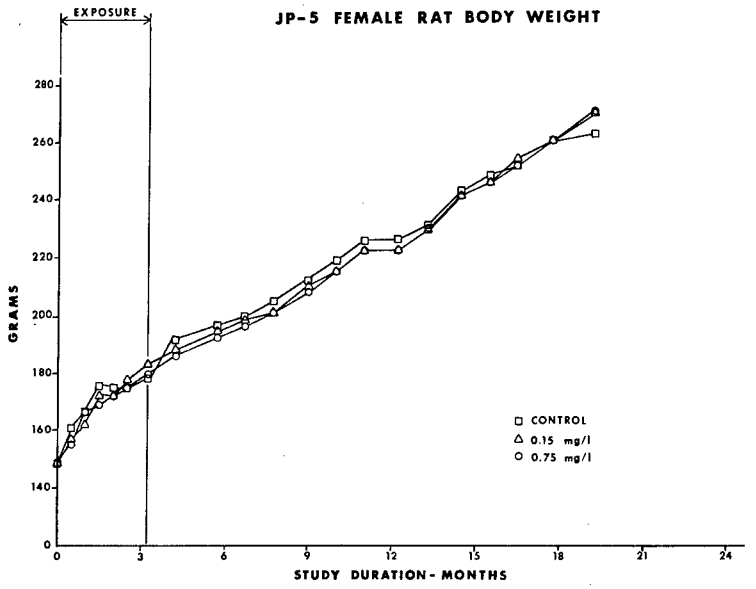

Figure 7. Effect of 90-day continuous exposure to JP-5 vapors on the growth of female rats.

A large number of the blood samples obtained from individual rats killed at the completion of the exposure were partially hemolyzed. The samples were withdrawn from the exposed brachial artery from anesthetized rats. Statistical analyses of the data from the hemolyzed and unhemolyzed samples were compared, and the means were found to be essentially identical. The data were, therefore, included for analysis of exposure effects and are shown in Table 2. Although a number of mean values for various clinical chemistry determinations were within normal biological ranges, there were statistically significant differences. These were thought to be clinically unimportant, with the exception of BUN and creatinine changes which may be related to histopathological kidney changes. Consistent with the changes seen in the dogs, albumin and A/G ratios of JP-5 exposed rats were significantly less than unexposed control rats. Analysis of rat organ weight/measurements revealed that the kidney/body weight ratios of the male rats exposed to 0.75 mg/liter JP-5 were significantly greater than those of the control male rats.

TABLE 2. HEMATOLOGY AND CLINICAL CHEMISTRY DETERMINATIONS ON FISCHER 344 RATS AFTER 90-DAY CONTINUOUS EXPOSURE TO JP-5 VAPORS

(Group Mean Values)

Clinical Measurement	Sex	Unexposed Controls	0.15 mg/ℓ Exposure Group	0.75 mg/ℓ Exposure Group
RBC (x 10^6)	♂	8.78	8.43*	8.41*
	♀	7.65	7.91**	8.05**
WBC (x 10^3)	♂	5.3	5.1	5.4
	♀	4.0	5.2	5.1
HCT (vol %)	♂	46	45*	42*
	♀	42	42	42
HGB (g %)	♂	15.3	15.3	14.7*
	♀	14.0	14.5**	14.3*
Sodium (mEq/ℓ)	♂	148	147	149
	♀	144	150**	148**
Potassium (mEq/ℓ)	♂	5.7	6.3	6.3
	♀	5.5	6.6**	6.2
Calcium (mEq/ℓ)	♂	11.0	10.4*	10.5*
	♀	10.4	10.1	9.8**
Glucose (mg/dl)	♂	171	147*	146*
	♀	139	130	114*
Total Protein (g/dl)	♂	6.4	6.2	6.5
	♀	6.2	6.0	6.3
Albumin (g/dl)	♂	4.6	4.3*	4.3*
	♀	4.5	4.2**	4.2**
Globulin (g/dl)	♂	1.9	1.9	2.1*
	♀	1.7	1.9**	2.1**
SGPT (IU/ℓ)	♂	52	58	53
	♀	54	50	49
Alkaline Phosphatase (IU/ℓ)	♂	16.1	16.4	13.8*
	♀	10.0	13.5	11.8
BUN (mg/dl)	♂	14.9	17.0*	18.5*
	♀	15.9	19.4**	19.6**
Creatinine (mg/dl)	♂	0.53	0.55	0.62*
	♀	0.43	0.46*	0.49**
A/G Ratio	♂	2.5	2.3*	2.0*
	♀	2.8	2.3*	2.0**

*Significant at the 0.05 level.
**Significant at the 0.01 level.

Histologic examination of rat tissue revealed some alveolar pneumonia and pulmonary congestion in female control rats which may have been related to the transient weight loss observed in this group of animals. Kidney injury consisting of nephropathy characterized by diffuse hyaline droplet formation, multifocal degeneration, and atrophy of proximal tubules, and the presence of dilated debris filled tubules at the junction was seen in both exposed groups of male rats. The lesions were more severe in the 0.75 mg/liter JP-5 exposure group. These changes were not seen in unexposed controls or in female rats and are consistent with the elevated BUN and creatinine levels as well as with the increase in kidney to body weight ratios observed in exposed male rats.

In mice, lesions that appeared related to JP-5 exposure were seen in the liver and kidney. Mild, diffuse fatty changes in the livers were seen with increased incidence in JP-5 exposed mice. Fatty accumulations in the epithelium of the proximal convoluted tubules of the kidney were found in 12% of the high level JP-5 exposure group. All of these mice also had fatty livers. These lesions probably represent mild reversible injury to subcellular organelles as a result of JP-5 exposure.

In control female rats and mice, there was a higher incidence of uterine hydrometra than seen in the JP-5 exposed rodents. Diffuse endometrial gland cysts were also seen in 22% of the control mice, but in none of the other mice. These changes may be related to environmental differences in the separate housing of the animals. However, there is a possibility that JP-5 exposure had an effect on ovarian function.

EFFECT ON ANIMALS: DFM

The dogs exposed to DFM vapors for 90 days were slightly heavier and gained weight at a slightly faster rate than control dogs. Hematology and chemistry values in the dogs were normal throughout the exposure period. Occasional statistically significant differences between exposed and control groups were found in some of the parameters, but the differences were never consistent from sample period to sample period and were never large enough to be considered clinically significant. One male dog exposed to 0.30 mg/liter DFM showed an elevated SGPT level approximately half-way through the exposure. This increase subsequently returned to normal by the conclusion of the exposure period. Figure 8 is a plot of dog red blood cell osmotic fragility values measured at the completion of the 90-day exposure. Increased RBC fragility in dogs exposed to DFM vapors is indicated in that both of the exposure group curves are elevated over controls and the curves are slightly flattened, especially between the 0.50% and 0.45% salt concentration points.

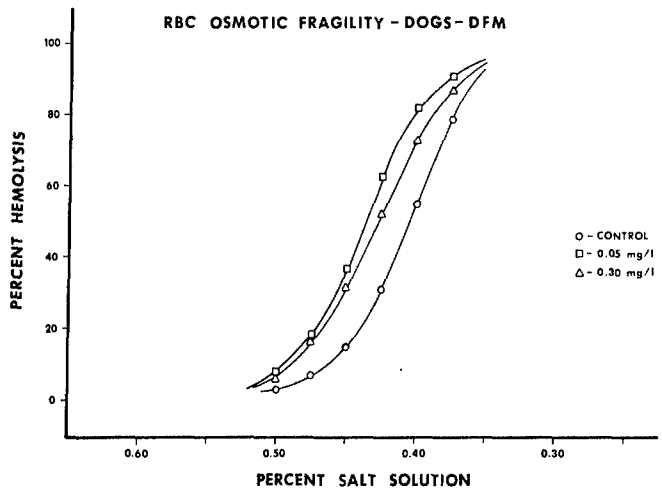

Figure 8. Effect of 90-day continuous exposure to DFM vapors on red blood osmotic fragility in beagle dogs.

The effect of DFM exposure on the growth of male Fischer 344 rats is shown in Figure 9. Significant decreases in the mean body weights of the DFM exposed male rats were apparent after 2 weeks of exposure. There was a dose response relationship in these body weight effects through the 14th month of the study. There was no significant effect of DFM exposure on female rat body weight until the 8th exposure week when the female rats exposed to 0.30 mg/liter DFM had a slight loss in mean body weight (Figure 10). The body weights in this group became significantly less than controls at that time and continued to be less than controls through the 14th month of the study. The body weights of the female rats exposed to 0.05 mg/liter DFM vapors did not become significantly different from controls until the seventh month of the study.

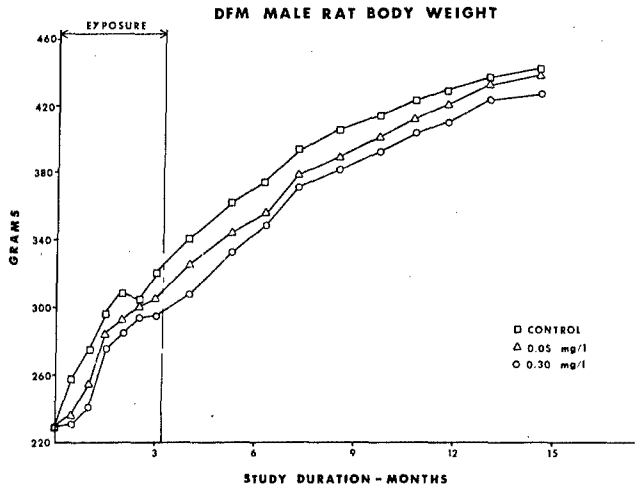

Figure 9. Effect of 90-day continuous exposure to DFM vapors on the growth of male rats.

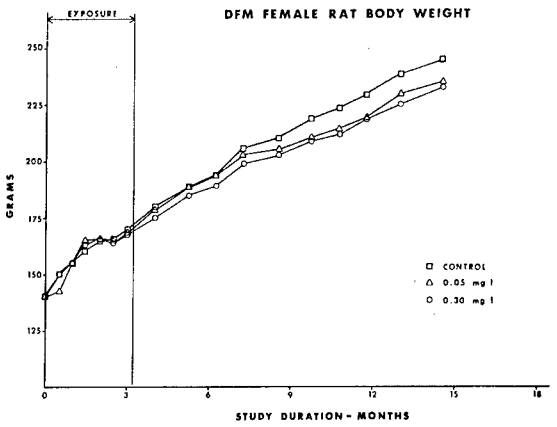

Figure 10. Effect of 90-day continuous exposure to DFM vapors on the growth of female rats.

Blood samples were taken from the rats at the end of the 90-day exposure. The samples were collected from anesthetized rats through the portal vein. The mean hematology and chemistry values are shown in Table 3. Leukopenia in both male and female rats exposed to 0.30 mg/liter DFM is evident. The WBC values obtained from these animal were at the extreme lower end of normal limits and could be considered biologically significant. Decreased WBC counts were also seen in the males exposed to 0.05 mg/liter DFM. The significance of decreased SGPT values in DFM exposed rats is not known. All other blood parameters were within normal limits. Increased liver/body weight ratios were noted in the male rats exposed to the high level of DFM, while decreased kidney weights were seen in females exposed to DFM vapors.

Histologic examination of the tissues obtained from the rats revealed exposure related lesions in the respiratory and urinary systems. Lymphoid hyperplasia of the bronchial submucosa, a nonspecific lesion denoting chronic irritation and seen early in cases of chronic respiratory disease, had an increased incidence in exposed rats (39% of controls, 62% at 0.05 mg/liter, and 54% at 0.30 mg/liter).

Hyaline degeneration of the cytoplasm of convoluted tubules was seen in 68% of low dose male rats and 84% of high dose male rats. The lesion was absent in exposed females and all controls. This lesion was considered a mild reversible degenerative process that resulted from accumulation of protein in the cytoplasm of convoluted tubule cells due to an incapacity of the cells to transfer resorbed blood protein from glomerular filtrate back into blood. An associated lesion (nephropathy) was seen in 96% of the high dose group male rats. High dose female rats plus all low dose and control rats were free of this change. The nephropathy was described as the presence of granular casts within viable collecting tubules localized at the corticomedullary junction of the kidney. This change was seen in connection with a variable and mild chronic interstitial nephritis.

TABLE 3. MEAN HEMATOLOGY AND CLINICAL CHEMISTRY DETERMINATIONS ON FISCHER 344 RATS AFTER 90-DAY CONTINUOUS EXPOSURE TO DFM VAPORS

Clinical Measurements	Sex	Unexposed Controls	0.05 mg/ℓ Exposure Group	0.30 mg/ℓ Exposure Group
RBC (x 10^6)	♂	8.4	8.2	7.7*
	♀	7.2	6.8*	5.3*
WBC (x 10^3)	♂	7.8	5.6*	5.2*
	♀	7.7	6.8	5.3*
HCT (Vol %)	♂	48	47	45*
	♀	43	41	42
HGB (g/dl)	♂	15.5	15.3	14.7*
	♀	14.5	14.1	14.2
Total Protein (g/dl)	♂	6.9	6.8	7.0
	♀	7.0	6.3	6.7
Albumin (g/dl)	♂	4.5	4.4	4.5
	♀	4.7	4.2	4.2*
Globulin (g/dl)	♂	2.3	2.4	2.5
	♀	2.3	2.1	2.5
Glucose (mg/dl)	♂	175	144*	130*
	♀	134	90*	99*
Potassium (mEq/ℓ)	♂	6.7	6.2	6.6
	♀	5.3	5.4	5.8
Calcium (mg/dl)	♂	12.2	11.9	12.1
	♀	12.5	11.5*	11.5*
Sodium (mEq/ℓ)	♂	152	149*	149*
	♀	150	147*	149
Total Bilirubin (mg/dl)	♂	0.44	0.44	0.44
	♀	0.62	0.62	0.51
BUN (mg/dl)	♂	17.0	17.0	17.0
	♀	16.9	17.0	17.4
Creatinine (mg/dl)	♂	0.69	0.65	0.67
	♀	0.56	0.49	0.56
SGPT (IU/ℓ)	♂	55	42*	34*
	♀	46	38	39
SGOT (IU/ℓ)	♂	96	87	86
	♀	96	103	90
Alkaline Phosphatase (IU/ℓ)	♂	16.0	17.1	16.2
	♀	9.2	10.9	14.4*

*Significant at the 0.01 level.

TABLE 3. FATE OF ^{14}C-METHYLENE CHLORIDE IN RATS AFTER A SINGLE 6-HOUR INHALATION EXPOSURE

	% Body Burden ($\bar{x}$ ± S.D., n = 3)		
	50 ppm	500 ppm	1500 ppm
Expired CH_2Cl_2	5.42±0.73	30.40±7.10	55.00±1.92
CO_2	26.20±1.21	22.54±4.57	13.61±1.20
CO	26.67±3.00	18.09±0.81	10.23±1.68
Urine	8.90±0.39	8.41±0.90	7.20±0.74
Feces	1.94±0.19	1.85±0.68	2.33±0.05
Carcass	23.26±1.62	11.56±1.87	7.24±0.65
Skin	6.85±1.37	6.72±0.13	3.97±0.15
Cage Wash	0.75±0.33	0.42±0.23	0.43±0.15

Concentrations of ^{14}C-activity in rat tissues at 48 hours after termination of the inhalation exposures are given in Table 4. Following all three exposures to $^{14}CH_2Cl_2$, the highest concentrations of ^{14}C-activity were found in the liver, kidney and lung. ^{14}C-activity in epididymal fat was consistently lower than that observed in either whole blood or the remaining carcass at 48 hours following the inhalation exposure. $^{14}CH_2Cl_2$ per se was not detected in any of the tissues assayed. Thus the observed radioactivity was presumed to represent non-volatile metabolites of $^{14}CH_2Cl_2$.

The time course for pulmonary elimination of $^{14}CO_2$ and ^{14}CO following $^{14}CH_2Cl_2$ inhalation exposure is shown in Figures 3 and 4, respectively. Despite the observed differences in the total percentage of the body burden exhaled as either $^{14}CO_2$ or ^{14}CO, there was no observable difference in the rates of elimination of these two metabolites at any of the $^{14}CH_2Cl_2$ exposure levels employed. For both metabolites, pulmonary elimination of ^{14}C-activity was greatest during the first 12 hours following $^{14}CH_2Cl_2$ exposure. The pharmacokinetic parameters for pulmonary elimination of $^{14}CO_2$ and ^{14}CO are summarized in Table 5.

TABLE 4. TISSUE DISTRIBUTION OF ^{14}C-ACTIVITY 48 HOURS AFTER INHALATION EXPOSURE OF RATS TO ^{14}C-METHYLENE CHLORIDE

	µgEq $^{14}CH_2Cl_2$/g Tissue		
	50 ppm	500 ppm	1500 ppm
Liver	8.4±1.5[a]	35.6±7.5	44.2±3.5
Kidney	3.3±0.1	16.2±2.4	30.5±0.2
Lung	1.9±0.2	11.0±1.3	16.5±1.6
Brain	0.8±0.3	4.2±1.3	6.7±0.2
Epididymal Fat	0.5±0.2	6.5±0.5	4.1±0.9
Skeletal Muscle	1.1±0.1	4.4±1.9	7.7±0.7
Testes	1.1±0.2	5.5±1.3	8.1±0.5
Whole Blood	1.1±0.2	8.1±1.9	8.9±1.7
Remaining Carcass	1.3±0.2	5.6±0.9	8.6±1.4

[a]Values are $\bar{x} \pm$ S.D., n=3.

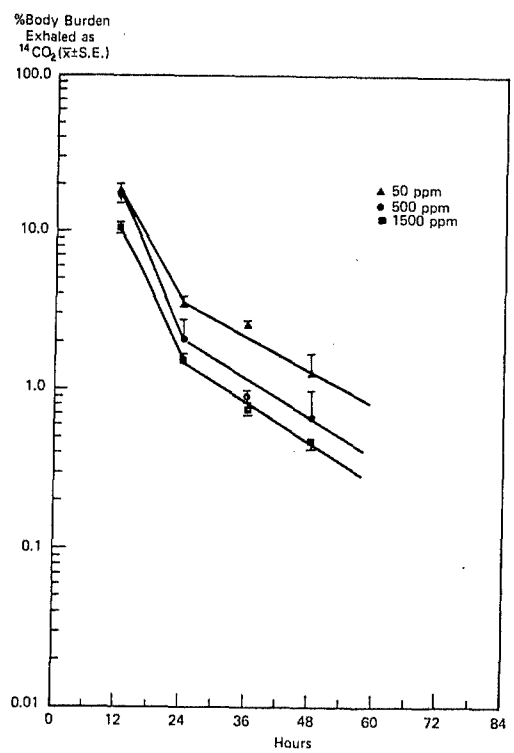

Figure 3. Time course for the pulmonary elimination of $^{14}CO_2$ by rats exposed to $^{14}CH_2Cl_2$ for 6 hours. Each point represents the $\bar{x} \pm$ S.E. for 3 rats.

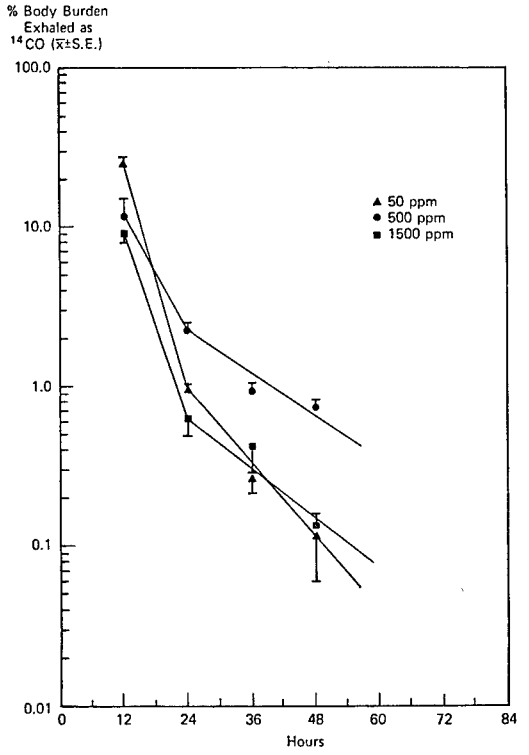

Figure 4. Time course for the pulmonary elimination of ^{14}CO by rats exposed to $^{14}CH_2Cl_2$ for 6 hours. Each point represents the $\bar{x} \pm$ S.E. for 3 rats.

TABLE 5. SUMMARY OF PHARMACOKINETIC PARAMETERS FOR THE EXCRETION OF ^{14}CO, $^{14}CO_2$ AND URINARY ^{14}C-ACTIVITY BY RATS AFTER INHALATION OF $^{14}CH_2Cl_2$

Metabolite		$^{14}CH_2Cl_2$ Exposure Concentration (ppm) 50	500	1500
$^{14}CO_2$[b]	α(hr$^-$)	0.358±0.134[a]	0.353±0.038	0.418±0.059
	t 1/2(hr)	1.94	1.96	1.66
	β(hr$^-$)	0.047±0.026	0.052±0.013	0.049±0.013
	t 1/2(hr)	14.7	13.3	14.1
^{14}CO[b]	α(hr$^-$)	0.393±0.060	0.288±0.126	0.472±0.098
	t 1/2(hr)	1.76	2.41	1.47
	β(hr$^-$)	0.104±0.054	0.047±0.007	0.061±0.014
	t 1/2(hr)	6.66	14.7	11.4
Urine ^{14}C[c]	α(hr$^-$)	0.088±0.009	0.083±0.007	0.086±0.025
	t 1/2(hr)	7.88	8.35	8.06

[a]Values represent the $\bar{x} \pm$ S.D. for 3 rats.
[b]First order rate constants for excretion of $^{14}CO_2$ or ^{14}CO assuming a 2 compartment open model.
[c]First order rate constant for urinary ^{14}C excretion for the first 36 hours after exposure.

The percentage of the body burden excreted in urine was constant over the entire range of $^{14}CH_2Cl_2$ exposures. Urinary ^{14}C-activity was devoid of any volatile components including unchanged $^{14}CH_2Cl_2$. Thus, urinary excretion of ^{14}C-activity appears to represent metabolized $^{14}CH_2Cl_2$. The elimination of ^{14}C-activity in rat urine following $^{14}CH_2Cl_2$ exposure is shown in Figure 5. The time course of urinary excretion of ^{14}C-activity by rats exposed to $^{14}CH_2Cl_2$ was similar following all three exposure concentrations. The data obtained for the first 36 hours postexposure were characterized by a single exponential decay. The pharmacokinetic parameters for ^{14}C-excretion in urine are summarized in Table 5.

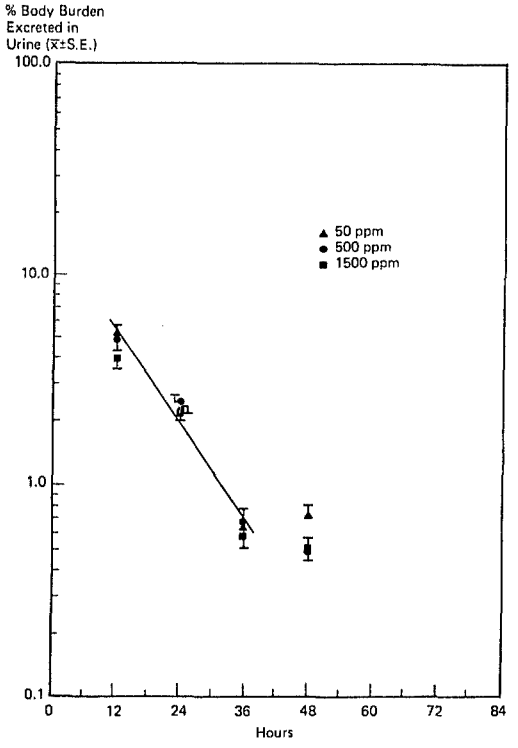

Figure 5. ^{14}C-activity in rat urine following a 6 hour inhalation exposure to $^{14}CH_2Cl_2$. Values are $\bar{x} \pm$ S.E., n=3.

MICHAELIS-MENTEN MODEL OF METHYLENE CHLORIDE METABOLISM

The data for total metabolism of CH_2Cl_2 at the three exposure concentrations (Table 2) suggested a saturation of CH_2Cl_2 metabolism with increasing exposure concentration which appeared to be in accordance with Michaelis-Menten kinetics. To ascertain whether Michaelis-Menten kinetics were applicable, the data in Table 2 were analyzed in accordance with the linear Wolf,

Augustinsen-Hofstee transformation of the Michaelis-Menten equation as described by Segel (1976). Appropriate parameter estimates for a Michaelis-Menten type model to predict the metabolism of CH_2Cl_2 by rats exposed for 6 hours to concentrations of 0-4,000 ppm were then obtained using a digital computer program. The curve obtained from this model and the superimposed experimental data are shown in Figure 6. A graphical estimate would indicate tht CH_2Cl_2 metabolism in the rat becomes disproportionately less with respect to exposure concentration greater than at about 250 ppm.

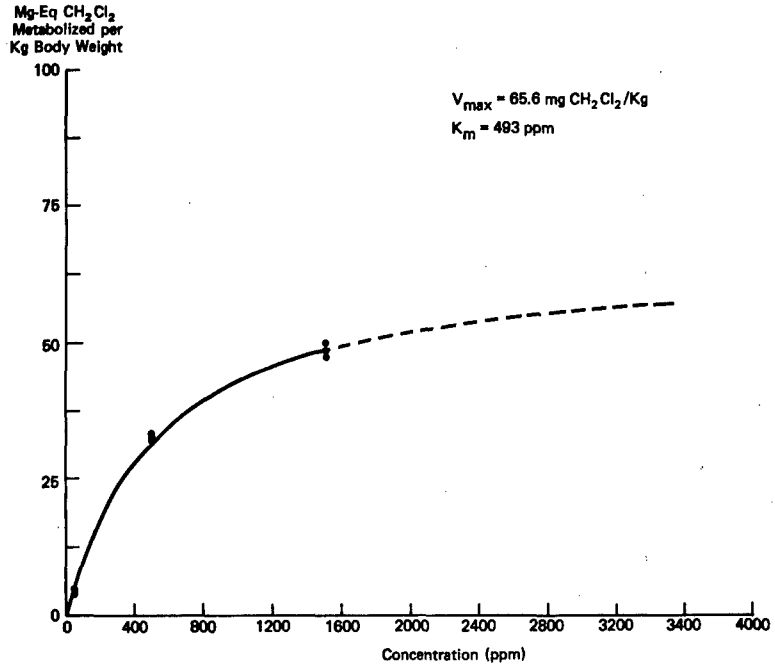

Figure 6. Michaelis-Menten model for the total metabolism of $^{14}CH_2Cl_2$ during a single 6 hour inhalation exposure. The points represent experimental data from individual rats. The curve represents the best fit of the model to the experimental data by a digital computer program.

Comparison of the observed and predicted values for CH_2Cl_2 metabolism following inhalation exposure of rats is shown in Table 6. The reliability of the model parameters was supported by the close comparison between observed and calculated values for CH_2Cl_2 metabolism. Estimates of the V_{max} and K_m obtained from the model directly were 65.55 $\pm$ 2.54 (SD) mg CH_2Cl_2 metabolized per kg and 493 $\pm$ 57 (D) ppm CH_2Cl_2, respectively.

TABLE 6. OBSERVED AND PREDICTED VALUES FOR METHYLENE CHLORIDE METABOLISM BY RATS DURING A SINGLE SIX-HOUR INHALATION EXPOSURE USING A MICHAELIS-MENTEN KINETIC MODEL

Exposure Conc. (ppm)	mgEq. CH_2Cl_2 Metabolized/kg Body Wt. Observed[a]	Predicted[b]
50	5.23	6.03
500	33.49	32.99
1500	49.08	49.32

[a] $\bar{x}$ for three animals per exposure concentration (see Table 2).

[b] Values obtained from a digital computer program used to obtain best-fit parameter estimates to the nonlinear Michaelis-Menten equation.

DISCUSSION

The evaluation of dose-response relationships in inhalation toxicity studies is often complicated by the observation that the response(s) of interest may not be directly related to the concentration of the test material in the inspired air. This is especially true in the case of materials which are extensively and rapidly metabolized during the inhalation exposure period. In such instances, a knowledge of the acquired body burden of the parent molecule and/or its metabolites resulting from exposure to a wide range of concentrations of the test material provides a more reliable estimate of the actual dose received by the experimental animal. Frequently the metabolism of inhaled materials contributes greatly to their accumulation in the animal. The biotransformation reactions involved are most often enzymatically mediated and, therefore, are concentration-dependent and saturable. The influence of such metabolic processes is often realized in dose-response relationships where an incremental response becomes diminishingly smaller with increasing exposure concentrations because biotransformation (and therefore accumulation) of the chemical follows apparent Michaelis-Menten rather than first-order kinetics.

The data reported in this study have provided evidence for the dose-dependent pharmacokinetics of inhaled CH_2Cl_2 in the rat. Further, these data indicate that the observed dose-dependency is due to the saturation of capacity-limited biotransformation processes for the solvent when inhaled at high concentrations for six hours. Evidence for the dose-dependent metabolism and pharmacokinetics of CH_2Cl_2 in rats after oral administration has been reported elsewhere (McKenna and Zempel, 1978).

Initially, evidence of the dose-dependent pharmacokinetics of inhaled CH_2Cl_2 was obtained from data for rat plasma CH_2Cl_2 levels and blood carboxyhemoglobin (HbCO) levels during and after inhalation exposure. Concentrations of both plasma CH_2Cl_2 and blood HbCO attained an apparent steady-state during exposure to CH_2Cl_2 (Figures 1 and 2). Steady-state plasma levels of CH_2Cl_2 were disproportionately higher with increasing exposure concentrations, suggesting a limited biotransformation on the parent molecule (Table 1). This was supported by the absence of an increase in HbCO levels when CH_2Cl_2 exposure was increased from 500 to 1500 ppm (Figure 2). Thus, at high CH_2Cl_2 exposure concentrations, metabolism to HbCO is limited and blood HbCO levels reach a maximum of 10-13% saturation. The resultant increase in circulating CH_2Cl_2 is greater than predicted by the increments in CH_2Cl_2 in the inspired air.

Postexposure clearance of CH_2Cl_2 from plasma (Figure 1 and Table 1) occurred very rapidly and was unaffected by the exposure concentration. However, the postexposure elimination of carboxyhemoglobin (Figure 2) was dependent upon the exposure concentration and showed a time course similar to that observed by Stewart et al. (1974) in experiments in man. After exposure to 50 ppm CH_2Cl_2, the postexposure decline in carboxyhemoglobin was apparently first-order with a half-life of 30 minutes. However, after exposure to 500 or 1500 ppm CH_2Cl_2, carboxyhemoglobin levels remained elevated for as long as 90 minutes and thereafter declined with a half-life similar to that observed at the lower exposure level. These data suggest that once a steady-state has been attained, termination of CH_2Cl_2 exposure may result in a redistribution of the solvent to the liver, thus providing an additional postexposure supply of CH_2Cl_2 for continued metabolism to carbon monoxide. Once this supply is exhausted or when respiratory clearance of CH_2Cl_2 exceeds this redistribution process, the blood levels of carboxyhemoglobin decline in the same manner as that observed following exposure to lower (50 ppm) concentrations of the solvent. It is important to note also that the half-life (30 minutes) for disappearance of carboxyhemoglobin from rats exposed to 50 ppm CH_2Cl_2 was identical to that obtained following inhalation exposure of rats to 100 ppm carbon monoxide. Thus the postexposure kinetics of carboxyhemoglobin were unchanged under these conditions, regardless of whether the source of carbon monoxide was exogenous or endogenous (i.e. produced by metabolism of CH_2Cl_2).

Additional evidence for the dose-dependent metabolism and pharmacokinetics of inhaled CH_2Cl_2 was obtained in experiments employing $^{14}CH_2Cl_2$. In these experiments, the net uptake and metabolism of $^{14}CH_2Cl_2$ for the 6 hour inhalation exposure was represented by the body burden of ^{14}C-activity in the rats at the end of the exposure period (Table 1). Subtracting the unchanged $^{14}CH_2Cl_2$ exhaled by the rats during the postexposure collection period from the body burden values yields an estimate of the total metabolism of $^{14}CH_2Cl_2$ during the experiment. These data clearly point out the influence of metabolism on the dose-dependent accumulation of ^{14}C-activity. The data for total metabolism of $^{14}CH_2Cl_2$ indicate a saturation of $^{14}CH_2Cl_2$ metabolism with increasing exposure concentration. The resultant effect of this capacity-limited metabolism of $^{14}CH_2Cl_2$ was observed as disproportionately lower values for end-exposure body burdens in rats exposed to either 500 or 1500 ppm when compared to those exposed to 50 ppm $^{14}CH_2Cl_2$.

Data for the disposition of inhaled $^{14}CH_2Cl_2$ in rats (Table 2) indicate that both of the major metabolic pathways for the solvent exhibit dose dependency. As the exposure concentration of $^{14}CH_2Cl_2$ was increased, the percentages of ^{14}CO and $^{14}CO_2$ exhaled as well as the ^{14}C-activity remaining in the carcass decreased. This was accompanied by an increased percentage of $^{14}CH_2Cl_2$ eliminated in expired air at the higher $^{14}CH_2Cl_2$ exposure concentrations. Urinary and fecal excretion of ^{14}C-activity were not affected by increasing the $^{14}CH_2Cl_2$ exposure concentration. Moreover, the rates of elimination of ^{14}C-activity as biotransformation products of $^{14}CH_2Cl_2$ were unaffected by the $^{14}CH_2Cl_2$ exposure concentration despite significant differences in the total fraction of the body burden metabolized (see Figures 3-5).

The tissue distribution of ^{14}C-activity was determined 48 hours after the inhalation exposure (Table 4). The greatest concentrations of ^{14}C-activity were in the liver and kidneys of animals from all three exposure groups. In view of the nature of the major biotransformation reactions for CH_2Cl_2 (i.e. to CO and CO_2), it is evident that appreciable potential exists for the incorporation of CH_2Cl_2 derived from carbon fragments into a variety of endogenous substrates involved in normal synthetic and metabolic pathways.

The relationship between CH_2Cl_2 metabolism and the toxicity of the solvent is not fully understood. Although evidence has been presented for saturation of the primary metabolic pathways for CH_2Cl_2, the significance of these pathways to the mediation

of an adverse response or the detoxification of the solvent remains unclear. In either case, the implications of the Michaelis-Menten kinetics of CH_2Cl_2 metabolism are extremely important to the design and interpretation of inhalation toxicity studies.

As is evident from Figure 6, increasing the CH_2Cl_2 exposure concentration above the apparent K_m results in diminishingly smaller increments in the metabolism of the solvent. At 2-3 times K_m no increase in metabolism is observed with increasing exposure concentration. Once saturation is achieved the amount metabolized will increase in a linear fashion with increasing duration of exposure rather than as a function of exposure concentration. Thus, in a hypothetical experiment, exposure of rats to CH_2Cl_2 concentrations in the range of 2-3 times K_m will result in no significant difference in the actual metabolized "dose" to the animals if the exposure time is held constant.

The concepts of dose-dependent metabolism and pharmacokinetics must be considered as an integral part of study design in inhalation toxicity studies. Not only do such data provide a more realistic estimate of the actual dose incurred during exposure, but also allow more meaningful dose response relationships to be obtained for use in safety evaluations.

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DR. HENDERSON (Olin Corporation): I have a question for Mr. Haun. In terms of your exposures with UDMH, did you do a materials balance to indicate the amount evaporated equals air flow in concentration coming out of the chambers? Do you know whether you've got any reaction within the chambers so that your exposure is the UDMH plus reaction products?

MR. HAUN (University of California, Irvine): No, I don't recall that. The sampling in the chamber was in the outlet line, I believe. But, no, we did not make that determination, at least to my knowledge.

DR. HENDERSON: So we don't know whether there could be reaction products to which the animals might be exposed in addition to the UDMH.

Dr. Phalen, the same type of question I direct to you because you mentioned that you measured the sulfuric acid by titratable acidity, and you also mentioned ammonia. It seems then that you had the chance of having a reaction of the sulfuric acid with ammonia and having a mixed sulfuric acid and ammonium sulfate exposure rather than just sulfuric acid.

DR. PHALEN (University of California, Irvine): Not only the chance, but that was the case. We characterized that exposure in three ways. We looked at sulfate ion, titratable acidity and total mass. You get three different numbers when you do.

DR. HENDERSON: With the sulfuric acid, it wasn't a straight sulfuric acid exposure.

DR. PHALEN: Roughly half of the sulfuric acid had been converted to ammonium sulfate.

MR. HAUN: I have a question for Dr. Henderson. Why would you expect any reaction from UDMH in the inhalation chambers?

DR. HENDERSON: I don't know. It's just that I know with other materials, we've run into this problem of reaction where the exposure had been to the material that we have been vaporizing and a reactive. I'm a little sensitized to it because we get into problems then in terms of effect attributed to the material evaporated that may be actually effects due to a reactant product.

MR. HAUN: We've had that kind of problem with the other hydrazines. But we didn't have that problem with UDMH although we didn't routinely measure input and outflow concentrations.

MR. VERNOT (University of California, Irvine): We didn't do a materials balance in the sense that you mentioned, but we do know how much we put into the chamber, and we do know our chamber concentration. In that sense, we know that we lose a measurable amount of UDMH going into the chamber. For different hydrazines, the loss is more or less significant. I think for UDMH the loss in the generation system and ducting to the chamber may amount to something like 30%. Whether that represents loss through chemical change, absorption on surfaces, I don't know. But we certainly don't get 100% of the vaporized UDMH into the chamber and that is why we conduct continuous chamber analyses for the UDMH concentrations.

MR. HAUN: Our experience in exposures with the other hydrazines showed that there were some losses due to absorption and adsorption.

DR. MAC EWEN: Mr. Vernot, wasn't it with UDMH that chamber air samples were taken for mass spectrometry analysis that was conducted at the School of Aerospace Medicine? Mr. Conkle, didn't you analyze some samples of chamber atmospheres of UDMH by mass spectrometry?

MR. CONKLE (USAF School of Aerospace Medicine): Yes.

DR. MAC EWEN: Were you able to identify any reaction products?

MR. CONKLE: The analysis of the chamber atmosphere was reported by Dr. Miller and appears in the "Proceedings of the 7th Annual Conference on Environmental Toxicology," AMRL-TR-76-125. Our experience has been that UDMH in the atmosphere may have NDMA associated with it.

DR. MAC EWEN: I would like to ask Dr. Phalen why he believed that ozone had a greater effect at higher chamber humidities? I'm not sure why this should occur since the lung already contains a saturated water vapor atmosphere.

DR. PHALEN: Yes, that makes two of us that don't know why it occurred. The first question one would ask that I can't answer is whether that would occur in an animal that's not furry and doesn't have to cool by evaporation into the chamber atmosphere. But I'm sorry, I can't answer that question.

DR. HAZLETT (Naval Research Laboratory): I would like to comment on the paper by Mr. Gaworski. From your GC traces of both the JP-5 and the DFM, it would appear that the bulk of the hydrocarbons present in the vapor were very small molecules. We've also looked at those same samples in an equilibrium vapor situation and see apparently the same molecules which we've identified as propane, isobutane, butane, isopentane and so forth. In comparing those vapor components with vapor from other JP-5 and DFM, it's not clear that this is really a typical vapor exposure for these types of fuels. We do see quite a range from the limited number of samples we've looked at thus far. So it seems to me that you need to make your conclusions with that in mind, and this points out the fact that additional tests need to be done with other samples of JP-5 and DFM.

MR. GAWORSKI (University of California, Irvine): The chromatogram I referred to is typical of the vapors in our chamber atmosphere. As you say, it might not be typical for other JP-5 samples. I really don't know. The chromatogram that was shown was only for the first 20 minutes of effluent from a specific GC column. If you follow it for a longer period of time, you would get more peaks coming off. With the generation system, we had to add heat to the evaporator column. By doing that, we got more of the lower boiling compounds.

DR. HAZLETT: That opens the question even further. If there is a variable ventilation rate or a varied ratio of liquid fuel to gas sweeping over, we could get quite a variety of exposures to the animals. It seems to me that we need some tests where we vary several parameters such as temperature and ventilation rate to get guidance on what sort of exposures should be used in animal tests.

MR. VERNOT: We thought about the problem of just what was the best way for use to introduce the material into the chamber. I know that some work that was done previously used aerosol introduction. But we felt this would not really simulate the actual condition of the great majority of conditions of exposure. We thought that certainly a vapor exposure would be more typical. Then in order to elicit the greatest effect we could get, we thought we'd just try to attain as high a concentration as we could get in the chamber without forming an aerosol. In

one instance, as Mr. Kinkead noted, when we did exceed saturated vapor pressures, we formed aerosols and we started getting severe acute effects in the animals. So we attempted to maximize the concentration of hydrocarbons in the chamber without getting an aerosol. And we found this to be the most convenient way to do it.

DR. CROCKER (University of California, Irvine): There was an episode of major toxicity when you introduced the fuel into the chamber at a higher temperature and then they condensed as they reached the cooler atmosphere of the chamber. There were aerosols formed at this time. The toxicity appeared to have been related to the existence of an aerosol. But I don't recall that you mentioned what kind of pathology resulted from the exposure in which an aerosol seems to have been presented. I presume this would have been a lipid pneumonia kind of pathology. That is, acute toxicity related to deposition of droplets of the material.

MR. GAWORSKI: Fifty percent of the mice died in that aborted experiment. The deaths happened over a period of 3 days.

DR. MAC EWEN: The deaths began on about the fourth day and we stopped the experiment on the following Monday because 50% had died.

DR. CROCKER: Just because of the question of the possible realistic occurrence of high temperature storage of the fuel and its emptying into an atmosphere at a lower temperature when condensation might, in fact, happen, is this a possible real user experience such that a condensed aerosol might occur? For example, I know that we often keep our fuel stored in open sunlight or in places where the temperature can be quite high and also that it's introduced from that place into other closed chambers or closed vessels. Is there any realistic likelihood that a condensate would occur under user conditions?

DR. MAC EWEN: Maybe we could ask some of the users. But I have never seen aerosol formed except from a leak in a pressurized line. I don't think it can stay in that form very long. It would be a very localized condition.

DR. CROCKER: In short, there is no reason to examine these fuels from the point of view of the presence of aerosol but only as vapor.

COMMANDER JENKINS (Naval Medical Research Institute): I'd like to try to respond to Dr. Crocker's question and then comment on Dr. Hazlett's comment. There is a possibility of an exposure to the release of both jet fuel and DFM that have been in a delivery pipe at a high temperature. In the upper levels of fire rooms on board ship, temperatures frequently exceed 120 or 130 degrees. This is of some concern since they have occasionally exceeded 140 degrees and these things flash at 140 degrees. This is, as you know, worth considerable more research and this will be done.

In answer to Dr. Hazlett's comments, he didn't point out that he is embarking on an analytical test scheme over the next year or so to try to define what real JP-5 is. If it turns out fortuitously or nonfortuitously, the batch that was used in the experiments that Mr. Gaworski reported on has a tremendous front end. There were a lot of low boiling hydrocarbons in that particular batch. In another JP-5 exposure that was done back at NMRI, the front end was almost totally nonexistent. So what Dr. Hazlett is going to do is actually go to the refineries, get batch after batch of JP-5, and see what it really is chemically. We don't know what a typical, standard JP-5 is. These are mil spec (military specification) fuels. There are no chemical specifications other than they can contain something like a maximum of 25% aromatics. The resultant mixture varies from refinery to refinery, and it also varies from batch to batch. It varies considerably in various sources of crude oil. I am just making the point clear that we really don't know that there is a standard JP-5 and hopefully, about a year from now, Dr. Hazlett will be able to tell us what it should contain.

DR. CROCKER: In the meantime, then, we should be conscious not only of vapor exposure but also of aerosol exposure.

COMMANDER JENKINS: Absolutely.

DR. CROCKER: There seems to be a very significant change in toxicity. The aerosol form is quickly lethal. The vapor is relatively nontoxic.

DR. MAC EWEN: The aerosol isn't immediately lethal. It took about 3 days to produce what appeared to be a chemical pneumonia.

COMMANDER JENKINS: I think it may also depend on the kind of aerosol and the way the aerosol is formed. We've done aerosol exposures in our labs on a couple of occasions and have not been able to mimic this. Now, it may also be a species difference because we had rats in the chamber, and Mr. Gaworski was using mice. These experiments tend to pose more questions than they answer.

DR. CROCKER: I'd like to ask a question of Dr. McKenna. I was particularly pleased with that presentation and would like to ask whether you might expect any toxic effects resulting from the nonmetabolized parent product? Is there any pharmacologic or toxicologic effect of the unaltered parent compound or does it simply circulate and not store itself in any fashion and undergo excretion by rebreathing or exhalation?

DR. MC KENNA (Dow Chemical, USA): We haven't been able to find stored methylene chloride. We terminated the collection portion of the experiment at 48 hours after the exposure. We could not find any methylene chloride in the body at that time. All of the ^{14}C activity that's left is non-methylene chloride ^{14}C activity. There's no urinary excretion of methylene chloride in the rat. There's very little urinary excretion in man. The only unaltered methylene chloride we recover is that which comes back out in expired air. Because it gets excreted so fast, it's hard for me to think of that being a cumulative type toxicity problem. In long-term inhalation studies that we've conducted, we ran into problems at exposures to 500 ppm methylene chloride that I don't think anybody really expected. We were surprised to find liver toxicity in rats due to methylene chloride. I would like to temper that remark and point out that we didn't find liver effects until after a year of exposure. The onset of the toxicity was first seen at the 12-month interim sacrifice. It wasn't there after 6 months exposure. The thing that makes me think that it might be a metabolite is that we see this same type of toxicity at all three dose levels. We didn't have a no-effect level in that experiment. I think it's going to be interesting, when all the histopathology is completed from that experiment, to see if we can't take either incidence data or some type of scoring procedure to characterize a little more quantitatively the liver lesion and find out if it correlates with a dose response relationship. I find it difficult to believe that it is methylene chloride that causes the toxic liver response. I'm not sure if we'll ever know for certain because the methylene chloride is just not a typical reactive alkylating chemical. Dr. Anders and his group have worked for about a year and a half now to try to demonstrate covalent macromolecular binding due to methylene chloride exposure and they finally found it. They had to look very hard. You can't find binding with nucleic acids. There's some in lipid and some in protein. But the binding itself seems to be associated with carbon dioxide metabolic pathways. It seems to go through formaldehyde formate and not the carbon monoxide pathway. We don't yet know how to handle chemicals that don't bind very well and don't produce effects until after months or years of exposure. It's not like the type of work

done with vinylidene chloride where we find a morphological effect almost immediately and we can correlate that with the biochemical change we see. Methylene chloride is an unusual chemical, and we probably don't know enough about that yet to be able to make a decision.

MR. BECKER (University of California, Irvine): Dr. McKenna, do you notice a change in the volume of distribution as you load the animals with methylene chloride?

DR. MC KENNA: You can't saturate the animals with methylene chloride per se but as you reach the limit of metabolism, blood levels get disproportionately higher with change. I don't know if the volume of distribution actually changes. I don't think we've seen any indication for that. We certainly get more methylene chloride in the various tissue compartments. However, there's no real difference in postexposure kinetics, so I'd have to say no.

MR. LEAHY (University of California, Irvine): Dr. McKenna, I wonder if these enzymes were inducible whether you had higher conversion or not.

DR. MC KENNA: In the two-year inhalation study, we did measure carboxyhemoglobin levels to give us an index to see if repeated exposure to methylene chloride would cause an accumulation. And it turned out that there was no difference between exposure levels. Carboxyhemoglobin levels ranged between 10 and 13%. That's true of the animals exposed to 500, 1500 or 3500 ppm methylene chloride. There was no statistical difference between them. I am not certain of the statistical relationship of the hamsters which was the other species used in the study. What we do know is kind of interesting. Hamsters produce a lot more carboxyhemoglobin than rats when they're exposed to the same concentrations of methylene chloride. They don't seem to have a dose response relationship that would be indicated by the exposure concentration of methylene chloride. But they do have carboxyhemoglobin levels on the order of 30%. This is interesting with regard to the hepatotoxicity because the hamsters were virtually unaffected by methylene chloride with regard to any kind of liver lesion. So it very well may be that carboxyhemoglobin formation is a detoxification pathway for methylene chloride if you're a rat or a hamster.

DR. COLLINS (Ohio State University): Dr. McKenna, the V_{max} and K_m values that you had for the in vivo rat experiment, are they comparable to in vitro values? And second, must you make any major assumptions in your computer analysis to get V_{max} and K_m values, anything different from an in vitro experiment?

DR. MC KENNA: I can't answer your first question because I don't really know whether they are different from the in vitro experiments or not. We have not conducted any in vitro metabolism studies on methylene chloride in our lab. In the near future, I hope to get together with Dr. Anders who has done a lot of work on both pathways in vitro and maybe we'll get some answers there. Secondly, I wouldn't want to over-emphasize the significance of those numbers. The only thing that they're really useful for is to characterize the curve. I don't know how good they are for correlation between methodologies. There weren't any major assumptions made other than we were looking at the net result of metabolism during the inhalation exposure. We didn't collect metabolites during the exposure. Other than that, it's an optimization routine, really, on a digital computer. It fits the linearized form of the standard Michaelis-Menten equation.

DR. MAC EWEN: Dr. McKenna, have you compared your carboxyhemoglobin elimination curve with those after brief exposures to carbon monoxide alone?

DR. MC KENNA: We did an experiment where we exposed rats to 100 ppm of carbon monoxide which gives them about a 10% carboxyhemoglobin and they have a half-life of about 30 minutes. It's a first order reaction, no shoulder on the curve; and that's exactly what we saw with the 50 ppm methylene chloride exposure. However, when we use higher concentrations then we get this shoulder and something that looks like it's a combination of a zero order and a first order reaction process. I think that we just don't shut off the input to that metabolic pathway when we shut off the exposure. That's why carboxyhemoglobin levels stay up. Stewart saw the same thing with people exposed to methylene chloride.

MR. VERNOT: I have a question for Dr. Phalen. You didn't seem to have too much confidence in your finding that sulfuric acid affected the lung clearance time. I wondered why this might be. Might that not be an expected consequence of exposure?

DR. PHALEN: Yes. The reason for the lack of confidence is that sulfuric acid didn't always affect clearance time. It didn't affect clearance time at all humidities, for example. Relative humidity seems to play a role that we don't understand with respect to sulfuric acid.

MR. VERNOT: You didn't get any pattern that you could relate to relative humidity? That is that effects got greater or lesser as relative humidity went up or down?

DR. PHALEN: Yes, it appeared that with sulfuric acid, effects were greater at lower humidity with respect to long-term clearance. That possibly relates to the fact that the acid concentration in the droplet would be higher. Sulfuric acid is always in equilibrium with the surrounding humidity. So the acidity would be higher. That's possibly the answer. I think that when one's dealing with a sulfuric acid exposure, control of the humidity is absolutely essential.

DR. MC KENNA: I'd like to add to that. We spent about 8 months trying to find out why we couldn't control particle size distribution in our inhalation chambers when we were trying to do a sulfuric acid mist teratology study. It's almost totally humidity dependent. It's extremely difficult to control particle size of sulfuric acid aerosol in a 5 cubic meter chamber, and the key factor is absolute control of relative humidity.

MR. LEAHY: Dr. Phalen, were the particles shown in the electron micrograph hollow or were they solid?

DR. PHALEN: They appear to be solid particles. Some of them are volatile under the heat and the vacuum of the electron microscope. But we didn't have evidence of hollow particles. They tend to be solid.

MR. LEAHY: Is the generated ozone input required to be higher in the higher humidity than in the lower humidity studies for arriving at the same concentration of ozone in the chamber?

DR. PHALEN: I'm sorry to say that we don't know. We merely adjusted our ozone generator output to get the desired level. Since we had adjustments both on flow rate and on voltage to our ozone generators, I don't know.

DR. MAC EWEN: There's one other comment I'd like to make about the UDMH study presented by Mr. Haun. There were statistically increased incidences of some tumors and almost all the common tumors were found in both control and test animals. There were only a couple of instances where there were very small increases of tumors between the highest exposure group and their controls. I don't believe we would have seen these small increases except for the very large numbers of animals that were used in these experiments. I'm not sure that it came through clearly that the numbers of animals used in this study were greater than most oncogenic studies usually contain. There were 400 mice, 200 rats and 200 hamsters in each exposure level. That's a very large number of animals, and you increase the probability of finding tumors when you use these larger numbers. I would not call this NDMA contaminated UDMH a strong carcinogen. At best, the tumor incidence in exposed animals was very slightly elevated over control animal tumor incidence.

SESSION IV

GENETIC TOXICOLOGY

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PAPER NO. 18

THE ROLE OF GENETIC TOXICOLOGY

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Genetic Toxicology is the subdivision of general toxicology which deals with toxicity directed specifically to the hereditary material, DNA. The importance of this specialized area of toxicology encompasses two types of data production and analysis (de Serres, 1976).

The first function of Genetic Toxicology is the idenfication of chemicals with mutagenic potential. These agents may be capable of inducing DNA alterations which lead to a variety of toxic endpoints including hereditary disease, cancer, or birth defects (Benditt, 1977; Brusick, 1978; Kalter, 1977). It is necessary to emphasize the word potential since the types of test methods employed in the detecting aspects of Genetic Toxicology are commonly submammalian, or in vitro mammalian assays. These tests, called short-term tests, are designed to be sensitive, rapid and inexpensive, but are not capable of indicating with certainty the expressivity of the defined genetic potential in vivo.

The second function of Genetic Toxicology involves the estimation of risk from exposure to chemicals defined as genetically active (genotoxic) by short-term detection tests. Risk assessment is generally confined to the quantification of the risk of transmissable (germ cell) mutations. Whole animal testing is necessary in risk assessment.

Figure 1 identifies the two primary forcing factors which relate to the use of genetic testing. The first, and probably least publicized, is an awareness that the genes carried in the present human population are the substrate upon which subsequent generations are built. The integrity of the genes in the human gene pool must be protected from alteration by environmental mutagens. If not, the genetic burden for future generations will be increased in terms of both the emotional and financial aspects of hereditary dysfunction

(Committee 17 of the Environmental Mutagen Society, 1975). The second forcing factor is the ability to predict carcinogenic activity on the basis of genetic testing (Bridges, 1976; Brusick, 1978; Magee, 1977; Purchase et al., 1976). This role of genetic toxicology has been widely applied and has received considerable attention from the private sector and the Federal regulatory agencies.

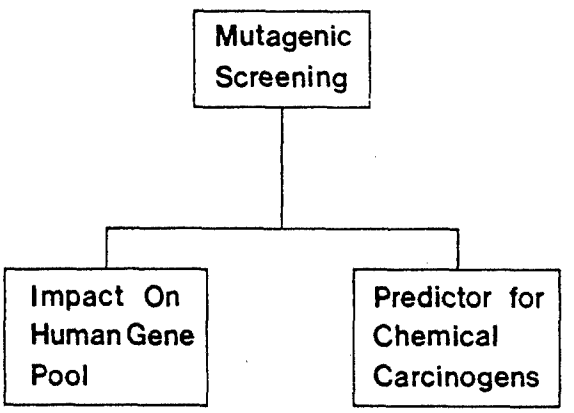

Figure 1. Forcing factors for Genetic Toxicology.

Screening studies are important components of both forcing factors whereas risk assessment in Genetic Toxicology only applies to the determination of mutagenic risk. Carcinogenic risk assessment cannot be developed directly from genetic testing.

Another area of investigation has recently seen considerable growth in the science of genetic testing. This area is population monitoring for exposure to genotoxic agents. Methods such as cytogenetic analysis of blood cells for aberrations and sister chromatid exchanges (SCEs) appear promising as techniques to establish human exposure to DNA modifying agents. These approaches may soon enable us to develop a good profile of exposure for occupational settings of potential high risk.

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PAPER NO. 19

THE DEVELOPMENT OF A CORE BATTERY APPROACH TO CHEMICAL EVALUATION FOR GENETIC EFFECTS

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Text of this presentation is not available for publication.

PAPER NO. 20

SISTER CHROMATID EXCHANGE IN ANIMALS EXPOSED TO MUTAGENIC CARCINOGENS - AN ASSAY FOR GENETIC DAMAGE IN HUMANS

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Recent progress in the field of sister chromatid exchanges (SCEs) was made possible by the development of new techniques for their detection. These techniques are generally based on exposure of cells to the thymidine analog 5-bromodeoxyuridine (BrdU) during two consecutive rounds of DNA replication. Following this exposure, the subsequently observed metaphase cells contain chromosomes with asymetrically-labeled chromatids. One chromatid is bifilarly substituted with BrdU, and its sister is unifilarly substituted (Figure 1). Such substituted chromatids stain differentially with fluorescent dyes such as Hoechst 33258 and also with a combination of Hoechst and Giemsa (the FPG technique of Wolff and Perry, 1974). In this way SCEs, which represent exchanges of homologous chromatid segments between sister chromatids, can readily be observed and counted.

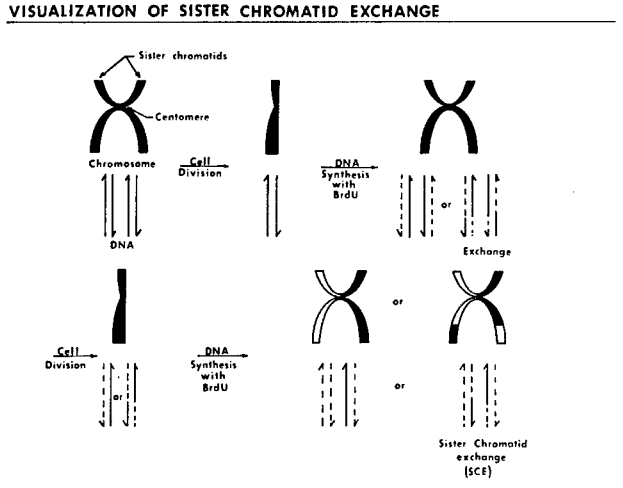


Figure 1. Brdu substitution patterns after two rounds of DNA replication in the presence of the thymidine analog, and staining patterns that result when the FPG technique is used to stain metaphase cells.

These new staining techniques were initially used for in vitro studies, and it was shown by Perry and Evans (1975), among others, that exposure of cells to known mutagenic-carcinogens caused significant increases in SCE frequency. Chemicals requiring metabolic activation to their mutagenic forms were predictably negative in this assay; but this obstacle was overcome by Stetka and Wolff (1976a) and Netarajan et al. (1976) with the addition of the S9 mix of Ames (1973). The S9 mix proved capable of converting otherwise inactive mutagens into a form capable of inducing SCEs (Figure 2).

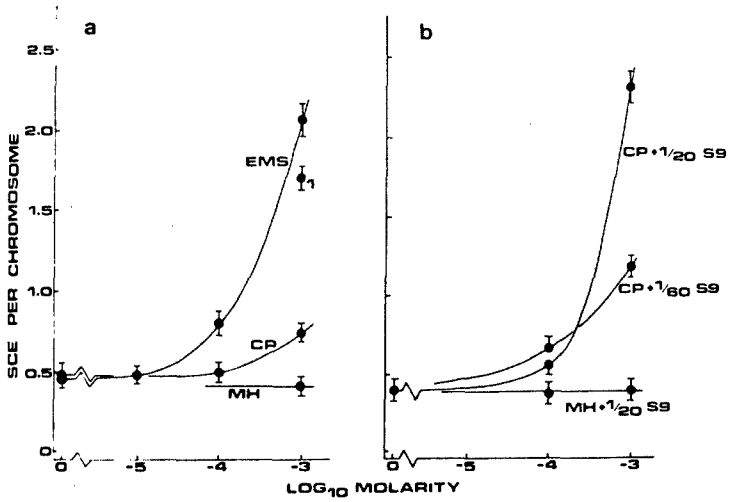


Figure 2. SCE frequencies in CHO cells exposed to EMS cyclophosphamide (CP) and maleic hydrazide (MH) with and without metabolic activation [the S9 mix of Ames et al. (1973)].

Several in vivo systems that allow detection of SCEs in animals following exposure to chemical mutagens have also been developed. One such system is that devised by Stetka and Wolff (1976b) in which chemicals injected into a rabbit (ip) induces SCEs that can be observed when the animal's lymphocytes are subsequently cultured in the presence of BrdU and then stained with the FPG technique. Results from control animals were quite consistent and indicated a background SCE frequency of approximately 0.13 SCE per chromosome (Table 1). Animals exposed to mutagens showed elevated SCE frequencies within one day after acute exposure. EMS and MMS, which do not require activation, and cyclophosphamide, which does, were all positive in this assay (Table 2 and Figure 3). The frequency of SCE then returned to control level within two weeks.

TABLE 1. THE FREQUENCY OF SCE IN CULTURED PERIPHERAL LYMPHOCYTES OF 8 UNTREATED CONTROL RABBITS

Rabbit No.	No. SCEs/No. Chromosomes	SCE/Chromosome ± S.D.
1	97/623	0.156 ± 0.016
2	110/663	0.166 ± 0.016
3	42/425	0.099 ± 0.015
4	173/1363	0.127 ± 0.010
5	58/497	0.117 ± 0.015
6	72/624	0.115 ± 0.014
7	56/496	0.113 ± 0.015
8	82/613	0.134 ± 0.015
Total	690/5304	0.130 ± 0.005

TABLE 2. THE FREQUENCY OF SCEs IN CULTURED PERIPHERAL LYMPHOCYTES FROM BLOOD DRAWN BEFORE (TIME 0) AND 1 DAY AFTER IP INJECTION OF RABBITS WITH TEST COMPOUNDS

Compound	Dose (mg/kg)	Time (days) Post-Treatment	Number SCEs/ Number Chromosomes	SCE/Chromosome ± S.D.	P*
CP	20	0	690/5304**	0.130±0.005	
		1	100/538	0.186±0.019	
	35	0	56/496	0.113±0.015	
		1	280/592	0.473±0.028	$<10^{-8}$
EMS	50	0	173/1363	0.127±0.010	
		1	206/1247	0.165±0.012	0.01
	200	0	51/449	0.114±0.016	
		1	274/870	0.315±0.019	10^{-4}
MMS	25	0	97/632	0.156±0.016	
		1	208/814	0.256±0.018	10^{-4}

*P value based on t-test between 0 and 1 day values.
**Values from Table 1, as control blood did not grow in this case.

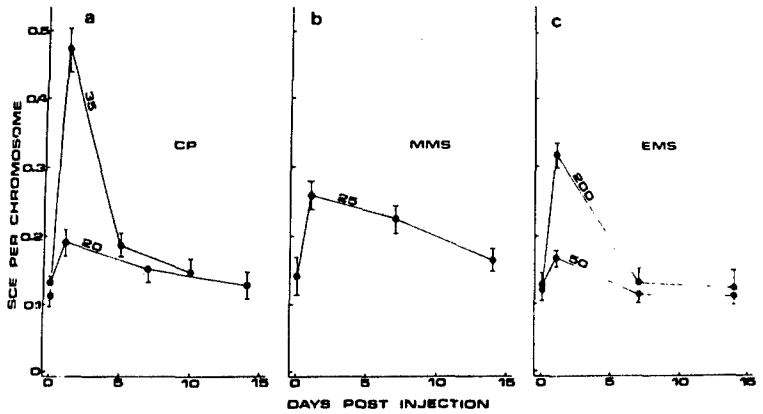


Figure 3. SCE frequency responses in cultured lymphocytes following acute (ip) exposure of rabbits to EMS, MMS, and CP. Blood was collected and cultured (with BrdU) at various times before and after injection of the mutagenic-carcinogens.

These results suggested that the culture of lymphocytes and determination of their SCE frequencies might be used as an assay for human exposure to mutagenic-carcinogens. The transient nature of the response, however, indicated that the individual would have to be available for blood sampling within one week of exposure for the test to be conclusive. Further experiments were therefore conducted by Stetka et al. (1978) to determine if another exposure regimen might induce longer-lived increases in SCE frequency. Three mutagens were used in these studies - methylcholanthrene, benz(α)pyrene, and mitomycin-C, and all were administered (ip) once a week for 6-8 weeks at low dose levels. With methylcholanthrene, the responses were erratic during the first few weeks but then, after a period of time, SCE frequencies were significantly elevated in all animals (Figure 4). More importantly, the SCE frequencies did not return to the normal, control level following treatment. Control frequencies occasionally approached the levels seen in these treated animals, but such higher frequencies were never sustained for more than one week in the controls. Results with benz(α)pyrene were less equivocal (Figure 5). Individual responses could be quite different during the first few weeks, but after this time, the SCE frequencies were clearly elevated and remained so for at least two months after termination of treatment. Similarly, mitomycin-C induced long-lived increases in SCE frequency that persisted for many months after the last exposure (Figure 6). Note also that with mitomycin-C the initial responses were quite consistent, i.e. SCE frequencies were elevated within one day of exposure and then returned to pre-exposure level within one week. Both animals deviated from this regular pattern after the fourth injection.

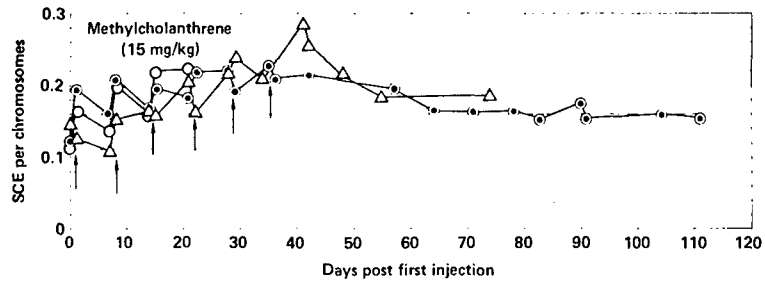
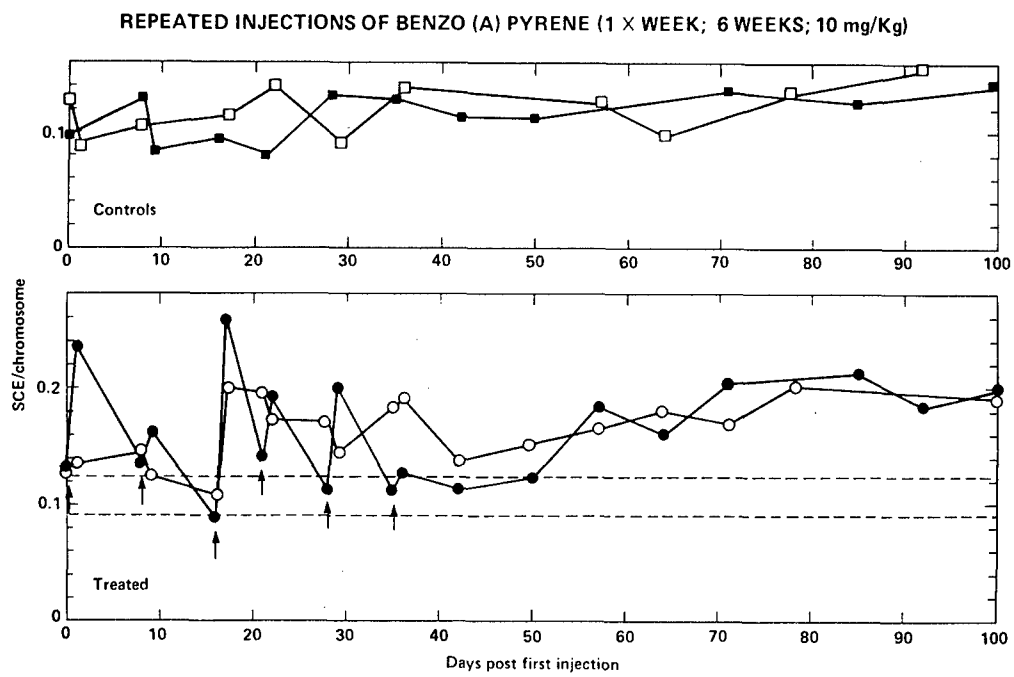
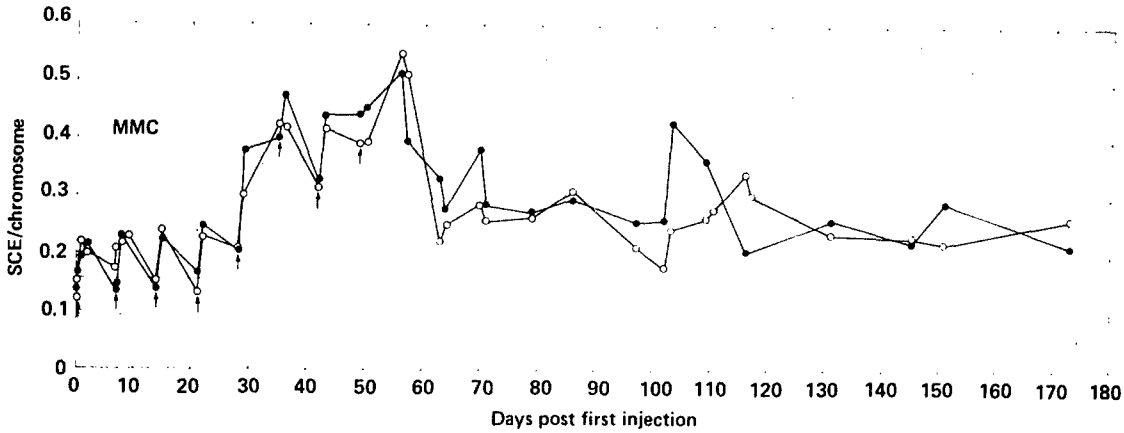


Figure 4. SCE frequencies of lymphocytes collected from rabbits during and after the period of subchronic exposure (weekly for 6 weeks) to methylcholanthrene (MC). Dashed lines delimit the control range. Control blood was drawn from two animals at all sampling times indicated by MC data points, and control animals were concurrently injected with corn oil (the vehicle for MC). Arrows indicate times of injection.



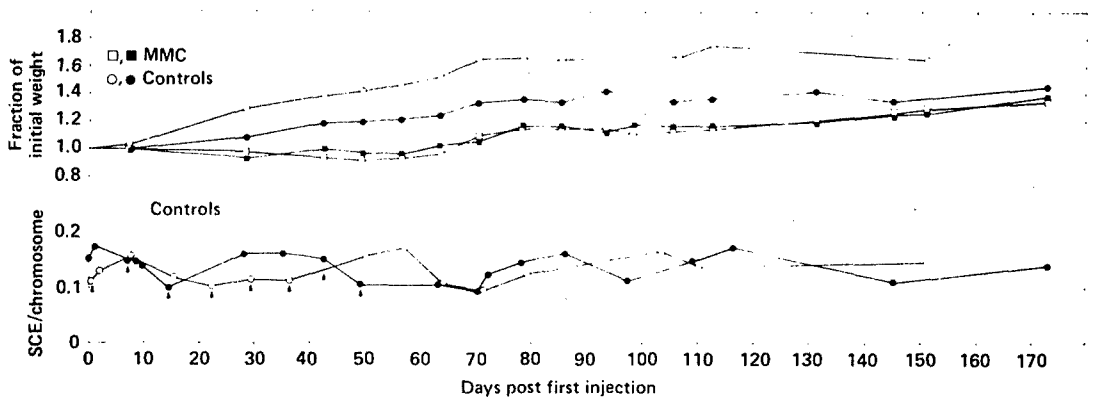
Repeated exposure to another chemical mutagen, benzo (α) pyrene, also induces persistent elevation of SCE frequency in rabbit lymphocytes. Note that one animal (open circles) failed to respond to this activation-requiring compound until after the third injection.

Figure 5. Temporal SCE response in rabbits exposed to benz-(α)pyrene (BP). See Figure 4 legend for details.



Repeated exposure induces persistent increases in SCE frequency after the 4th ip injection. Frequencies are still elevated long after the final injection, contrary to the response following single, acute exposure to the same total dose.

Figure 6a. Frequency of SCEs at various times during and after repeated injections with mitomycin-C (MMC) (0.5 mg/kg). Animals were injected once a week for 8 weeks.



Growth rates are retarded during MMC treatment; they return to normal after treatments. Control SCE frequencies remain constant throughout.

Figure 6b. Upper curves represent growth rates during and after treatment with MMC. Bottom curves show constancy of control SCE frequencies throughout 173 days.

The different initial response with methylcholanthrene and benz(α)pyrene are not necessarily surprising since these compounds require metabolic activation by liver enzymes. The spontaneous levels of such enzymes, as well as their inducibility, are known to vary between animals. Thus, the different response may be related to genotype differences. It is important, however, that eventually all animals do respond in terms of SCE frequency, to nearly the same extent. First exposures of nonresponders apparently cause sensitization (by inducing enzymes, perhaps) so that these animals do respond to subsequent exposures. It is significant that all compounds tested to date that are capable of inducing SCEs in vitro without activation also induce SCEs in lymphocytes within one day of the first exposure.

Despite the differences in initial lymphocyte responses to activation-requiring mutagens, it is nevertheless clear that all 3 compounds (methylcholanthrene, benz(α)pyrene, and mitomycin-C did induce persistent, long-lived increases in SCE frequency with subchronic exposure. This suggested that the test system might work very well as an assay for repeated, low-level mutagen exposure in humans. Indeed, Drs. Carrano (Lawrence Livermore Laboratory) and Stetka (Litton Bionetics) have initiated a pilot study of SCE frequencies in lymphocytes of petrochemical workers. Dr. Carrano has recently reported that SCE frequencies do appear to be elevated in workers exposed to a variety of presumably mutagenic chemicals over a period of years. No other short-term assay has the sensitivity to detect such low levels of exposure.

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PAPER NO. 21

USING THE SISTER CHROMATID EXCHANGE TEST AND THE MICRONUCLEUS TEST IN VITRO AND IN VIVO TO ESTIMATE LONG TERM GENOTOXIC POTENTIAL

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It has been estimated that there are now 65,000 different chemicals in our environment and new ones are being introduced every day. The American people have demanded, through their elected representatives, that chemicals be tested to determine whether they are capable of causing harm. Through mandates by the regulating arms of the government such as the Food and Drug Administration, the Environmental Protection Agency, and the Occupational Safety and Health Administration, almost all new chemicals must now be tested for their potential toxicity. But what kind of tests ought to be done? Traditionally, animals, primarily rodents, have been exposed to chemicals acutely and chronically and effects observed. These animal results have then been extrapolated (with some uncertainty) to humans. For toxic effects such as carcinogenicity or mutagenicity, long, expensive, testing protocols have traditionally been used. Clearly, however, resources of industry and government are in no way sufficient to test even a significant fraction of 65,000 chemicals for carcinogenicity or mutagenicity using the traditional procedures that cost upwards of $250,000 per chemical and take upwards of three years to test per chemical.

What has been recognized as being needed are faster, less expensive tests that can jointly give the same information as the traditional long-term tests (in the ideal case), or at least have a very high probability of doing so (in the realistic case).

Many rapid, inexpensive tests have been proposed in the last few years as possibilities for fulfilling these requirements. The best known of these is the Ames Test, an assay for the induction of a particular reverse mutation in the bacterium Salmonella. But conceptual problems have been raised with the

Ames Test and with all in vitro tests similar to it which use other bacteria, yeast, fungi, or mammalian tissue culture cells. There are no comfortable relationships between these tests and whole animal exposure experience; unrealistically high exposure doses are used to obtain test results and then a large extrapolation is made to more realistic exposure levels; the results found with these in vitro tests of chemicals never correlate perfectly with long-term, whole animal study results for each chemical tested; cells grown in vitro are just not people walking around.

With these problems in mind, we have chosen two of the newly suggested, rapid, relatively inexpensive tests to use for development and evaluation here at the Toxic Hazards Research Unit operated by the University of California, Irvine. We have chosen the Micronucleus Test, a very simple assay for chromosome breakage, and the Sister-Chromatid Exchange Test, a very sensitive assay for chromosome rearrangement. Both of these tests can be done directly with samples taken from living mammals, even humans, answering most objections about relevancy. They can be done with samples taken from animals exposed chronically by almost any exposure route to low dose levels of test chemical, answering most objections to unrealistic exposure routes and levels. The animals are exposed in a highly controlled environment at levels and for times that humans are or would be exposed. The same animals can be used for both the long-term traditional carcinogenesis studies and for these short-term tests for chromosome damage. No animal sacrifice is necessary in doing the tests we have chosen. Because of this, we can directly and truly validate these tests as short-term results, and long-term results done with the same animals can be compared.

Specifically, the tissues we are using in our tests are peripheral lymphocytes and bone marrow cells. Our tests have been done primarily with these tissues derived from dogs and rats, but we have also done some limited work with humans and Rhesus monkeys. We have tested some cells exposed in vitro as well as others exposed in vivo after dosing by inhalation, and soon by intraperitoneal injection. We have tested the well known alkylating agents ethylmethanesulfonate, methylmethanesulfonate, and Mitomycin C as positive controls. We have tested the water soluble fuels hydrazine, monomethylhydrazine, 1,2-dimethylhydrazine, and 1,1-dimethylhydrazine as well as a contaminant of the latter, dimethylnitrosamine. And we have tested the water insoluble fuels and fuel components, jet propellants-4, -5, and -10, marine diesel fuel, decahydronaphthalene, and methylcyclohexane. We would like

to test other tissues, species, exposure routes and chemicals, but these are the ones we have chosen to begin with. We have by no means come even close to testing all possible combinations of everything listed.

What we have looked at is the cytogenetic, chromosome damage induced in blood cells by several chemicals, and we are waiting to compare our short-term results with the lifetime experience of the same animals we have tested.

In Figure 1, the possible fates of a broken chromosome are shown: (1) The broken piece may be repaired at its original site. This kind of event can be assayed by detecting the DNA synthesis that takes place when the piece is rejoined; (2) The broken piece may be repaired at its original site but it can be exchanged with its sister chromatid in the process of being repaired. This kind of repair can be assayed with the sister chromatid exchange test as described by Dr. Stetka in the previous talk; (3) The broken piece may be repaired but at a different place, being exchanged for a broken piece of a completely different chromosome. This reciprocal translocation can be detected by looking for abnormal chromosomes in well-spread metaphase chromosomes; (4) The broken piece may remain unrepaired. This piece can be seen in well-spread metaphases, but it can also be detected much more easily and rapidly by simply looking for the broken pieces among interphase cell nuclei. This is the micronucleus assay, and I'd like to talk about this in some detail.

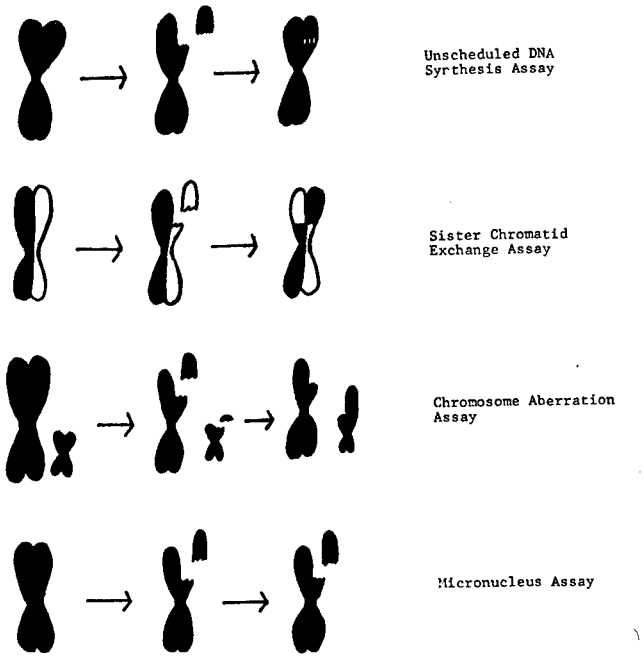

Figure 1. Possible fates of broken chromosomes and assays to detect each fate.

Figure 2 illustrates the principle upon which the micronucleus test works. In the left hand side of the figure, the fate of the contents of a normal cell nucleus through one division is shown. The chromatin, dispersed throughout the interphase nucleus, becomes condensed until individual chromosomes can clearly be seen. These chromosomes line up during metaphase and the two sister chromatids of each chromosome are drawn apart by the attached spindle fibers. Each daughter chromosome group then decondenses, forming two new interphase nuclei.

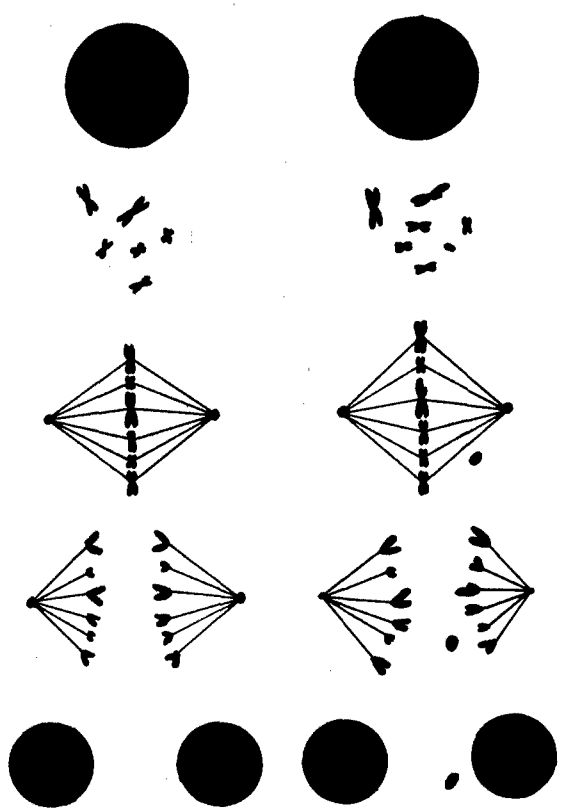

Figure 2. Left-side: normal cell division. Right-side: cell division with chromosome fragment present.

In the right-hand side of the figure, we can see what happens if the DNA is broken during the first interphase and remains unrepaired. When the chromatin condenses to form individual chromosomes, there is a fragment present. This fragment has no spindle attachment site and so when the chromatids are drawn into the daughter nuclei, the fragment can be left out of both nuclei (although retained in the cytoplasm of one of the daughter cells). To assay this kind of

damage, we simply look at random populations of cells that have divided at least once since treatment and count the small, "micro"-nuclei.

Figure 3 shows an example of a canine peripheral lymphocyte nucleus with a micronucleus nearby.

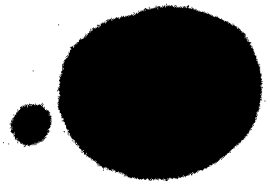

Figure 3. Canine peripheral lymphocyte nucleus and micronucleus.

We have tested a number of chemicals for their ability to induce micronucleus formation, i.e., to break chromosomes. The next three figures show typical positive results. The first (Figure 4) shows the effects of ethylmethanesulfonate (EMS), a well known DNA monofunctional alkylating agent, on canine peripheral lymphocytes exposed in vitro. With increased dose of EMS, the number of chromosome breaks increases, until at even higher doses EMS becomes cytotoxic, inhibiting cell division. As I explained before, a cell division is necessary for micronuclei to be seen. As fewer cells are capable of dividing at higher doses of EMS, fewer micronuclei can be seen even though the amount of chromosome breakage, presumably, continues to increase.

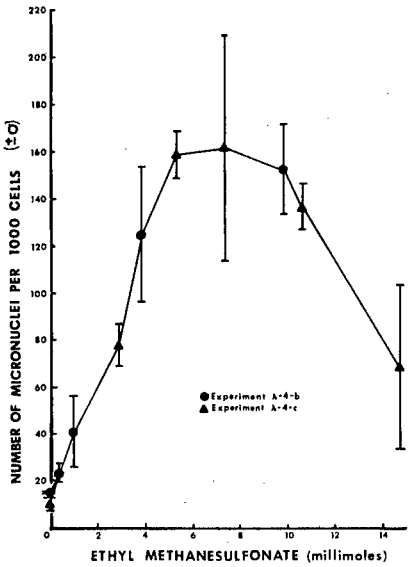

Figure 4. Micronuclei induced by in vitro exposure of canine peripheral lymphocytes to ethyl-methanesulfonate.

In Figure 5, a similar curve can be seen resulting from exposure to methylmethanesulfonate.

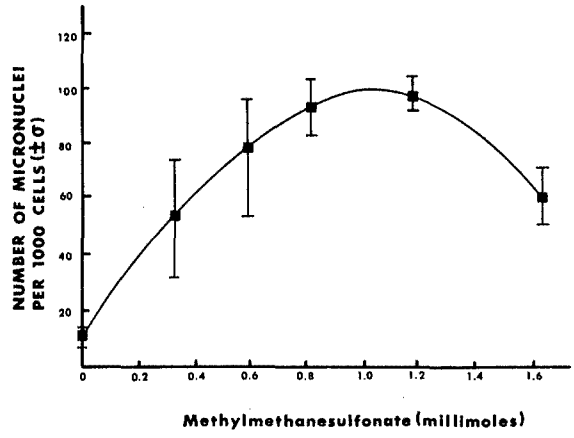

Figure 5. Micronuclei induced by in vitro exposure of canine peripheral lymphocytes to methylmethanesulfonate.

In Figure 6, results can be seen of in vitro exposures of canine peripheral lymphocytes to Mitomycin C (MMC), a bifunctional DNA alkylating agent. Notice that as little as 250 nM of MMC gives a clearly positive response in this test.

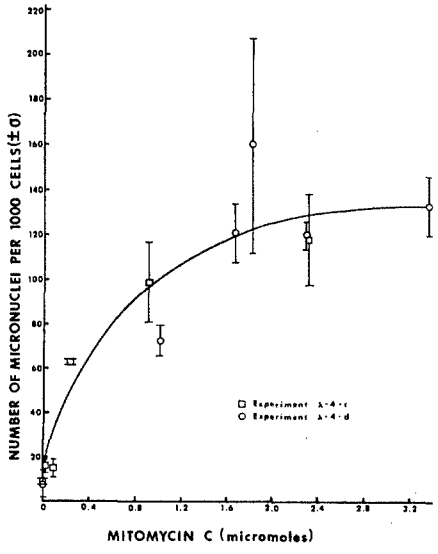

Figure 6. Micronuclei induced by in vitro exposure of canine peripheral lymphocytes to Mitomycin C.

Figures 7-9 show the result of canine peripheral lymphocyte, being exposed in vitro to hydrazine (HZ), monomethylhydrazine (MMH), and 1,2-dimethylhydrazine (SDMH). HZ and MMH show no significant induction of micronuclei while SDMH is possibly positive. The latter chemical is being retested.

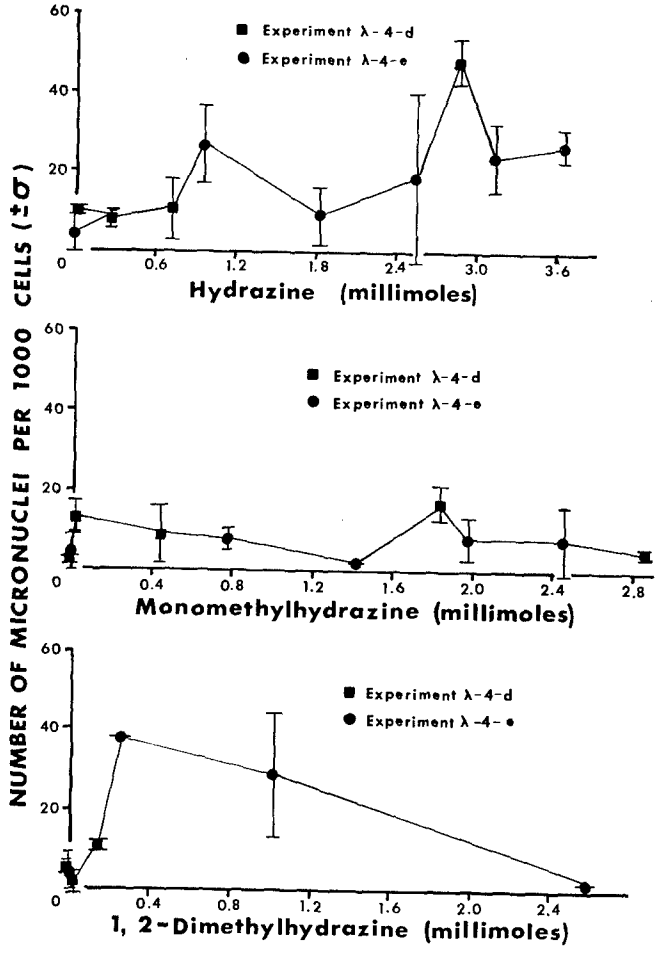

Figure 7. Micronuclei induced by in vitro exposure of canine peripheral lymphocytes to hydrazine, ...

Figure 8. ... to monomethylhydrazine, ...

Figure 9. ... or to 1,2-dimethylhydrazine.

Figures 10 and 11 show negative results for 1,1-dimethylhydrazine (UDMH) and, separately, its contaminant, dimethylnitrosamine (DMNA).

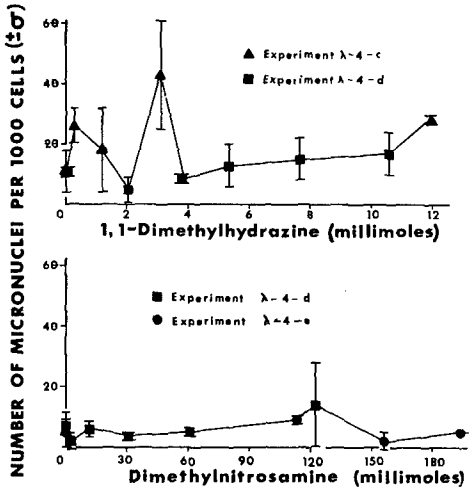

Figure 10. Micronuclei induced by in vitro exposure of canine peripheral lymphocytes to 1,1-dimethylhydrazine, ...

Figure 11. ... or to dimethylnitrosamine.

The cells in all cases were not "activated" in vitro. We intend to use whole animals themselves as "activators".

The next figures (12-14) show the results of analyses of peripheral blood from dogs exposed by inhalation in Thomas Domes by methods described in papers given here this morning. Results are consistently negative for dogs exposed on a workday basis to MMH for up to 52 weeks, and on a continuous basis to jet propellant number (JP-5) and marine diesel fuel (DFM) for up to 90 days. Figure 15 shows equally negative results for continuous exposure to decahydronaphthalene (decalin). (Although we took samples, we did not score the zero time slides for decalin when we saw the 8 and 12-week results.)

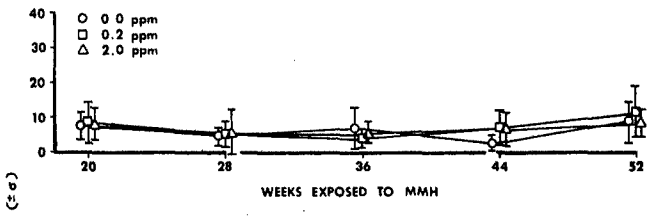

Figure 12. Micronuclei induced in canine peripheral lymphocytes taken from dogs exposed in vivo to MMH, ...

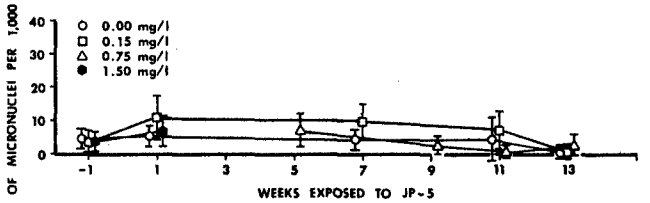

Figure 13. ... to JP-5, ...

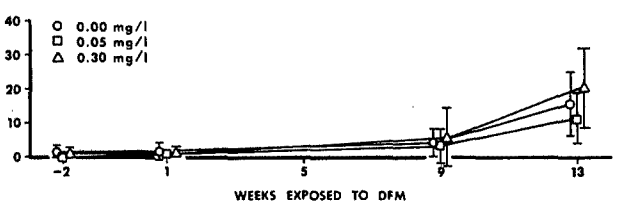

Figure 14. ... or to DFM.

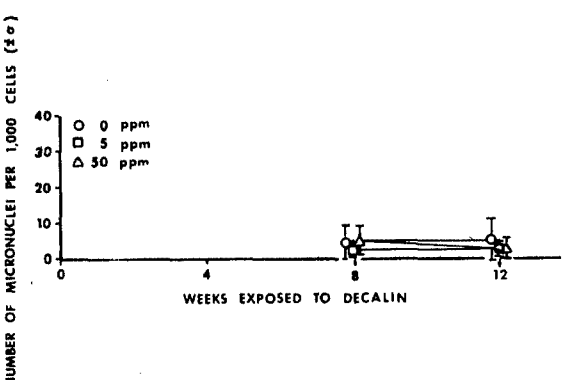

Figure 15. Micronuclei induced in canine peripheral lymphocytes taken from dogs exposed in vivo to decalin.

We have not yet done positive controls for inhalation studies. We will do these if the micronucleus test appears to be sufficiently sensitive to make this worthwhile. We will first test positive controls using animals injected intraperitoneally (I.P.) with known chromosome damaging agents to establish the test's sensitivity.

To sum up our current progress with the micronucleus test: three chemicals expected to break chromosomes do induce micronuclei in canine peripheral lymphocytes exposed in vitro without any activating agent, while three water-soluble military fuels plus a normal contaminant of one of them appear not to induce micronuclei. One water-soluble fuel, SDMH, may be positive. It is currently being retested. No micronuclei over background levels can be seen in lymphocytes taken from animals exposed in vivo by inhalation to any of four military fuels. In vitro testing of water insoluble fuels is now beginning and positive and test chemicals administered I.P. will soon begin.

The other relatively rapid, inexpensive test we are using is the Sister Chromatid Exchange Test. This test is slower and technically more difficult to do than the Micronucleus Test as we are required to obtain well-spread metaphase chromosomes, but it is a very sensitive test for induced chromatid rearrangement.

In the previous paper, Dr. Stetka explained how sister chromatid exchanges (SCE's) are visualized. Figure 16 shows a normal human peripheral lymphycyte metaphase spread stained to show SCE's which were obtained in our laboratory. Figure 17 shows what can happen if the cell is exposed to a DNA damaging agent.

Figure 16. Human peripheral lymphocyte chromosome stained to show sister chromatid exchanges - control.

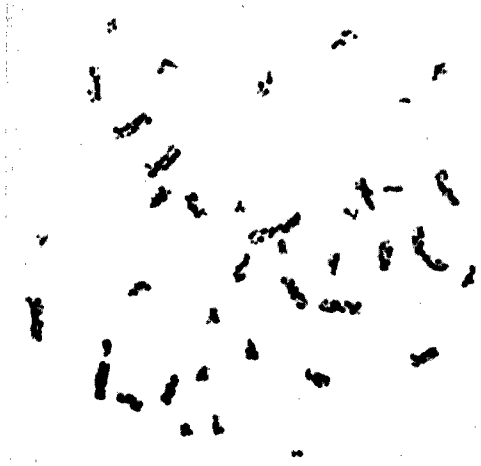

Figure 17. Human peripheral lymphocyte chromosomes stained to show sister chromatid exchanges - exposed.

Figure 18 shows a rat peripheral lymphocyte metaphase and Figure 19 shows a canine peripheral lymphocyte metaphase stained to show SCE's.

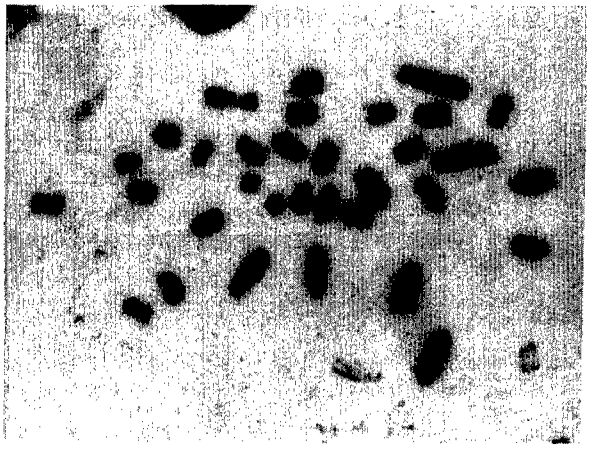

Figure 18. Rat peripheral lymphocyte chromosomes stained to show sister chromatid exchanges.

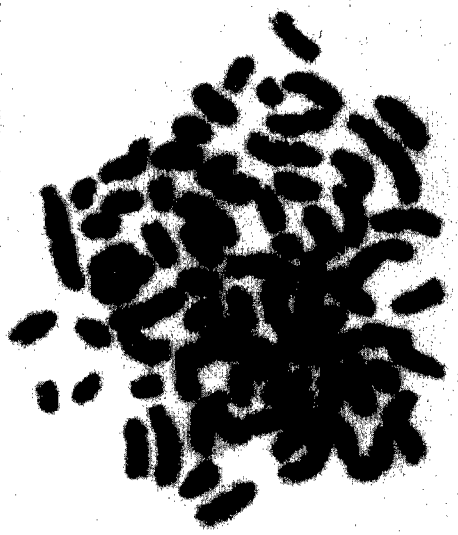

Figure 19. Canine peripheral lymphocyte chromosomes stained to show sister chromatid exchanges.

We had great difficulty in obtaining well-spread canine lymphocyte metaphases and so we have little complete data to show for this test at this time. Figures 20 and 21 show the results of analyses for SCE's of peripheral lymphocytes taken from dogs exposed continuously in vivo by inhalation to decalin and DFM. What results we were able to get are negative for these chemicals. We have done no positive controls as yet for these studies.

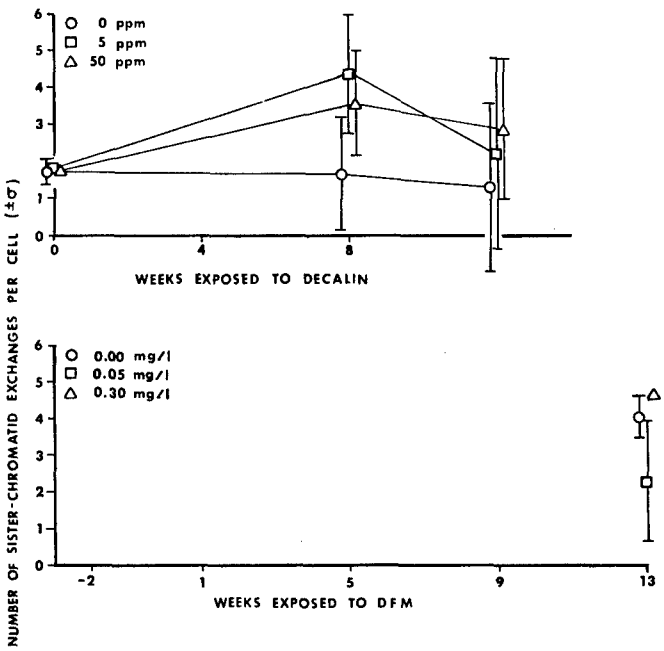

Figure 20. Sister chromatid exchanges induced in canine peripheral lymphocytes taken from dogs exposed in vivo to decalin, ...

Figure 21. ... or to DFM.

Because of the technical problems in working with canine lymphocytes, we have recently turned our full attention to the rat.

We have acquired the techniques of obtaining peripheral blood and bone marrow from rats without killing the animals. We are just completing some early in vitro exposures of these tissues to the known chromosome damaging chemical MMC. We will soon begin repeating and extending work such as Dr. Stetka has done with rabbits, doing chronic in vivo exposures to rats to known positive chemicals and chemicals untested in this way. The rats will be exposed at first by I.P. injection, and marrow cells and peripheral lymphocytes will be analyzed for the

induction of SCE's and micronuclei. If either of the tests appears to be sufficiently sensitive, animals exposed by inhalation will also be tested.

Finally, both tests will be validated by observing the long-term fate of animals exposed to the various chemicals tested, comparing the short-term cytogenetic test results with the ultimate health of the same animals.

PAPER NO. 22

ANIMAL MODELS USED IN GENETIC RISK ESTIMATION

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Washington, D. C.

INTRODUCTION

It is now recognized that among the myriad of chemicals currently in use, a reasonably small number of them possess the ability to alter the genetic material of experimental biological systems. However, it is either undesirable or impractical to cease all production and use of such chemicals. This calls up, then, the process most commonly referred to as risk estimation. There are many factors involved in generating an estimate of potential genetic harm to humans from mutagenic chemicals and much has been written on the subject. In this brief presentation, I shall focus only on a discussion of the nature of human genetic harm and the types of animal model systems that may be employed to generate data to be used in risk estimation.

GENETIC RISK IN HUMANS

While 70 years have passed since Garrod published his treatise, "Inborn Errors of Metabolism", the field of medical genetics has received concentrated effort only in the last 10-15 years. Additionally, often the mode of inheritance is complex and a large number of cases of a particular disease are required before a clear familial pattern can be established. Currently, it is conservatively estimated that about 10 percent of live borns will manifest sometime during their lifetime a variety of serious genetic disorders. In terms of cost, from a composite of several limited studies, it is apparent that at least 15 percent of all pediatric admissions involve disease of clearly genetic origin, or stated differently, one to two times as many health dollars are associated with childhood genetic diseases than all other causes combined.

The above represent the current estimate of the incidence of genetic disease and that is necessary information for use in a risk estimation procedure. But in environmental mutagenesis, our real concern is whether our chemical environment is increasing the genetic disease burden of future generations. That we are dealing with diseases in generations to come brings to focus probably the most difficult aspect of the risk assessment problem. When we make any type of estimation of potential or real events in the future, it is important that when those particular conditions are reached, we have a method to evaluate the accuracy of our predictions. Only a fraction of the genetic diseases are inherited in such a simple way that a new mutation (disease) could be detected in the immediate offspring from exposed individuals. Also, while the total number of different genetic diseases exceeds 4000, at the molecular level there are but a few mechanisms by which mutations arise. Furthermore, we know that not all mutations cause disease; indeed, probably most go undetected. For these and other reasons, it is highly improbable that we will clearly identify a particular chemical as a human mutagen. It must be stressed though that the inability to detect an event is not equivalent to the lack of occurrence of that event. Certainly one of the more important areas of current research is the development of more sensitive methods for human monitoring for mutational events.

LABORATORY ANIMAL SYSTEMS

Before embarking on a search of laboratory systems for suitable models, it is necessary to have a clear statement of the problem.

For genetic risk estimation, this is to obtain a statement of heritable mutations as a function of dose, i.e., mutagenic potency. The DHEW document on mutagenesis further amplifies this definition:

> "The mutagenic potency of a chemical is a function of: (1) the overall absorption, metabolism and excretion of the administered chemical; (2) the transport of the chemical or its metabolites across the membrane of the target germ cell; (3) metabolism within the target cell; (4) the interaction of the (activated) chemical with DNA to form a premutational lesion; and (5) the fixation of the lesion as a stable alteration in the DNA through various forms of repair or replication. The first three factors can be grouped together as the pharmacological disposition of the chemical while the latter two are referred to as its intrinsic mutagenicity."

In other areas of toxicology, especially cancer, animal models for risk assessment generally focus upon a particular organ, that is liver cancer in animals as predictors for liver cancer in humans. However, in genetics we have already noted that there are over 4000 different genetic diseases, and there is not a single organ system which is not affected by one genetic disease or another. To obtain an animal model for every genetic disease would be an insurmountable task. But, since all of these diseases arise from molecular events in the genetic material of the gonads, we have immediately restricted the target of concern. Also, our fundamental knowledge of the universality of genetic material and mechanisms of inheritance enable meaningful application of numerous nonhuman test systems for ascertaining the potential of a chemical to induce mutations in humans. In all multicellular organisms, including humans, two major types of mutation are possible, i.e., chromosomal and gene (point) mutation. For each of these there are several adequate systems that have been successfully employed to detect genetically active chemicals.

For convenience, I have defined two approaches for generating animal data for risk estimations. One is a simple or what I shall refer to as the direct approach in which the pharmacological disposition and the intrinsic mutagenicity are both measured as a single endpoint. This is in contrast to the integrated approach which combines independent biochemical information on gonadal dose with mutational data obtained from a variety of tests.

DIRECT APPROACH

The first application of animal systems for the estimation of human genetic risk was for ionizing radiation (Bear, 1956). In this effort, the data used were from Drosophila and the mouse specific locus test. At that time, there was little recognition of diseases associated with chromosome mutations; indeed, the correct number of human chromosomes was not known. Today, both the specific locus test in the mouse and the recessive lethal test in Drosophila remain as the only proven methods for measuring gene mutations in whole animals. The mouse test, while simple in performance, in that the mutations are easily detected, is time consuming and costly. In over 25 years of its existence, only three laboratories have used it; and a total of only 14 chemicals have been examined (Ehling, 1978). Since tens of thousands of animals are required to detect the activity of any but potent mutagens, it is not likely that its use will spread in the future.

The Drosophila sex-linked recessive lethal test is by far the oldest mutational assay and the same basic protocol has been in use for over 50 years. Because the X-chromosome constitutes about 20 percent of the entire genome, it is considerably more sensitive than the mouse. The relatively high regard for its use by geneticists is based not only on the test itself but also because more is known of the genetics of Drosophila than any other higher eukaryote. However, it is an insect, and in spite of the fact that it possesses a mixed function oxidase system, a direct extrapolation to humans in the absence of pharmacological and physiological considerations is judged to be untenable for chemical mutagens.

Over the past twenty years, much has been learned about the importance of chromosome mutations in genetic disease. Loss or gain of a single chromosome as well as alterations of chromosome structure (deletions or exchanges) are the source of a number of severe genetic diseases. The ability to detect such events by direct microscopic observation has also been significantly advanced. It is now possible, though certainly not yet feasible, to totally automate, with computer detection, the scoring of cells for chromosome alterations. However, the systems that are currently available for use in a direct risk estimation procedure are the heritable translocation tests in mice and Drosophila and the chromosome loss test in Drosophila. The procedures for performing these tests in the mouse are not universally accepted as yet since the data are so limited. In terms of time and sensitivity, direct cytological examination of gonadal tissue from the treated animal is preferred. However, heritability is not ascertained and postmeiotic stages are not sampled in this procedure. The indirect heritable translocation test is designed to overcome these particular problems but is more time consuming since the presumed translocation is detected by reduced fertility of F_1 males and is then subsequently confirmed by cytological methods.

While the above represent current methodology for use in a direct risk estimation procedure, current research efforts to develop new or to modify existing procedures offer promise of reducing the cost and/or improving the sensitivity of whole animal systems. The primary advantage of the direct approach is that a meiotic system is used, i.e., the mutations are heritable. Not only does the heritability have relevance to the concern for future generations but also it provides mutant animals that can be the basis for study regarding the impact of particular mutations on individual and population fitness. This research effort is of special interest in the new biochemical gene mutation assays which measure mutations in enzymes in mice that are comparable to enzymes in humans now used in human population monitoring (Malling and Valcovic, 1978).

Clearly, the main disadvantages are lack of sensitivity in detection and the lack of a measure of dose at the target. In essence, this places the direct approach for genetic risk estimation in a comparable position with carcinogenic risk estimation using the rodent bioassay.

INTEGRATED APPROACH

The process of calculating dose was a relatively easy task for ionizing radiation. With chemicals, a seemingly infinite number of barriers, both physical and chemical, preclude a simple calculation of gonadal dose based only on exposure information. There are, however, two factors which greatly simplify the problem in the area of heritable genetic risk estimation. First, in contrast with other toxicological endpoints such as cancer and birth defects, the molecular target of the mutagenic chemical as well as the specific tissue (gonads) and cell types at risk are known in both laboratory animals and humans. Second, the majority of mutagenic chemicals are alkylating agents or can be metabolically transformed to alkylating agents. Each of these factors is being exploited to obtain more accurate expressions of mutation induction as a function of target dose. As examples, I shall briefly describe two methods that have been employed in this context.

The first employs radioactively labelled mutagen and subsequent isolation of DNA from the target cells to express the dose as a function of alkylations per nucleotide. This, in fact, is the procedure that was used over 20 years ago to indicate that chemical mutagens bind to DNA to form stable alkylated ligands. A key feature of this method is that it can be used in the same system that one uses for the mutational assay, either in vitro or in vivo. This means that it is possible to compare exposure with target dose across a variety of test systems to get an understanding of metabolism of the chemical and also compare target dose with mutational yield to get information about the mutational process and the expression of mutations. Naturally, the utility of such comparisons varies as a function of the understanding of the genetic and physiological differences of the biological systems used. While some research is being done with these techniques, I am not aware of their use for risk calculations. Aaron (1979) recently reported on a project to compare a variety of test systems using alkylations of DNA as a measure of dose. He found a 100-fold variation in the detection index (a ratio of mutation to alkylations) among the in vitro tests employed. The significance of these results is not yet clear; one important question relates to which DNA alkylations are best correlated with mutation.

The second approach combines chemical kinetics, metabolism, and genetic studies. This is best exemplified with the work on ethylene oxide done by Ehrenberg (1974). Reaction rate constants can be determined for a variety of biological nucleophiles, and for structurally simple alkylating agents such as ethylene oxide, it is shown that hemoglobin rate constants are similar to DNA rate constants. Hence, for these chemicals which are rapidly distributed throughout body tissues, gonadal dose can be calculated from alkylation of histidine in hemoglobin. This is important especially since the procedure can be applied to humans who are occupationally exposed. Ehrenberg has combined these biochemical data with ethylene oxide-induced mutation frequencies from a variety of systems and generated a risk estimation for worker exposure to ethylene oxide. This approach seems quite applicable to chemicals that rapidly spread throughout the body and are not metabolically altered at specific sites.

CONCLUSIONS

I have very briefly outlined some of the available procedures for obtaining data from laboratory animals that have a scientific basis for use in the risk estimation procedure. But, what do we know of their use in a real situation? As stated earlier, we do not have a human mutagen against which they can be measured. Furthermore, at the present time we do not have available animal data to determine whether the various methods will yield similar estimates. This information will soon be available for ethylene oxide; studies are in progress using the dominant lethal, heritable translocation and specific locus tests in the mouse. But more needs to be done. The total data on mutagenicity of chemicals are numerous (over 6000 chemicals have been tested in at least one system) but much of the data have been generated in a research setting in individual laboratories. It is important now to coordinate testing efforts - find out where information gaps exist and begin to fill them. We are aware that the current systems are probably far from optimum, especially in the areas of gene mutations, metabolism, low dose effects and, of course, human genetic monitoring. However, we must fully evaluate (or rather calibrate) the systems that are now available in order to compare them with future approaches.

To conclude, existing methodology can be utilized to derive risk estimates for human exposure to chemical mutagens In fact, the current scientific basis is stronger than that which existed for regulatory decisions to limit ionizing radiation exposure decades ago. However, such estimates,

like all extrapolations, consist of certain unavoidable assumptions and undefined levels of uncertainty that can be reduced with future efforts. Some areas of need are: (1) a more accurate estimate of the spontaneous human mutation rate, (2) identification of population subgroups whose genetic constitution leads to heightened response, (3) the development of in vivo mammalian systems that discriminate gene mutations from small deletions, and (4) information on differential sensitivity of mammalian germ cell stages.

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DR. PHALEN (University of California, Irvine): This question is for Dr. Benz. I learned a tremendous amount from the talk and was especially impressed with the appearance of some of the smears. I think the techniques appear superb. But in your curves where you were plotting, for example, the number of micronuclei versus dose and indicating whether there was a positive effect or not, on what criteria is that kind of a decision made?

DR. BENZ (University of California, Irvine): I was essentially looking for at least a doubling of the background levels. That has been accepted as a standard increment for considering a compound to be mutagenic for the Ames test and other procedures. There is some discussion of modifying that approach but that's the basis for my comment.

DR. PHALEN: Were the error bars standard deviations?

DR. BENZ: Yes.

LT. COL. CALCAGNI (OEHL, Brooks Air Force Base): I have a question for Dr. Brusick or Dr. Benz. You described a sister chromatid exchange test basically in terms of screening or use in chemical testing. Has this progressed at all to the point where it would be of any value in human cross sectional or longitudinal epidemiologic studies?

DR. BRUSICK (Litton Bionetics, Inc.): I indicated one pilot study. Actually, there were two pilot studies conducted on a cross sectional basis. The first one originally started with sampling of individuals on chemotherapeutic drugs which are alkylating agents. The methodology was worked out for acquiring the sample and developing the staining techniques on these individuals. In many cases, they are used as positive control groups for other studies. The other is the ongoing study with petrochemical workers which was just initiated, and I think only 30 or 40 people have been looked at thus far. This study includes, in addition to SCEs, some other types of health effect testing related to urine cytology and other things that are going on in conjunction with the SCEs. In this preliminary or pilot study, they are finding significant increases in SCEs in the workers that have been exposed to these chemicals compared to a control group. Another study on hair dyes has been proposed, but I don't know if it's been initiated. This will be conducted in Great Britian and is a longitudinal study. The same individuals would be controls

for themselves before and after hair dyeing. I don't know if the study has been initiated, finished, or what the results are. It had been proposed, and I think it was one that was going to be conducted. So there are studies that are going on this way, and I think it offers an opportunity to do some monitoring. But there are still a lot of questions, a lot of methodology problems and certainly interpretation problems as to what it means if you do see SCE induction. That's really the biggest problem.

DR. BERCZ (Environmental Protection Agency): We have a more recent project going on in Nevada, testing the population of a geographical area exposed to arsenic in their water using sister chromatid exchange as an indicator of effect. There was a recent report in the literature describing elevated SCEs in people who have received arsenic as a medication. We are following a group of people who are exposed to naturally occurring ambient arsenic levels. The study is going on now and the data are now complete. But what I would like to do is address a question to Dr. Brusick. You have eloquently highlighted the batting average of the Ames test in identifying mutagens in chemicals that are known carcinogens. Would you care to comment on what percentage of all the known mutagens are carcinogens? That is a reverse question now. I think there is a gross discrepancy that the audience is not aware of.

DR. BRUSICK: I can give you my opinion, and maybe some of the other panel members would like to voice their opinion as well. There's an inherent bias in the way we look at correlations. We look at correlations starting with chemicals that have proven carcinogenic activity or nonactivity in rodents. And then we test them for genetic activity and develop a comparison and find a certain percentage of correct answers. If you start with a given random sample of chemicals and pass them through an animal and look for a toxicological endpoint, you will screen out those chemicals which are active in vivo. There are probably a certain percentage of these compounds which may potentially have activity in vivo but for one reason or another, instability, inadequate dosage, for whatever reason, they are incapable of expressing an effect in an in vivo situation. Therefore, they are negative. In an in vitro assay system, the same group of chemicals may give a high number of positive responses because you're measuring with very sensitive methodologies. There is a discrepancy in the correlation of carcinogens in known mutagens when compared with the ratio of mutagens in known carcinogens. You will have a higher proportion of mutagens not being carcinogens than you would have misses of carcinogens being mutagens. This is my interpretation of what's going on.

DR. BERCZ: I would like to ask about the use of the mutagen in vitro tests in the evaluation of complex mixtures. All of the propositions that we have heard today were directly related to testing individual pure chemicals. I would like to hear your opinions with regard to the strategy in dealing with highly complex mixtures such as shale oil extracts, tobacco smoke, water extracts, and so on.

DR. BRUSICK: I think that the short-term tests offer a good opportunity to look at complex mixtures because they're amenable to dosing with various complex mixtures; and when you begin to separate or pull apart complex mixtures, the only practical solution for individualized testing on subfractions is with a very rapid, short-term test.

DR. BERCZ: There has been quite a bit of evidence showing up in the various laboratories that we have under contract and have given grants. There are synergistic and inhibitory effects in mixtures that are in interplay and that we, at the present time, cannot totally understand. We have no mechanistic explanation. It appears, for example, that the Ames test is totally worthless for telling which organic mixture of a group is potentially more hazardous.

DR. RAY (Pfizer Pharmaceuticals): Could I comment on that? For years there have been procedures for gross separation of mixtures, particularly of organic natural substances. Rather than condemn an assay for its inability to detect a mutagen in the presence of something that tends to negate the effect, it might be worthwhile to perhaps pull out some initial separations. It may not be feasible on a routine basis, but I think that if you look at the options you have for toxicological assessment, some of the simpler in vitro procedures offer you almost the only way to get cheap and easy answers. You can't go with the whole animals clearly. You can't go with very complex mammalian cell culture studies initially. Perhaps a procedure could evolve whereby some initial fractionation of those mixtures could be made for testing.

DR. BERCZ: Even with the most extensive fractionation, we still find subfractions that, like the whole mixture, are negative and appear to contain inhibitory compounds.

DR. RAY: Inhibitory in what sense?

DR. BERCZ: Inhibitory in the sense the fractionation will yield mutagenic subfractions whether the total mixture and primary subfractions are mutagenic or not.

DR. BRUSICK: Some of those mutagenic subfractions may have been generated by the fractionation treatment. It's been shown quite clearly that when you begin to fractionate and extract materials with various solvents, you can generate mutagens in the process. I think that there is great danger in running separations of mixtures in finding misleading false positives. But, as I said, I think if one wants to test the fractions, short-term tests are one way to do it. The other approach, of course, is to look at fractionating techniques and see whether there are better ways of separating complex mixtures. But it is a problem. The only program that I am intimately aware of or familiar with is one that's being conducted by another group at the IERL Laboratories of EPA in which they are looking at complex mixtures. They are doing chemical analysis first followed by biological testing. They are calculating an anticipated degree of hazard based on certain key components in the chemical analysis and then following that up with biologic testing. Although not a perfect index, there is good correlation between those mixtures calculated to have a high degree of chemical hazard with high mutagenic and other toxic responses. This is again another approach. It doesn't require fractionation. It just requires chemical analysis of a complex mixture, calculation of what you think ought to be the biological response and then running the in vitro assay for correlation.

DR. BENZ: I think, too, what you do depends on exactly what it is you want to know. If you want to know what the effects of individual components are, then you're going to have to fractionate and recombine and conduct a large variety of tests. If what you're really concerned about is an immediate or acute effect and you're not really worried about how it works, then I think testing mixtures is the only way to go because that's what people are actually exposed to. The selection of method depends on what kind of information you really want.

DR. PECK (Merck Institute for Therapeutic Research): I want to direct some comments to Dr. Ray. There are several compounds which are identified as carcinogens which I sort of lump together such as selenium, chloroform, carbon tetrachloride, and phenacetin. These can all produce tumors, most of them in the liver but phenacetin in the kidney. I think these tumors are related primarily to necrosis. The question is, what is the mechanism there? Benzimidazoles will produce nondysjunction in fungi by interfering with microtubules. Is this same type of action occurring in the liver or the kidney so that you get nondysjunction? My question is, while this dysjunction may lead to mutagenic effects, can it lead to carcinogenic effects?

DR. RAY: I don't believe we have a sufficient data base to deal with that in a structured scientific sense. I do think that wherein you have substances which, when administered at high levels over long periods of time, may be carcinogenic but which may not show up to be mutagenic or genotoxic in some of these in vitro screening systems that it's reasonable to think about mechanisms which are involved in the process of necrosis and regeneration. The process of forcing organs with very low mitotic indices into rapid proliferation may have a hormonal effect and effects on mitosis which could result in a carcinogenic state over a long period of time. That's why I prefaced my presentation by saying that I believe short-term tests are valuable in terms of identification of mutagens and direct acting carcinogens or substances which in vivo are converted to electrophilic substances. I doubt sincerely whether the short-term tests would be of value in identifying all substances which will be designated as carcinogens by long-term rodent studies.

DR. PECK: I'm referring only to those chemicals that produce necrosis because this, I think, may be the mechanism.

DR. RAY: If it's a liver damage where you have a regenerative process which follows it, that could be the result of producing mitosis in an organ in which it's a very low phenomenon normally.

DR. PECK: It is my understanding that with nondysjunction in fungi, there are threshold levels with the benzimidazoles.

DR. RAY: There are dose levels at which we do not see microtubule destruction. I am aware that a number of these fungicides do act through that mechanism. But it's very interesting to note, however, that most of these chemicals, if tested in an in vitro cytogenetic system and the mitotic index was measured, you would see a jump in the mitotic index. You're talking about the percentage of those cells in metaphase at the time of examination. Many of these substances that you just mentioned produce a marked increase in the number of cells in that metaphase. That's why I believe that in vitro cytogenetic systems are of value in detecting such events without going to the more elaborate nondysjunction kind of assays.

DR. CAMPBELL (Environmental Protection Agency): I want to ask Dr. Benz or Dr. Brusick to comment on the appropriate statistical test of significance for SCE in vivo tests used in animals. Is the individual animal the basis for a statistical bit and then you compare incidence in groups of animals or do you approach it from the sense of the cell being the unit? You're looking at exchanges per cell and getting an average of

the exchanges per cell but it would seem to me that the cells from an animal in a given exposure to a toxicant would not be independent but that the animals in the group used would be independent in a statistical sense. What is being used as the appropriate statistical test of significance for in vivo tests?

DR. BENZ: The methods are evolving. Initially, there was no statistical treatment of data. It was just looking for a dosage that doubled the number of mutagenic events. Most SCE studies have been done in tissue culture so you're looking at what is supposedly a clone population of all the same cells. So you don't have that kind of problem. Looking at animals or humans, we can almost go to another extreme. Dr. Carano and Dr. Stetka have been conducting SCE studies on humans, and they are finding that there is a normal SCE level for an individual that varies plus or minus 10%. What that really means is that when you are talking about background levels of 8 or 7 SCE's, then 1 more is significant. There is a fairly narrow range for a normal individual. That means that if I test your cells today and again 3 months from now, you'll have the same number of SCE's plus or minus 10%. Between individuals, it's almost that close. There's not that much difference among people unless they are receiving different treatments or have specific disease syndromes. Even looking at people who smoke cigarettes versus those that don't, you're still only talking about counts of 8 versus 10 or something like that. The values are very close in a normal population. So you can't look for anything like a doubling level in a population study. You can lose some possible useful information if you do. I haven't really answered your question because it's an evolving problem.

DR. CAMPBELL: What you just said then raises the question of what becomes biologically or medically significant as opposed to statistical significance. But my question related to the case where if we're wishing to use cells other than you can obtain nondestructively from the same animal in time, that is a case in which you can use the animal as his own control. If you're using groups of animals to compare to one another because of that, then what do you think would be the appropriate statistical test?

DR. BRUSICK: We don't use the animal as a unit. In fact, in some of Dr. Stetka's studies, they did a comparison between sister chromatid exchanges per cell using cells with complete complements of chromosomes versus measuring SCE's per chromosome where you may not even have a complete complement to determine whether or not there is randomness. In other words, does any unit of chromosome have an equal opportunity of undergoing exchange? The comparison was extremely close so that now we

use an analysis based on that sister chromatid exchange per chromosome. The chromosome is really the target, and therefore, that is the statistical unit in measuring.

DR. CAMPBELL: Then you could presumably do a test on one experimental animal versus one control animal?

DR. BRUSICK: The data that I showed you with the rabbits was using, I think, 3 or 4 rabbits at the most. Each animal serves as its own control and you follow it against time. But the number of animals was 2 or 3 or 4.

DR. CAMPBELL: Again, that was the case where the animal was used as its own control. I'm talking about a situation where that cannot be, where we cannot test the animals cells of interest. In this case, I'm talking about lung cells before and after treatment or exposure of the intact animal.

DR. BRUSICK: I don't know how to handle that statistically.

MR. JONMAIRE (Uniroyal, Inc.): Dr. Ray, in view of the increasing awareness of promoter substances and considering what we heard yesterday about in vitro methods of measuring promotion of mutagenesis caused by chemicals or UV radiation, when do you think an in vitro bioassay system will be included in this bank of tests to study the promotion of mutagenesis?

DR. RAY: That's very difficult to answer. One of the proposals is to include an in vitro transformation model in the battery as it now exists. There are efforts to use such models to detect activity of promoters. If we try to go about this intelligently, though, and to have a sufficient experience with a variety of substances before we incorporate it into assays, then it will take considerable time indeed. There are diverse views on the definition of a promoter. I just don't think that the information that we have with in vitro systems at the moment has developed to that point where they would certainly be used routinely. If it's in a research phase, then it's very difficult in my view to project how much of that will have to go on before we have a validated system. The unfortunate thing in mutagenesis work is that we started to use systems in the practical or commercial way before we had the data base, before the test methods were validated. As a consequence, we are now running into problems which we can't readily sort out and a mixed finding or conflicting results appear between systems. We may have encountered these problems anyway but had we had a sufficient idea of how the tests respond over broad classes of chemical substances, I think we'd be in a much better position to interpret what we see.

DR. CONAWAY (Texaco, Inc.): I'd like to ask Dr. Brusick a question regarding the in vivo test where we gave 3-methylcholanthrene to rabbits, and we got a rise in SCE's over a period of time when this material was given at several doses. I'm wondering if you can explain to me what happens in your opinion regarding these cells. We have cells which are possibly mutated in vivo and continue on until they reach their normal lifespan and then they die. They are supposedly replaced by cells which are produced by cell division. Can you explain how these continued high SCE levels occur?

DR. BRUSICK: Probably not to any great satisfaction. This is something that is being studied. I don't think that Dr. Stetka and the people who have done this work really understand the dynamics of this. But there appeared to be some populations with higher levels of SCE's which might indicate that there are altered stem cells and that the stem cells that produce these cells will continue to proliferate and form cells with a higher level of SCE's. That's just conjecture at the moment.

DR. CONAWAY: When you take leukocytes from humans and test them in vitro, is there any indication of selection during the process of culturing these cells or in the addition of mitogens?

DR. BENZ: I would like to speak to your first question. In the process of replicating DNA, it has to be broken. It's just too long and too wound up a molecule to release its replicate. If you try to build a model of DNA replicating, you've got to break it many times to get the new DNA molecules separated. It physically won't work otherwise. It's believed that DNA is broken all the time and undergoes normal repair. Occasionally, it's not repaired; or if the repair isn't quick enough, there might be enough time for another system to produce a sister chromatid exchange. That's why you get a background of sister chromatid exchanges. What might be happening to produce a continued level of higher SCE's is that a certain population of the stem cells are mutated because their repair system is depleted.

DR. MILO (Ohio State University): In answer to your question about modification of the lymphocytes of any given tissue that you put in culture, the answer is yes, you do modify them. In other words, the cell changes. There are many publications in the literature about mixed function aryl hydrocarbon hydroxylase actually being diminished as it goes along in culture. This has also been shown by a number of people on a number of different human tissues. You are going to find those changes once you use tissue culture for long periods of time.

SESSION V

ENVIRONMENTAL STUDIES

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PAPER NO. 23

THE EFFECTS OF AIRBORNE FLUORIDES ON AGRICULTURE AND FORESTRY

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INTRODUCTION

Fluoride (F) is the thirteenth most abundant element in the earth's crust, comprising between 0.065 and 0.78% (Banks and Goldwhite, 1966; Emeleus, 1950). The average soil contains only about 0.020% F, but topsoils in the U.S. have been found to range between 0.002 and 0.164%. Most of this is contributed by particles of biotite, muscovite, horneblends, tourmaline, and other minerals. Phosphatic soils are generally very rich in F (MacIntire et al., 1949). Fluorine is also present in natural waters and appreciable amounts are often added to soils in fertilizers.

The plant-damaging potential of airborne F was first recognized in 1883 by Schroeder and Reuss near a copper smelter (Thomas and Alther, 1966), shortly thereafter by Mayrhofer (1893), Rhode (1895), and Wislicenus (1898) in the vicinity of superphosphate plants, and by Ost (1907) near both glass and fertilizer factories. Fluorine-containing minerals, such as fluorspar (CaF_2), have been used as metallurgical fluxes from at least the end of the 15th century and, mixed with sulfuric acid, for etching glass in the 17th century (Weinstein and McCune, 1970).

The recognition of F as a serious problem to agriculture in the U.S. came with the expansion of the aluminum industry during and shortly after World War II (de Ong, 1946) and with extensive mining and processing of phosphate deposits in Florida and Tennessee (MacIntire et al., 1949). The problem has grown as a result of expanded use of aluminum, coal, and fertilizer.

Among the common air pollutants, F is ranked fifth in importance with respect to the amount of plant damage produced in the U.S. The four pollutants ranked higher are ozone, sulfur dioxide, oxidants other than ozone (peroxyacyl nitrates, nitrogen oxides), and pesticides (Heck et al., 1973). But F is the most phytotoxic of these pollutants, and it may cause injury to susceptible plant species at atmospheric concentrations 10 to 1000 times lower than the others (less than 1 ppb or ca. 0.8 $\mu gF \cdot m^{-3}$). Fluorine has one other important characteristic - it accumulates in the plant.

In recent years, several reviews have been published on the effects of F on plants (Chang, 1975; Groth, 1975; McCune, 1976; McCune and Weinstein, 1971; Weinstein 1977; Weinstein and McCune, 1970; Weinstein and McCune, 1971; National Academy of Sciences, 1971) and much of this review has been taken from one of them (Weinstein, 1977).

THE NATURE AND SOURCES OF FLUORIDE

Gaseous and particulate F compounds are emitted to the atmosphere from a number of industrial processes by which they are manufactured, used as catalysts of fluxes, or are present as contaminants in one of the raw materials. These industrial applications include production of hydrofluoric acid (HF); use of HF as an alkylating agent in gasoline production; various industrial uses of elemental fluorine; manufacture of phosphate fertilizers, brick, ceramics, glass, enamel frit, beryllium; smelting of aluminum, steel and nonferrous ores; and combustion of coal (Thomas and Alther, 1966).

The predominant gaseous forms of F emitted from these processes are HF and silicon tetrafluoride (SiF_4). Nontoxic fluorocarbons are also evolved in the manufacture and use of refrigerants and spray propellants and as tetrafluoromethane (CF_4) from aluminum smelting under some conditions (Schmitt, 1963). The extremely high reactivity of elemental F precludes its existence in the atmosphere for more than transitory periods. No definitive determinations have been published of the various particulate forms of F emitted from industrial processes, but cryolite (Na_3AlF_6) and aluminum fluoride (AlF_3) are evolved from aluminum smelting and calcium fluoride (CaF_2) and fluorapatite ($CaF_2 \cdot 3Ca_3P_2O_8$) from phosphate manufacture, among other compounds.

EFFECTS OF F ON PLANTS

EXTERNAL SYMPTOMS

Foliar symptoms are the best documented response of plants to F, but other organs of the plant, including flowers and fruits, can also exhibit symptoms. Unfortunately, although these symptoms are characteristic of F, they are often not specific (Brandt and Heck, 1968; National Academy of Sciences, 1971; Treshow and Pack, 1970). Most of the following description is taken from Weinstein and MuCune (1971).

On most monocotyledonous (narrow-leaved) plants, the initial symptom is the development of chlorosis at the tips and margins of elongating leaves, usually followed by necrosis. Gladiolus leaves exhibit tip necrosis which progresses more or less evenly downward with low concentrations of F and is distributed more irregularly over the leaf at higher concentrations. The color of the necrotic area ranges from ivory or light tan through various shades of brown, but it is characteristically light in color with a dark brown margin. This line of demarcation is very useful in identifying multiple exposures. The necrotic area is sharply delineated from the healthy portion of the leaf blade by a narrow band of chlorotic tissue sometimes streaked with red. Flower bracts of gladiolus are also very sensitive and will exhibit necrosis at the tips and along the margins. The symptoms produced on corn, sorghum, and some other grasses begin as scattered chlorotic flecks at the tips and upper margins of middle-aged leaves. As the symptoms progress, the flecking becomes more intense and extends downward, especially along the margins. The amount of chlorosis diminishes from the tip downward and from the margins toward the midrib. At high F concentrations, there is less chlorotic flecking and a greater tendency for tip, marginal, and interveinal necrosis, or a transverse necrotic band at the arch of the leaf. On sorghum foliage, necrosis is often accompanied by a red coloration.

Ponderosa pine needles first exhibit a lightening in color which turns light brown to reddish-brown at the tip and progresses basipetally. The discoloration is often accompanied by narrow dark zonate areas which may be the result of intermittent exposures to F at different times. Dark bands may also occur at the interface of necrotic and healthy tissues. Needles within a fascicle tend to be marked to the same extent.

The initial symptom of F on leaves of dicotyledonous (broad-leaved) plants is usually chlorosis of the leaf tip, which later extends downward along the margins and inward toward the midrib.

This chlorosis becomes more intense and extensive with prolonged exposure until the midrib and some veins appear as a green arborescent figure on a chlorotic background. With continued exposure, the chlorotic leaf tip may become necrotic and fall out, leaving the leaf notched. Longitudinal expansion of the leaf may be inhibited resulting in a savoyed and/or cupped appearance. If no further injurious exposures occur, subsequent growth will be normal. At higher atmospheric concentratins or upon prolonged exposures at lower concentrations, the chlorotic areas may become necrotic with symptoms progressing from the tip downward along the margins and inward toward the midrib. On plants such as Chinese apricot and Italian prune, the initial symptom is usually wilting and then desiccation of tissue at the tip or along the margins of the leaf which then develops into light to dark brown necrotic areas. These lesions become brittle and frequently fall from the leaf giving it a tattered appearance.

It is possible that the entire leaf area may become necrotic through single or multiple exposures and the leaves and developing fruits may abscind. But in some instances, foliar abscission may occur with relatively small amounts of necrosis or in the absence of foliar lesions.

Both lichens and mosses are susceptible to injury by fluoride, but the algal symbiont appears to be more susceptible than the fungal symbiont in lichens (Nash, 1971; Sidhu and Roberts, 1976).

An unusual disorder induced by F is "suture red spot" or "soft suture" of peach fruits characterized by premature ripening of the flesh on one or both sides of the suture toward the stylar (blossom) end of the fruit. The ripening of this tissue considerably precedes that of the normal fruit and is often accompanied by splitting of the flesh along the suture. At harvest, the affected areas are soft and often decomposing.

Recent studies* have shown that the fruit is most susceptible after pit-hardening and that the symptoms can be induced after long-term, continuous exposures to relatively low concentrations of HF.

Necrotic patches or dispersed lesions on several species of tree fruits (Bolay et al., 1971) and a condition described as "snub nose" or "shrivel tip" of sweet cherry (Treshow and Pack, 1970) have been reported after exposure to high atmospheric F concentrations.

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*MacLean, D. C., L. H. Weinstein, D. C. McCune, and R. E. Schneider, unpublished.

MIMICKING SYMPTOMS

There is often a degreee of confusion and uncertainty in the evaluation of F induced injury in the field. This is due to the fact that most vegetation will display symptoms of one form or another as a result of a number of environmental and pathologic conditions (Weinstein, 1961; Weinstein and McCune, 1970). Foremost among the agents or factors that mimic F-induced injury are other air pollutants such as sulfur dioxide, ozone, chloride, and others, and the degree to which they resemble F injury will depend largely upon the plant species and the pollutant dose. Symptoms of deficiencies of manganese, zinc, magnesium, calcium, potassium, boron, and sometimes iron, may resemble F injury. Confusion may also result from an excess of fertilizer or of individual nutrient elements; heat, cold, or drought; herbicides and other pesticides; infestation of insects or other pests, including mites, scale insects, leaf or needle miners, borers, etc.; diseases caused by viruses, mycoplasma, bacteria, and fungi; genetic mottle; and natural senescence (Treshow and Pack, 1970).

Abnormal suture diseases of peach, which have symptoms closely resembling those of suture red spot have been ascribed to herbicides of the phenoxyacetic acid type (Benson, 1959), physiologic stress (Dorsey and McMunn, 1944), viruses (Richards and Cochran, 1957), and to unknown etiology (Hildebrand, 1943).

GROWTH, YIELD, AND REPRODUCTION

Most of our information on the parameters of exposure that relate F to specific plant responses is derived from experimental fumigations in which plants have been exposed to known concentrations of gaseous F usually as HF, for different periods of time. Unfortunately, much of that information is limited, for technical and other reasons, by: (1) relatively short exposure periods, (2) a limited range of species and varieties of plants tested, (3) an elementary knowledge of the influence of climatic and edaphic factors on plant growth and reproduction, (4) the difficulties inherent in comparing results between laboratories because of differences in equipment, methods of analysis, etc., and (5) only a rudimentary knowledge of the spatial and temporal characteristics of emissions from industrial sources. Furthermore, the credibility of these experiments in relation to problems of industrial fluoride emission is reduced by the difficulties in reproducing the temporal distribution of fluoride or variations in climatic and edaphic conditions found in the field. On the other hand, many of the field investigations that have been reported suffer from questionable procedures in selection of experimental materials, subjective bias in evaluation of results, and lack of appropriate air monitoring.

As is the case with foliar symptoms, the occurrence of effects will depend on differences in species and varieties, the concentration of F, length of exposure, and climatic and edaphic factors. These effects can best be discussed with relation to the intended use of the plant. For example, if a pine tree grown for use as lumber exhibits an amount of needle tip burn insufficient to affect the growth of the tree, there has been no economic loss. The same pine growing as an ornamental specimen in a park or at a private residence would have suffered aesthetically and thereby its worth would have been reduced. Gladoli with F-induced markings on the leaves and flower bracts are unsalable, but if they are being grown for corm production, the foliar effects may have been insufficient to reduce corm yield or quality.

Growth can be defined as an irreversible increase in mass, or as any dimensional change including length, area, or volume. A number of these changes that have been associated with atmospheric fluoride include a decrease in tree growth, biomass of leaves, stem, and roots, and numbers and quality of flowers and fruits (Bunce, 1978; MacLean et al., 1977; Pack and Sulzbach, 1976; Weinstein, 1977). These effects represent a broad range of atmospheric F concentrations, durations of exposure, times of exposure in relation to plant development, and external factors, and they also represent field and chamber studies. Because of the range of conditions and species of plants investigated, it is not surprising to find that there are also many reports where F exposures have not had significant effects on growth and development. A considerable amount of evidence has been published indicating that exposure to F may stimulate growth as in Douglas fir needles and shoots (Treshow et al., 1967); total linear growth of citrus, roses (Brewer et al., 1967; Brewer et al., 1960a; Brewer et al., 1960b) and tomato (MacLean et al., 1977); seed head production in sorghum (Hitchcock et al., 1963); and growth of alfalfa (Hitchcock et al., 1971; Treshow and Harner, 1968) and bean (Treshow and Harner, 1968). It would be naive to accept all of these responses as evidence for the beneficial effects of fluoride to plants since they can be better ascribed to a more twiggy growth in rose and citrus or taller but not necessarily sturdier bean and tomato plants. Perhaps under some limited set of conditions, there is a beneficial growth enhancement.

Pack and Sulzbach (1976) have conducted the most extensive investigations on the response of plant fruiting to HF exposure. The most frequent fruiting response found was a reduction in seed production, especially in those crops grown for seed, such as pea, sweet corn, soybean, sorghum, oat, and wheat. Flowering was inhibited in both corn and pepper. Reduced seed production was ascribed to the effects of F on

pollen germination or pollen tube growth as reported by these authors earlier (Sulzbach and Pack, 1972), for tomato and cucumber, and by Facteau et al. (1973) for sweet cherry.

A less subtle effect of F on fruiting is the direct injury reported on the fruit of peach, apricot, pear and cherry by high concentrations of airborne F (Benson, 1959; Bolay et al., 1971; Treshow and Pack, 1970).

ACCUMULATION OF F AND PLANT RESPONSE

F is a natural constituent of the atmosphere because it is found in all soils and natural waters. In some Tennessee soils (MacIntire et al., 1949), concentrations may range from trace amounts to 8300 ppm, and up to 2800 ppm in water.

One effect that is peculiar to F is its accumulation in plants from both the atmosphere and soils. This accumulation of F has a significance that transcends its effects on plant growth and yield (Applegate and Adams, 1960). The plant becomes the vehicle for the concentration and transfer of F to herbivores and the potential problems of dental or skeletal fluorosis associated with F ingestion of vegetation that may otherwise appear normal.

The major site of F accumulation in the plant is the leaf and it is taken up more readily than SO_2, O_3, NO_2, and NO (Bennett and Hill, 1973). Once accumulated by a leaf, it is generally assumed that there is little subsequent translocation to other parts of the plant (Benedict et al., 1965; Jacobson et al., 1966) although some investigators have reported otherwise (Garber, 1962; Keller, 1974).

Gaseous forms of F enter the plant mainly through the stomata of the leaf and perhaps through lenticels on twigs. Some penetration of soluble particulate forms of F undoubtedly occurs through the cuticle and epidermis. Fluoride enters the leaf, dissolves in plant liquids, and moves via the transpirational stream toward the tips and margins of leaves where it accumulates (Jacobson et al., 1966; Rommel, 1941). Because F can be removed from some leaves by repeated mild washing, transport of F from the interior to the surface of the leaf must occur (Jacobson et al., 1966; Laurence, 1978). Thus, neither the distribution nor the content of F in the leaf is constant under the influence of normal weathering conditions.

Although the leaf is the major organ of accumulation by plants, other parts of the plant also may contain measurable amounts of F, especially the roots (Leone et al., 1956). Fruits are generally low in F (Treshow and Pack, 1970) but large amounts can accumulate after exposure to high concentrations of F (Bolay et al., 1971; Garber, 1967). Accumulation and distribution of F in field corn grown in a polluted area was recently reported by Kronberger and Halbwachs (1978). Large differences were found in the F content of organs with development of the plant, but seeds remained low throughout the growing period.

The "normal" F content of leaves has been the subject of considerable disagreement, but it generally ranges from 2 to 20 ppm on a dry weight basis (Garber, 1967a, 1967b; Garber et al., 1967). It will vary with the species (and even the variety) of plant, the age of the leaf, characteristics of the soil, use of fertilizer, irrigation, etc. This "background" F is derived mostly from the soil which normally contains from 20-500 ppm although small amounts of F in the atmosphere probably contribute to the background. A survey of the F concentrations of 107 samples of alfalfa from many areas of the U.S. assumed to be free of industrial pollution ranged from 0.8 to 36.5 ppm, with a mean of 3.6 (Suttie, 1969). Some plants, notably the Theaceae (teas, camellias, etc.), are efficient accumulators of environmental F and usually contain from 50 to several hundred parts per million (Zimmerman and Hitchcock, 1956; Zimmerman et al., 1957).

Experimental evidence is fairly conclusive that the amount of F normally accumulated from the soil is small and there is little relationshp between the concentration of F in the soil and that of the plant (Adams, 1956; Garber, 1966; Garber et al., 1967; Hansen et al., 1958; Jacobson, 1966; MacIntire et al., 1949; Prince et al., 1949). One exception may be that plants grown in more acidic soils tend to have a somewhat higher F content. There is also the possibility that long-term exposure of some soils to acidic precipitation may increase the solubility of many elements such as heavy metals (Barrons, 1966), perhaps including an increase in the background F content of plants grown on these soils (Daines et al., 1967). Application of lime reduced F uptake from the soil and this practice would lessen any concern of acidic precipitation in agricultural soils (Prince, 1949).

The deposition of airborne F on soil has been reported to have little or no effect on the F content of the plant (Israel, 1974; McClenahen, 1976; Oelschlager, 1971). Although others agree with this conclusion (Weinstein, 1977), it is certainly not a unanimous one. The presence of significant amounts of "available" F was reported in soil litter but uptake by plants was not measured (Sidhu and Roberts, 1976). In most studies,

soil amendments of relatively insoluble forms of F, including fluorapatite, calcium fluoride, cryolite, or slags had no important effects on F uptake or injury in vegetation even when added at high rates of application. Soluble F salts increase F uptake by vegetation but the amount of accumulation depended upon the amount added, the soil type, and the crop (Daines et al., 1952; Garber, 1968; Hitchcock et al., 1971; Prince, 1949).

A general conclusion of most soil studies is that F that might be added to soils as a contaminant in fertilizer would have little or no effect on the F content of the crop (Oelschlager, 1971). But Bovay, Bolay, and their coworkers (Bolay et al., 1971; Bovay, 1969; Bovay et al., 1969a, 1969b), have reported remarkable increases in the accumulation of F from soils treated with fertilizers containing potassium fluoroborate (KBF_4).

Most of the information on F accumulation in plants has been determined for forage crops. The aggregate of these data suggests that the concentration of F is proportional to the dose, i.e., to the product of the concentration of gaseous F (usually HF) and the duration of exposure (McCune and Hitchcock, 1970). The relationship can be expressed by the dose-rate equation: $\Delta F = KCT$ where Δ represents the F concentration in the tissue after correction for the background concentration. C is the mean concentration of atmospheric F. T is the exposure time in days, and K is the accumulation coefficient (ppm F $\mu g^{-1} m^3$ day^{-1}). But MacLean et al. (1969) have shown that F accumulation in forage is not the same for equivalent doses. Rather, the pollutant concentration is more important than the duration of exposure.

Fluorine accumulation depends upon many factors, among which are the form of airborne F, concentration of F, duration and frequency of exposure, light intensity, relative humidity, air and soil temperatures, soil moisture, nutrition, and precipitation, species and stage of development of the plant (Figure 1). Accumulation includes uptake by the plant and penetration into the leaf through stomata, the cuticle, or other avenues of entry. Distribution includes both the F that has penetrated the plant and that on the exterior surfaces. Elimination includes F lost from the foliage through leaching, other types of weathering, by foliar abscission, and perhaps, volatilization (Hitchcock et al., 1964; Kanbe, 1970; Weinstein, 1969).

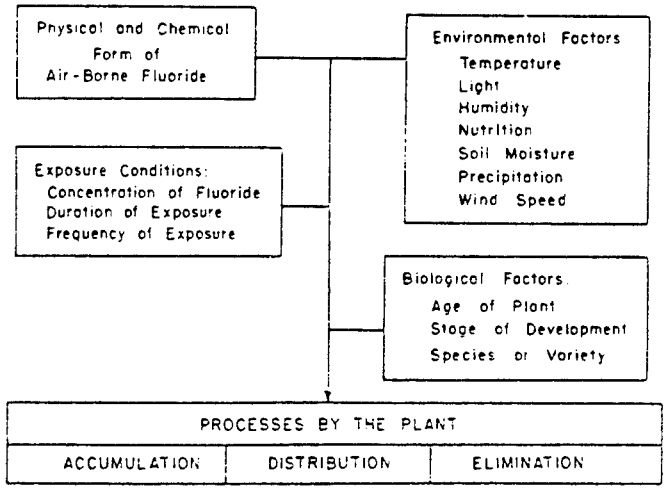

Figure 1. Some factors that determine the action and fate of F in plants.

Accumulation coefficients (ppm F $\mu g^{-1} m^3$ day^{-1}) of about 2 to 4 for alfalfa and about 1 for orchard grass were found for fumigation experiments conducted in the field over many years (Hitchcock et al., 1955; McCune and Hitchcock, 1970). The concentration of F in alfalfa decreased by 50% during a postexposure period of 8 to 22 days which represented losses through weathering and foliar abscission. Accumulation coefficients of about 4 have been reported for continuous exposure of a Timothy-red clover mixture, but for intermittent exposures at the same dose, the coefficients were about one-half (MacLean and Schneider, 1973). Alfalfa and orchard grass exposed to atmospheric F in the field had a mean coefficient of 3.8 ± 0.8 ppm $\mu g^{-1} m^3$day (Israel, 1974). This value is remarkably close to those reported for alfalfa (Guderian, 1969; Hitchcock et al., 1971; McCune and Hitchcock, 1970) and other forage crops (Benedict et al., 1964; Benedict et al, 1965; Guderian, 1969; MacLean and Schneider, 1973) but higher than the values reported for orchard grass (Benedict et al., 1964; Benedict et al., 1965; Guderian, 1969; Hitchcock et al., 1971; McCune and Hitchcock, 1970). Other investigations(Davison and Blakemore, 1976; Less et al., 1975) found that equilibration between airborne and grass F was rapid and therefore independent of time.

Plants exhibit a broad range of tolerances to foliar injury by accumulated F. Susceptible cultivars of gladiolus will develop necrosis when the tissue concentration exceeds 20 ppm, whereas cotton may show no evidence of injury with more than 4000 ppm in the foliar tissues (Jacobson et al., 1966).

Although there is no clear relationship between relative susceptibility and F accumulation within a species, there may be between different species, with the more resistant species tending to accumulate more F from a given dose than susceptible ones, although there are exceptions (Hitchcock et al., 1963).

FACTORS AFFECTING PLANT RESPONSE TO AIRBORNE F

The conditions that determine the accumulation of F in plants also control the response of the plant to F and they may be grouped as characteristics of the environment, the pollutant, and the biologic receptor.

ENVIRONMENTAL FACTORS

Although the atmospheric concentration of F, the duration and frequency of exposure, age or stage of development of the plant, and species and variety are the major factors which determine plant susceptibility, environmental conditions also exert important effects on plant response. These conditions may be classed as climatic, such as wind speed, temperature, humidity, and light, and edaphic, such as soil moisture and mineral nutrition (Figure 1). Little is known of the effects of light intensity and quality, and length of the photoperiod on the response of plants to airborne F and the effects of light itself are ambiguous.

Plants fumigated in darkness are injured to about the same (Adams et al., 1957) or to a lesser degree (Daines et al., 1952) than plants exposed in the light. Shaded plants have been reported as more (Hitchcock et al., 1964) or less (Wiebe and Poovaiah, 1973) susceptible than those in direct sunlight. Using a F susceptible strain of Jerusalem cherry, different light-dark sequences were found to have a marked effect on susceptibility to HF (MacLean et al., 1978).

An increased temperature from 16 C to 21 C resulted in a sharp increase in the degree of tip necrosis in gladiolus, but at 26 C there was no further injury (MacLean and Schneider, 1971). The severity of foliar injury was increased in soybean when high temperatures were imposed after fumigation with F (Wiebe and Poovaiah, 1973). Phytotoxicity was also found to be greater with increasing relative humidities (Daines et al., 1952; Maclean et al., 1973).

Plants grown under conditions of water deficit are reportedly less susceptible to F injury than those receiving adequate water (Applegate and Adams, 1960; Benedict and Breen, 1955; Daines et al., 1952; Zimmerman and Hitchcock, 1956) but in soybean this occurred only when the water stress was imposed before

or during HF fumigation; when the stress was imposed after fumigation, foliar injury was accentuated markedly (Wiebe and Poovaiah, 1973).

As was the case for F accumulation, the relationship between the nutritional status of the plant and susceptibility to F injury is uncertain. Low nutrient levels of Ca, K, P, Mg, and N have been reported to enhance (Adams and Sulzbach, 1961; MacLean et al., 1976; MacLean et al., 1969; McCune and Hitchcock, 1970; Pack, 1966) and low nutrient levels of N, Ca, and P to reduce (Brennan et al., 1950; McCune and Hitchcock, 1970) foliar injury even though the atmospheric concentration of F was unchanged.

Pollutants themselves must also be regarded as environmental factors because phytotoxic air pollutants rarely, if ever, occur alone under ambient conditions. Hydrogen fluoride or fluorosilicic acid in combination with hydrocarbons produced less foliar injury in sorghum and gladiolus than did F alone (Hitchcock et al., 1971; Hitchcock et al., 1962). No synergistic or antagonistic effects between HF and SO_2 were found for several species of plants (Hitchcock et al., 1962) or for citrus (Matsushima and Brewer, 1972), but fumigation of barley and corn plants with relatively low concentrations of HF and SO_2 resulted in an enhancement of foliar lesions which was greater than the sum of the effects of the two pollutants alone (Mandl et al., 1975). The presence of SO_2 was also found to reduce F accumulation in corn (Mandl et al., 1975) and in other species*. The mixture also reduced sulfur accumulation and increased diffusive resistance in alfalfa leaves, decreased foliar injury in gladiolus and sunflower, reduced F accumulation in gladiolus and ryegrass, and reduced yield in sweet corn. The presence of photochemical oxidants has also been shown to reduce F accumulation in alfalfa. Whether an interactive effect occurs depends not only on the pollutant mixture, the concentrations of the components and the plant species, but also on the effect being measured. For example, there were greater-than-additive and less-than-additive effects on sweet corn depending on whether the effect measured was foliar lesions or F accumulation.

THE POLLUTANT AND EXPOSURE CONDITIONS

The phytotoxicity of airborne F is influenced by its physical and chemical characteristics. HF, SiF_4, and F_2 are the most phytotoxic gaseous forms, but only HF has been studied extensively under experimental conditions.

*Unpublished results of J. S. Jacobson and R. H. Mandl of the Boyce Thompson Institute.

Fluoride-containing particulate compounds are generally much less toxic than the gaseous forms and their toxicity is related to solubility. The low phytotoxicity of cryolite (Na_3AlF_6) was confirmed by McCune et al. (1977) but the effect of cryolite with respect to both foliar injury and F accumulation depended upon the relative humidity of the atmosphere and the presence or absence of free water on the foliar surfaces. Injury from both HF and cryolite increased with increasing relative humidity and in the presence of free water.

The exact nature of the relationship between degree of effect and the concentration of airborne F or duration of exposure is not known. The simplest assumption is that the degree of injury has the same relation to concentration and time as does F accumulation.

In general, for the threshold for some effect, a hyperbolic curve would describe the relationship between concentration and exposure, where one asymptote would be called the "threshold concentration" and the other asymptote the "minimum time" required for any concentration to produce an effect (Figure 2) (Weinstein and McCune, 1970). Both these asymptotic values depend upon many factors. For example, Tm, the minimum time to produce an effect, or the presentation time, would reflect the rate of diffusion from the atmosphere to the leaf surface and the penetration from the leaf surface to the interior cells. As such, it would be a boundary condition determined mainly by physical-chemical considerations. From a practical standpoint, the value would be hard to describe experimentally because the concentration of F required would be extremely high and the time short. On the other hand, the threshold concentration, Ct, is of great significance since its determination would allow one to specify air quality objectives in terms of concentrations that will produce no effect. The application of results from experimental fumigations to field problems is limited, because constant concentrations over long durations are not encountered in the field, and the effects of recurrent fumigations have not been studied extensively. Adams and Emerson (1961) concluded that the sequence of exposures may outweigh in importance the actual level of fluoride used within a range of concentrations, and MacLean et al. (1969) and MacLean and Schneider (1973) found that recurrence of exposure was as important as concentration and duration.

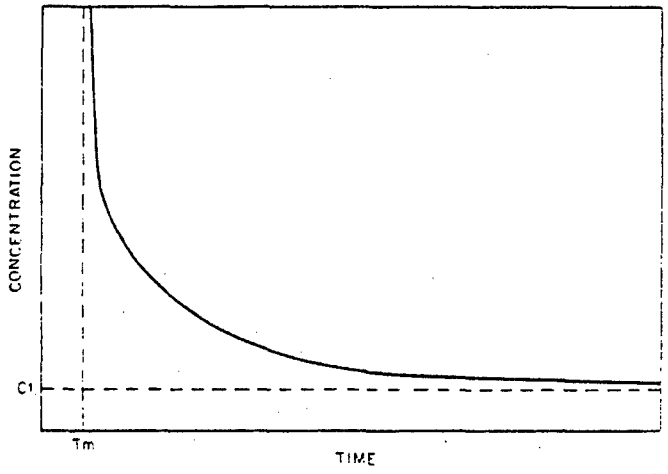

Figure 2. Threshold exposure to produce a given effect. Asymptotic values for concentration (Ct) and time (Tm) are indicated by dashed lines.

The line shown in Figure 2 might be taken as a typical example of one which describes any single effect, such as chlorosis, an effect on photosynthesis, or an effect on growth. A series of curves can also be constructed which describe thresholds for several effects such as those on growth and yield, visible injury, or metabolism (Figure 3), but as was the case for Figure 2, the shape of each curve will change according to all the variables which control and modify the effect of F on the plant (Weinstein and McCune, 1970). Thus, as the exposure to F increases, different kinds of effects occur and the magnitude of the effect increases.

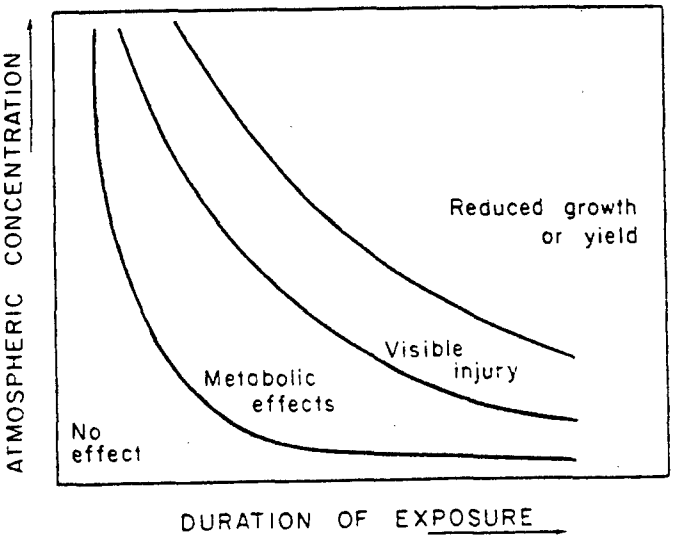

Figure 3. Dose-response of atmospheric F and effects produced.

BIOLOGIC FACTORS

The biologic factors that modify the susceptibility of plants to F may be classed as ontogenetic and genetic. The former category would include the stages of development of the plant, and the latter category, differences between species and cultivars, and the phenotypic variants that occur in natural populations of many species.

Stages of Development

Stage of development of the plant may refer to the age of the plant, i.e., seedling or mature plant; a period in the life cycle of the plant, such as flowering; or a stage in the life cycle of an organ, such as a leaf. Flowering is truly a stage of development if one considers annual or biennial plants, but no perennial plants, which repeat the cycle each year. The development of an organ of the plant, such as a leaf, is directly related to the life cycle of the parent plant.

There have been a number of published reports on the effects of F on the yield of plants grown in chambers or greenhouses. Reduced fruit yields have been reported for citrus (Brewer et al., 1960b) and a number of other plant species (Pack, 1966; Pack and Sulzbach, 1976). Studies of crops under field conditions perhaps provide more realistic data. Exposure of sorghum to F during the period of tassel-shooting and anthesis reduced the yield of seeds and above-ground portions of the plant; the exposures before and after this short period of development were without effect (Hitchcock et al., 1963). The stage of development of the gladiolus plant at the time of F exposure is as important for subsequent corm yield (Hitchcock et al., 1962) as was tassel-shooting and anthesis for seed production in sorghum. Thus, a gladiolus plant fumigated at an early stage of development may exhibit the amount of foliar necrosis required to reduce corm yield, but subsequent foliage will not be injured and the percentage of the total leaf mass that is necrotic would be less by the time corm production is initiated. Exposure at a later stage of development or continued exposures during growth might result in destruction in leaf mass during the period of corm production sufficient to reduce yield.

MacLean et al. (1977) found that field-grown beans exposed to a low concentration of HF for 43 days had reduced number and weight of marketable bean fruits of about 20 and 25%, respectively. There were no effects on the number and weight of tomato fruits grown in the same plots and exposed for 93 days. These results emphasize two important points. First, the

occurrence of the flowering period may be of prime importance in evaluating the potential impact of fluoride on yield. Second, there is only limited value in the use of foliar lesions as the major determinant in ranking plants for susceptibility.

For reasons that are best explained metabolically, the immature leaf is almost always the most susceptible tissue, as has been clearly shown for ponderosa pine (Adams et al., 1956) and many other conifers (Treshow and Pack, 1970), where elongating needles are highly susceptible and mature needles are very resistant. Unfortunately, this clear distinction is blurred by the latency of some F effects, and it is not unusual to observe the expression of necrosis on fully elongated pine needles where they have accumulated enough F to be injured when environmental stresses, such as high temperatures and drought, occur later in the season.

Species and Cultivars

As is the case for all air pollutants, plants exhibit a broad range of tolerances to F. Fluoride susceptibility is not restricted to species of higher plants but is found among the bryophytes and epiphytic lichens as well (Comeau and LeBlanc, 1972; LeBlanc et al., 1972; Nash, 1971). Among the higher plants, the great differences in susceptibility found between species are exemplified by such highly tolerant and diverse species as camellia, cotton, cabbage, juniper, Chinese elm, and balsam popular, and such highly susceptible ones as Oregon grape, goatweed, blueberry, gladiolus, Chinese apricot, Italian prune, and young pine needles. This differential response is found to a lesser degree among cultivars of gladiolus (Daines et al., 1952; Hitchcock et al., 1962), corn (Hitchcock et al., 1964), sorghum (Hitchcock et al., 1963), citrus (Brewer et al., 1960a), and rose (Brewer et al., 1967). Great differences in response to F have also been shown by individual variants among a natural population, as is commonly seen among conifers. Populations of some grasses native to soils with a high F content appear to be much more tolerant than nonindigenous populations (Cooke and Johnson, 1978). The diversity in response of plants to F suggests the existence of a threshold for injury for each species that is controlled by genetic factors, but can also be influenced by external factors. These differences can be measured objectively by physiological methods and are contrary to the simplistic views of Carlson et al. (1979).

The criteria used to establish differences in susceptibility between species and cultivars have been based traditionally upon either the amount of injury produced by a given dose of F (Benedict and Breen, 1955; Daines et al., 1952; Guderian et al., 1969; Zimmerman and Hitchcock, 1956); the dose

required to reach the threshold for foliar injury (Adams et al., 1957); the amount of fluoride present in injured leaves (Bolay and Bovay, 1965); or the amount of F accumulated that could be hazardous to livestock (McCune et al., 1974). In the latter case, a forage crop such as alfalfa, which will accumulate from 2 to 4 times as much F as orchard grass from a given dose of gaseous F, would be the more susceptible crop. The greatest confusion can occur when a relatively narrow groups of plants, such as legumes, grains, and grasses are ranked according to their relative susceptibilities (e.g., 50). The most "susceptible" plants in these groups may be classed as much more tolerant on larger, more objective lists. The different means of estimating susceptibility account at least in part for the anomalous positions of several species in the many tabulations that have been published (Daines et al., 1952; National Academy of Sciences, 1971; Guderian et al., 1969; Thomas and Alther, 1966; Treshow and Pack, 1970; Wolting, 1975; Zimmerman and Hitchcock, 1957) although the lists are similar in many respects. No attempt has been made to classify plants according to effects on fruit production or timber volume, condition of foliage (as in spinach, lettuce, etc.), or any other objective criteria related to the intended use of the plant. By these criteria, bean would be more susceptible to F than tomato (MacLean et al., 1976) but the reverse would be true when the basis used is foliar susceptibility. A compilation of relative susceptibilities for North American species of plants based mainly upon the production of foliar injury is given in Table 1. As many species as possible have been ranked according to observed susceptilities in the field, but often the observations were made in such widely differing areas as Florida, the Ohio Valley, the Northwest, and eastern and western Canada. Other entries are based upon controlled fumigation studies in which the author was a participant or observer. For these reasons, the list should be used only as a guide to the species catalogued. Species with characteristics that make them well-suited for planting on the sites of F emitting industries are indicated.

TABLE 1. RELATIVE SUSCEPTIBILITIES OF PLANTS TO ATMOSPHERIC FLUORIDE

Flowering Shrubs and Ornamental Plants

Susceptible	Intermediate	Tolerant
Crocus	Aster	*Barberry, Japanese
Laurel, Sheep	Azalea	Bearberry (Kinnikinick)
Oregon grape	Barberry, Warty	Bridalwreath
Tulip	Barberry, Wintergreen	*Bunchberry
	Boxwood, Common	Camellia
	Cherry, Flowering	Chrysanthemum
	Lilac	Cotoneaster
	Narcissus	Dahlia
	Peony	*Deutzia, Slender
		*Deutzia, Fuzzy

TABLE 1. RELATIVE SUSCEPTIBILITIES OF PLANTS TO ATMOSPHERIC FLUORIDE (continued)

Susceptible	Intermediate	Tolerant
	Rhododendron	Dogwood, Flowering
	Rose (hybrid tea, rambler, multiflora)	Dogwood, Red Osier
	Serviceberry	*Lobelia
	Violet	*Marigold
		*Mock-orange
		Periwinkle
		*Pinks
		*Portulaca
		Privet
		*Quince, Flowering
		*Snowberry
		*Spirea, Anthony Waterer
		*Spirea, Vanhoutte
		*Spurge, Japanese
		*Stonecrop
		Sunflower
		*Sweet William
		*Virginia creeper
		*Yucca, Adam's Needle
	Weeds	
Crabgrass	Chickweed	Burdock
	Pigweed	Cinquefoil, Shrubby
	Ragweed	Dock
	Smartweed	Nightshade
		Plantain
		Purslane
	Conifers	
Pine, Eastern White, Mugo, Loblolly, Lodgepole, Ponderosa, Scotch (young needles)	Pine, Western White	*Arborvitae
	Pine, Jack	*Cedar, Eastern Red
	Pine, Austrian	*Cedar, Western Red
	Spruce, Bird's Nest, Black, Blue, Englemann, White (young needles)	*Cypress, False
		Pine, Eastern White, Mugo, Loblolly, Lodgepole, Ponderosa, Scotch (old needles)
		Spruce, Bird's Nest, Black, Blue, Engelmann, White (old needles)
	Yew	

TABLE 1. RELATIVE SUSCEPTIBILITIES OF PLANTS TO ATMOSPHERIC FLUORIDE (continued)

Broad-leaved Trees and Shrubs

Susceptible	Intermediate	Tolerant
Box Elder (Manitoba Maple)	Alder, Red	Birch, Black, Cutleaf, White
	Alder, Speckled	Black-haw
	Ash, Green	*Cottonwood, Black
	Aspen, Trembling	Elderberry
	Cherry, Choke	Elm, American, Chinese
	Linden, American	*Locust, Black
	Linden, Littleleaf	Locust, Honey
	Maidenhair	*Oak
	Maple, Hedge	Planetree, London
	Maple, Norway	Poplar, Balsam
	Maple, Red	*Poplar, Carolina
	Maple, Silver	*Poplar, Lombardy
	Maple, Sugar	*Poplar, Silver-leaved
	Mountain Ash, European	*Russian olive
	Mulberry	*Sweet gum
	Sumac, Smooth	*Sycamore
	Sumac, Staghorn	*Tree-of-Heaven
	Viburnum, Doublefile	*Tulip tree
	Walnut, Black	Viburnum, Arrowwood
	Walnut, English	*Viburnum, Leatherleaf
		*Viburnum, Siebold
		*Weigela, Pink
		*Willow, Goat
		*Willow, Laurelleaf
		*Willow, Weeping

Fruit Trees and Shrubs

Apricot, Chinese	Apple	*Blackberry
Blueberry	Apricot (Moorpark and Tilton)	Currant
Peach (fruit)	Cherry, Bing and Royal Ann	Pear
Plum, Bradshaw	Lemon	*Raspberry, Red
Prune, Italian	Orange	
	Peach (foliage)	
	Plum, Flowering	
	Tangerine	

TABLE 1. RELATIVE SUSCEPTIBILITIES OF PLANTS TO ATMOSPHERIC FLUORIDE (continued)

Field and Garden Crops

Susceptible	Intermediate	Tolerant
Barley (young plant)	Barley (mature plant)	Alfalfa
Corn (Sweet) (some cultivars)	Clover, Crimson, White	Asparagus
Oat (young plants)	Corn, Field	Bean
Rye (young plants)	Oat (mature plants)	Cabbage
Sorghum (some cultivars)	Pepper	Carrot
Wheat (young plant)	Rice	Cauliflower
	Rye (mature plants)	Celery
	Spinach	Coffee
	Tomato	Cotton
	Wheat (mature plant)	Cucumber
		Eggplant
		Pea
		Potato, White
		Soybean
		Squash
		Strawberry
		Sugar Cane
		Tobacco

*Suitable for planting near fluoride-emitting industries.

INTERACTIONS IN THE BIOTIC ENVIRONMENT

It is possible that F may alter the plant disease and plant-insect interaction in the field, affecting both the environmental transfer F and ecologic balance, and resulting in decreased yield or increased cost of production to agriculture or forestry. These relationships are just beginning to be elucidated and much work remains to be done to understand these effects as they occur in the field.

PLANT PATHOGENS

The incidence or severity of several diseases of plants is changed when the host is exposed to certain air pollutants, and the pollutant-plant-pathogen interaction and its epidemiological consequences were reviewed recently (Laurence, 1978). The number of local lesions produced by tobacco mosaic virus on pinto bean was found to be increased when tobacco leaves contained 200-300 ppm F, and decreased when leaves contained more than 500 ppm F, when compared with unfumigated control leaves. HF fumigation resulted in a consistent reduction in

bean powdery mildew (Erysiphe polygoni D.C.), probably indicating that HF was affecting the infectivity of the pathogen itself, because the reduction in disease was proportional to the length of the exposure period, infection was continuous during the exposure period, and the pathogen itself is epiphytic. Pre- and post-inoculation exposures of bean plants to HF reduced the number of bean rust [Uromyces phaseoli (Pers.) Wint.] uredia and it was postulated that the results may have been due to accumulated F in the leaf. When Freesia cultivars susceptible to "leaf necrosis," a disease of unknown etiology, were exposed to low concentrations of HF, symptoms of "leaf necrosis" developed earlier and increased in severity with length of HF exposure (Wolting, 1975).

INSECTS

In several geographic areas, an increased incidence is associated with injury due to air pollutants in indigenous vegetation (Bunce, 1978). But it is not known whether the meteorologic or topographic features that determine pollutant dispersal in these areas also created a favorable environment for insects, or whether there was some causal relationship between the two. Carlson and Dewey (1971) believed that increased pine needle scale infestation was related to a greater F content of needles. No relationship was found between larch case bearer infestation and distance from a F emitting source. In a later study, Carlson et al. (1974) reported a statistical relationship between foliar F concentration and pine needle sheath miner and pine needle miner, but their data may indicate strong evidence of a weak association. Pfeffer (1962-1963) observed that bark beetles and fir leaf roller infestations on fir often appeared before development of F injury symptoms. Compton et al. (1961) and others (Edmunds and Allen, 1958; Johnson, 1950) found no relationship between the F content of ponderosa pine needles and the incidence or severity of pine needle scale or bark beetles and Edmunds (1973) questioned the results of Carlson and Dewey (1971). There is an obvious need for impartial research of high quality.

Studies on growth and fecundity of the Mexican bean beetle on HF-fumigated bean plants provide documented information on the indirect effects of airborne F on an insect (Weinstein et al,, 1973). Three broad characteristics of the plant-insect system were changed by exposure of the plant to HF: the growth, development, and reproduction of the insects were affected detrimentally when they were cultured for up to five generations on plants that had been exposed to the pollutant; the mobility and feeding activity of the insects were reduced with culture on pollutant-containing foliage; and insects that had been cultured on fumigated plants retained less than 0.1% of the total fluoride they processed.

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PAPER NO. 24

THE CHANGING CHEMISTRY OF ATMOSPHERIC DEPOSITION AND ITS BENEFICIAL AND DETRIMENTAL EFFECTS ON BIOLOGICAL SYSTEMS

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Many atmospheric trace constituents are at least partially soluble in water and can thus be absorbed or assimilated by plants when precipitation is intercepted by vegetation. Since some of the substances dissolved in precipitation are beneficial while others are injurious to plants and animals, the net effect of atmospheric deposition can be beneficial or injurious depending on the chemical composition of the deposited matter, the duration and intensity of deposition episodes, the species and genetic characteristics of the organisms on which the substances are deposited, and the physiological condition, structure, phenology, and stage of maturity of the organisms (Galloway et al., 1978).

Direct injury to living organisms is most likely when a particularly vulnerable life form (for example, lichens or fish) is exposed at a particularly vulnerable life stage (for example, early stages of reproduction), and is growing in a poorly buffered environment (for example, sandy soils or oligotrophic lakes and bogs) during a season of the year when acid precipitation is most likely (for example, spring rains) (Galloway et al., 1978; Heck et al., 1977).

Rain and snow change in chemical composition within as well as between precipitation events. In cold climates, acid substances accumulate in the snowpak where they are released in concentrated form with the first melt water and thus cause very sudden increases in acidity of soils, vegetation, and surface waters (Ambio, 1976). Thus a given organism or ecosystem may be subject to beneficial atmospheric influences at one time and to negative influences at another time within a given day, month,

growing season, or the years of its development in the case of perennial plants and animals (Tamm and Cowling, 1976). Even a given molecular species such as SO_2 or NO_2 may be absorbed and utilized as a beneficial nutrient at one concentration; at another, higher concentration, even on the same day, the same substance may be absorbed and found to be toxic or even injurious to the very same plant, animal or microorganism.

Herbaceous and perennial plants are subject to changes in atmospheric deposition within a given growing season. Trees and fish, on the other hand, persist for many years (or even decades in the base of trees); they are subject to long-term as well as episodic changes in the chemistry of the atmosphere and precipitation.

For all of these reasons, it is difficult to assess the effects of acid substances in precipitation in isolation from the general chemistry of the atmosphere. The effects of acidic precipitation on terrestrial and aquatic ecosystems are only one facet of the much larger subject of atmosphere-plant-soil-surface water interactions. Acidity in precipitation should be understood as a reflection not only of the amounts of substances yielding hydrogen ions (such as sulfuric, nitric, hydrochloric and organic acids) but also as a reflection of the total ionic balance between all the cations and anions dissolved in precipitation.

Also, the effects of a given "acid rain" or a prevailing condition of "acid rains" are very complex, variable in time, and involve significant interactions which are only partially understood.

POTENTIAL EFFECTS OF ACID PRECIPITATION ON VEGETATION

A partial list of theoretical effects of acid precipitation on vegetation was developed by Tamm and Cowling (1976) and is reproduced in Table 1. The effects are classified as either direct or indirect, although most direct effects will have many indirect consequences as well. A decreased rate of growth would be the expected consequence of most of the effects postulated in Table 1 but unequivocal evidence of significant growth effects has yet to be demonstrated in agricultural, forest, or range ecosystems. Specific biological effects which have been proven to occur in at least one experimental plant are marked with an asterisk (*) next to the numbers in Table 1.

TABLE 1. POTENTIAL EFFECTS OF ACID PRECIPITATION ON VEGETATION (FROM TAMM AND COWLING, 1976)

DIRECT EFFECTS

*1. Damage to protective surface structures such as cuticle.

Damage to surface structures may occur due to accelerated erosion of the cuticular layer that protects most foliar organs. It also could result from direct injury to surface cells by high concentrations of sulfuric acid and other harmful substances that are concentrated by evaporation or adherence of soot particles on plant surfaces.

*2. Interference with normal functioning of guard cells.

Malfunction of guard cells will lead to loss of control of stomata and thus altered rates of transpiration and gas-exchange processes and possibly increased susceptibility to penetration by foliar plant pathogens.

*3. Poisoning of plant cells after diffusion of acidic substances through stomata or cuticle.

This could lead to development of deep necrotic or senescent spots on foliar organs including leaves, flowers, twigs, and branches.

*4. Disturbance of normal metabolism or growth processes without necrosis of plant cells.

Such disturbances may lead to decreased photosynthetic efficiency, altered respiratory patterns and intermediary metabolism, as well as abnormal development or premature senescence of leaves or other organs.

*5. Alteration of leaf- and root-exudation processes.

Such alterations may lead to changes in populations of foliar-surface and root-surface microflora and microfauna, including nitrogen-fixing organisms.

*6. Interference with reproduction processes.

Such interference may be achieved by decreasing the viability of pollen, interference with fertilization, decreased fruit or seed production, decreased germinability of seeds, etc.

TABLE 1. (continued)

*7. Synergistic interaction with other environmental stress factors.

Such reinforcing interactions may occur with gaseous sulfur dioxide, ozone, fluoride, soot particles, and other air pollutants as well as drought, flooding, etc.

INDIRECT EFFECTS

*1. Accelerated leaching of substances from foliar organs.

Damage to cuticle and surface cells may lead to accelerated leaching of mineral elements and organic substances from leaves, twigs, branches, and stems.

2. Increased susceptibility to drought and other environmental stress factors.

*3. Alteration of symbiotic associations.

Changes in leaf- and root-exudation processes and accelerated leaching of organic and inorganic substances from plants may affect the formation, development, balance, and function of symbiotic associations such as mycorrhizae, nitrogen-fixing organisms, lichens, etc.

*4. Alteration of host-parasite interactions.

Resistance and/or susceptibility to biotic pathogens, parasites, and insects may be altered by subjecting plants to any environmental stress. Acid precipitation may increase the susceptibility of plants to these injurious agents, alter their capacity to tolerate disease or injury, or alter the virulence of pathogens. The effects of acid precipitation may vary with the following: the nature of the pathogen involved (whether a fungus, bacterium, mycoplasma, virus, nematode, parasitic seed plant, insect, or multiple-pathogen complex); the species, age, and physiological status of the host; and the stage in the disease cycle in which the acidic stress is applied. For example, acid rain might decrease the infective capacity of bacteria before infection and increase the susceptibility of the host to disease development after infection.

THE AVAILABILITY OF ESSENTIAL AND INJURIOUS ELEMENTS FROM THE ATMOSPHERE

Plant life and animal life as we know it would be impossible without atmospheric sources of carbon for photosynthesis, nitrogen for biological fixation and protein synthesis, oxygen for respiration and synthesis of carbohydrates, and water for transpiration of plants. Many essential elements and injurious substances are present in the gaseous, particulate, and aerosol emissions from natural and anthropogenic sources. All of the vast amounts of these emissions into the atmosphere eventually return to the surface of the earth where they are deposited into ecosystems by one of four major processes: (1) precipitation of dissolved, suspended or adsorbed substances in raindrops and snowflakes, (2) deposition of windblown dust and particulate matter, (3) impaction of aerosols, and (4) absorption of gases.

For these reasons many terrestrial and aquatic ecosystems are influenced by deposition of nutrient and toxic elements from the atmosphere. Among the essential elements, carbon, hydrogen, and oxygen are rarely limiting. The major fertilizer elements - nitrogen, phosphorus and potassium - are often limiting. Sulfur and calcium are sometimes limiting. Magnesium is only rarely limiting as are the so-called micronutrients - iron, copper, zinc, magnesium, molybenum, and boron.

Some elements are both essential and injurious to organisms. For example, small amounts of sulfur and large amounts of nitrogen are needed for synthesis of proteins, nucleic acids, and other substances in plants and animals, but gaseous sulfur and nitrogen oxides and sulfuric and nitric acid aerosols are injurious to many organisms at very low concentrations. Similarly, excess amounts of the micronutrient elements and heavy metals can be injurious. Atmospheric fluoride is toxic to plants at 25-50 ppm. Aluminum is the most abundant toxic metal in the earth's crust. The availability and toxicity of aluminum and other metal ions is influenced greatly by the acidity of soils which in turn is influenced by the abundance of acid precipitation. Estimates of the threshold concentrations for detectable injury or economic damage by air pollutants including NO_2, SO_2, O_3, toxic metal ions, and acid precipitation require substantial additional research with a great variety of organisms and ecosystems.

Previously, it was believed that essential nutrients were taken up by plants almost entirely from the soil solution. Now, it is recognized that airborne gases, particulate matter, and aerosols significantly augment the supply of both essential and injurious elements. In fact, all of the nutrient elements essential for growth of plants can be taken up readily through foliar organs as well as by absorption by roots from the soil solution (Sharma, 1976; Tamm, 1958; Wittwer and Bukovac, 1969).

Much larger amounts of essential nutrients are required for sustained yield agriculture than for sustained yield range management or forestry. This is true in rangelands because biomass yields are very low, and in forests because the part of trees that usually is harvested - the wood and bark of tree stems - contains very much less of most essential elements than the seeds and fruits that are commonly harvested in agriculture. This is a major reason why fertilization is so common in agriculture and so rare in forestry and range management. In some forested and natural grasslands, atmospheric deposition is adequate to permit sustained-yield cropping of the land without fertilization. This is much less likely to remain so as more and more of the nutrient-rich branches, foliage and roots of trees are harvested in "whole tree chipping" and other modern forestry practices.

Many scientists believe that acid rain and snow are deposited directly onto soils where acid substances could be neutralized in well-buffered soils or by applications of lime. This is true on some agricultural lands, especially after harvesting of crops. But this is not true in forests, rangelands, or even on most agricultural lands during the growing season. Most raindrops are intercepted by the foliage of plants where substances dissolved in the rain can induce various physiological changes before reaching the soil. In a mature forest, for example, rain will wash over three tiers of foliage before reaching the soil. Most forests and rangelands in the United States are rarely fertilized and almost never limed to neutralize excess acidity. Furthermore, forests cover about half the total land area of the United States and develop very large canopies of leaves and branches that extend high into the air. Thus, forests provide a very large surface for deposition and assimilation of all the beneficial and injurious substances dispersed in the atmosphere and deposited as precipitation (Tamm, 1958; Tamm and Cowling, 1976; Witter and Bukovac, 1969).

SOURCES, AMOUNTS, AND DISTANCE OF TRANSPORT OF BENEFICIAL AND INJURIOUS SUBSTANCES IN THE ATMOSPHERE

There are three major reasons why agricultural, forest, and aquatic biologists are becoming increasingly concerned about atmospheric transport and deposition of both nutritionally beneficial and potentially injurious substances: (1) vegetation, soils, and surface waters are the primary deposition sites for precipitation and airborne particulate matter of all types; (2) atmospheric deposition constitutes an important source of nutrients and potentially injurious substances that affect the productivity and stability of agricultural, forest, and aquatic ecosystems; and (3) human activities are steadily increasing the amounts and variety of substances dispersed in the atmosphere (Bolin et al., 1971; Oden, 1968).

Previously, it was believed that most anthropogenic emissions were removed from the atmosphere near the site of emission. Now it is recognized, particularly with increased use of tall stacks in power plants, that atmospheric processes can lead to extensive mixing, and both chemical and physical interactions and transformations of atmospheric particles, aerosols and gases. Furthermore, these substances and their reaction products are dispersed by meteorological processes and finally enter the biosphere in fields of deposition that extend hundreds or even thousands of kilometers from the original sources of emission. The recent fallout of radioactive materials in the eastern United States as the result of atomic explosions in the Peoples Republic of China provides a dramatic reminder of the long-distance transport and deposition of pollutants.

The amounts of various substances introduced deliberately or inadvertently by man into the biosphere of the earth are becoming so large that man is becoming a major force in the biogeochemistry of the earth (Kovda, 1975). This is shown in Table 2 which contains a tabulation of data on annual output of fertilizers, industrial dusts, garbage and other urban wastes and byproducts, mine refuse, and discharges of aerosols and gases mainly from combustion of fossil fuels. All of these categories of matter are becoming comparable in magnitude with the discharges of dissolved and suspended substances in all the rivers of the world, the annual yield of photosynthetic products, or the cycling of inorganic elements in the earth as a whole. Anthropogenic emissions into the atmosphere are also very large as shown in Table 3. Most gases, carbon oxides, and aerosols result from combustion of fossil fuels. A very large fraction of these global emissions was produced in the United States.

TABLE 2. BIOGEOCHEMICAL AND TECHNOLOGICAL FORCES IN THE BIOSPHERE OF THE EARTH (DATA OF KOVDA, 1975)

Biosphere Components	Tons Per Year
Biogeochemical Processes:	
Yield of photomass	1×10^{10}
Cycle of inorganic elements	1×10^{10}
River discharges:	
Dissolved substances	3×10^{9}
Suspended substances	2×10^{10}
Anthropogenic Sources:	
Output of fertilizers	3×10^{8}
Industrial dust	3×10^{8}
Garbage, urban wastes and byproducts	2×10^{10}
Mine refuse	5×10^{9}
Aerosols and gas discharges	1×10^{9}

TABLE 3. ANTHROPOGENIC EMISSIONS INTO THE ATMOSPHERE (DATA OF KOVDA, 1975)

Type of Emission	Tons Per Year
Dust	2.5×10^{8}
Gases (mainly SO_2, HC, and NO_x)	6.5×10^{8}
Carbon oxides ($CO + CO_2$)	2.0×10^{9}
Aerosols	1.0×10^{9}

NOTE: Doubling about every 7-10 years.

If the United States is to so greatly augment the amounts of substance dispersed in the atmosphere and deposited into the biosphere of the earth, it is essential that we should measure the amount and chemical form of the deposited matter and understand the biological consequences of that deposition. Regrettably, our understanding of these processes in the United States is very fragmentary. Fortunately, however, more extensive measurements of atmospheric deposition and its biological consequences have been made in Europe where an atmospheric deposition network has been maintained since the late 1940's (Oden, 1968).

The European Air Chemistry Network began in Sweden and has gradually spread to include most of western Europe and parts of eastern Europe including Poland and the Soviet Union. Since the mid 1950's, a network of about 100 stations has provided monthly measurements of changes in the chemistry of precipitation. The substances analyzed at most of these stations include the following major cations and anions: NH_4, Na, Ca, K, Mg, SO_4, No_3, PO_4, Cl as well as pH, conductivity, and titratable acidity and alkalinity. These data have shown long-term trends in the chemistry of precipitation. For example, the amount of nitrate nitrogen in precipitation (an important fertilizer element) increased markedly in many parts of Europe during the 15 years between 1955 and 1970. Nitrate nitrogen helps plants grow. Thus, the nitrogen added in precipitation probably increased yields of agricultural and forest crops.

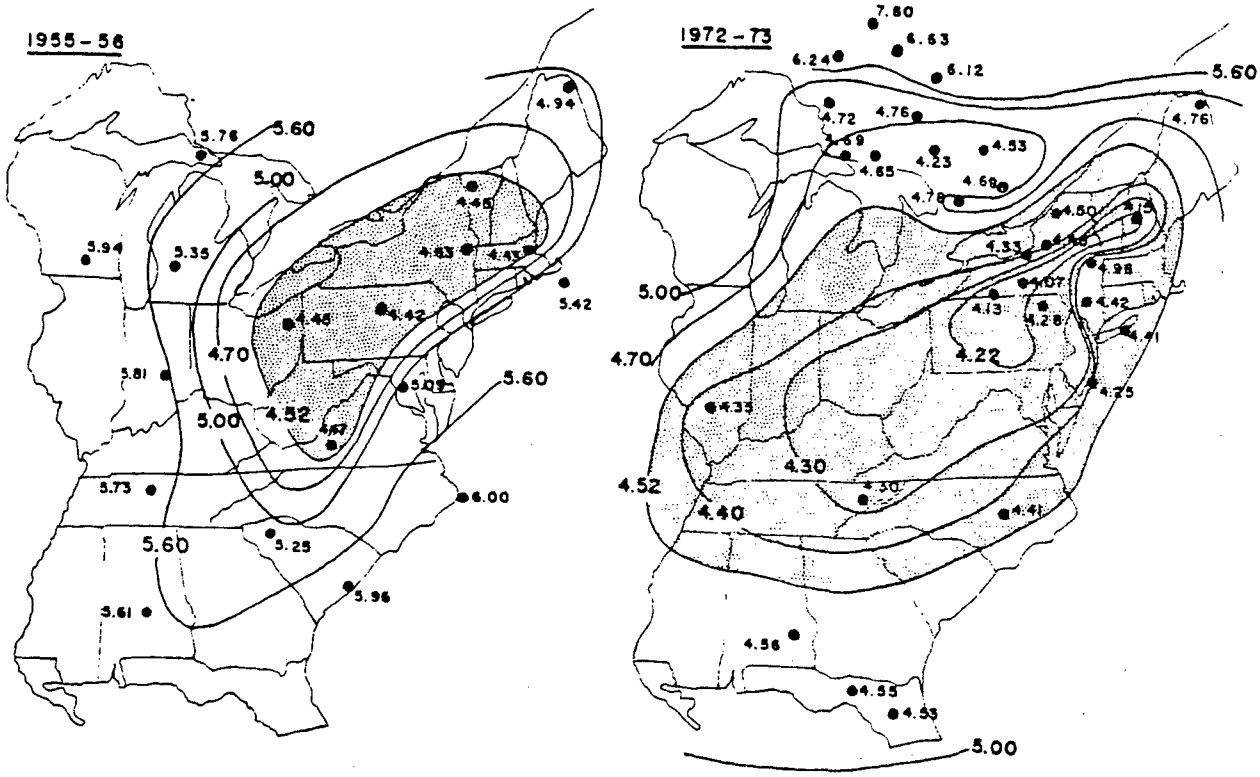

Figure 1. Changes in the weighted annual average pH of precipitation in the eastern United States in 1955-56 and 1972-73. Adapted from Likens (1976) and used by permission of Chemical and Engineering News.

But not all the substances detected in precipitation were beneficial. Long-term trends of injurious sulfate and hydrogen ions also were detected. These changes were attributed to strong acids formed in the atmosphere, mainly from oxides of sulfur and nitrogen produced during combustion of fossil fuels. More recent data show that these trends of increasing acidity are continuing although the relative contribution of sulfuric and nitric acids is changing (Ambio, 1976; Oden, 1968; Oden, 1976).

CHANGES IN THE CHEMISTRY OF PRECIPITATION IN THE UNITED STATES

Some monitoring of the chemistry of precipitation has also been carried on in the United States (Feth et al., 1964; Lodge et al., 1968). A few of these studies provide excellent and reliable information about the acidity of precipitation. But most studies in this country have suffered from three major shortcomings: (1) The data were collected for a limited land area - typically only a single point or a few points in one or two states (Gambell and Fisher, 1966); (2) The data were collected for very limited periods of time - typically only one or two years; and (3) very few direct measurements of pH have been made. There is only one location in the United States - at the Hubbard Brook Experiment Forest in New Hampshire - where the acidity of rain has been measured directly and consistently for more than 10 years. The longest term national monitoring program was operated by the National Center for Atmospheric Research for six years from 1960 to 1966 (Lodge et al., 1968).

Using fragmentary bits of information, obtained indirectly and in limited areas and periods of time, Cogbill and Likens (1974) managed to calculate the probable changes in average acidity of rainfall in various parts of the eastern United States from 1955-1973. As shown in Figure 1 (Likens, 1976), precipitation in a large portion of the eastern United States was less than pH 5.6 in 1955-56; the zone of greatest acidity (lowest pH) was generally consistent with the zone where sulfur emissions were high - parts of Ohio, Pennsylvania, West Virginia, New York, and New England. By 1972-1973, however, the area with an average pH of rain below 4.5 had extended to include parts of Missouri, Arkansas, Mississippi, Alabama, Georgia, South Carolina; North Carolina, Virginia, Kentucky, Illinois, Michigan, and further north into New England and Canada. Essentially, it embraced most of the area east of the Mississippi River. Individual rainstorms with pH values below 3.6 have been reported in New York, Illinois, Indiana, New Hampshire, Massachusetts and North Carolina - in some cases many hundreds of kilometers from major sources of air pollution (Likens, 1976).

The relative contribution of sulfate and nitrate to the total acidity of precipitation apparently changed markedly during the years since 1964-65. At Hubbard Brook, New Hampshire, the ratio of sulfate to nitrate changed from 83:15 in 1964 to 66:30 in 1974. During this same decade total input of hydrogen ions increased by 36%. Thus, most of this increase appears to be due to increased deposition of nitric acid (Likens, 1976).

EFFECTS OF ACID PRECIPITATION ON BIOLOGICAL SYSTEMS

Research on the effects of change in the chemistry of precipitation on terrestrial and aquatic ecosystems has been conducted in Europe since about 1955 and in North America mainly since about 1966. Several publications are worthy of special notice in this connection. The pioneering researches by Eville Gorham first in England, later in Canada, and most recently in the United States dealt with effects on lake waters, aquatic vegetation, terrestrial vegetation and human health (Gorham, 1958; Gorham, 1976). Oden published his famous Ecological Committee Report in 1968 (Oden, 1968). In 1971, Bolin and coworkers completed the Swedish Cast Study contribution to the United Nations Conference on the Human Environment (Bolin et al., 1971). In 1972, the Norwegian government established a special Manhattan-Style research project called "Acid Precipitation: Effects on Forests and Fish" with an annual budget of 10,000,000 Norwegian Kroner (about $2,000,000 U.S.). This huge project was initiated with two specific objectives:

1. To establish as precisely as possible the effects of acid precipitation on forests and freshwater fish; and
2. To investigate the effects of air pollutants on soil, vegetation and water, to the extent required to support the primary objective.

The first International Symposium on Acid Precipitation and the Forest Ecosystem was held at Ohio State University at Columbus in May of 1975 (Dochinger and Seliga, 1976). In June of 1976, an International Conference on Effects of Acid Precipitation was held at Telemark, Norway, and the major papers assembled for this meeting published by Braekke (1976), and in a special issue of Ambio (1976). In November 1976, Likens published his summary report in Chemical and Engineering News (Likens, 1976). In 1977, the Organization for Economic Cooperation and Development (OECD, 1977) published comprehensive findings regarding the international exchange of sulfur compounds among the various nations of Europe.

In May, 1978, a NATO Advanced Research Institute on Ecological Effects of Acid Precipitation was held at Toronto, Canada. In September, 1978, the Central Electricity Generating Board in England hosted an international symposium on the biological effects of acid precipitation. The proceedings of these last two meetings are scheduled for publication in 1979.

EFFECTS ON AQUATIC ORGANISMS

The effects of acid precipitation on fish populations and aquatic ecosystems have been very pronounced in various regions of northern Europe and eastern North America. The most important of these effects were summarized in the Report from the International Congress on the Effects of Acid Precipitation held at Telemark, Norway, in June, 1976 (Ambio, 1976). That report reads in part:

> "Water quality has changed during the last decades in numerous lakes and rivers in southern Scandinavia and eastern North America, pH often falling below 5, with sulfate becoming the most important anion. There is strong evidence that this change is due to acid precipitation. It is associated with the loss of buffering capacity, and the occurrence of additional short-term decreases in pH, related to meltwater from snow or episodic inputs of acid precipitation from polluted air masses.
>
> The acidification of freshwater ecosystems leads to many changes, most of which involve decreases in biological activity and important changes in nutrient cycling. For example, decomposer organisms are less active in acid waters, resulting in increased accumulations of organic matter. When the pH drops below 6, numbers of species in several groups of organisms (phytoplankton and zooplankton, bottom fauna and several other groups of invertebrates) decrease considerably, thus affecting the variety of food for fish and other animals depending on freshwater ecosystems. Shifts have been observed from higher aquatic plants toward mosses, and this will influence not only the bottom fauna but also nutrient exchange with the sediments.
>
> High acidity (pH <5.5) seriously affects fish populations, particularly when occurring in waters of low ionic strength. Rapid extinction rates of fish populations inhabiting acidified waters have been observed during the past few decades in southern Scandinavia as well as in parts of eastern North America.

Case studies of several fish populations and experiments clearly indicate that the elimination of fish is often a result of chronic reproductive failure in acid conditions and of damage done to sensitive stages, especially the newly hatched larvae. Such a process is insidious and not readily evident in terms of fishery yield until extinction is imminent.

In lakes and streams with soft waters, acid stress has also been shown both experimentally and in field studies to cause mortality among adult fish as a result of interference with physiological mechanisms regulating active ion exchange across gill membranes. In this case, factors such as size, age, acclimation history, genetic background and ionic strength of the water interact in complex ways to determine the relative acid tolerance of the fish.

There is strong evidence that the increased acidity of precipitation is now the main cause of these extensive losses of salmonid fish stocks as well as other populations of economic importance both in southern Scandinavia and the northeastern part of the United States and parts of southeastern Canada.

Sensitivity is related to the tolerance of the differing species (reflecting their genetic diversity), to the timing of episodic acid precipitation in relation to the stage in the life cycle, and to the influence of the geological environment, eg lakes and rivers on bedrock, overburden and soils highly resistant to chemical weathering. This may also be the case in geological areas of this type elsewhere in the world.

Special consideration should be given to the preservation of unique gene pools and habitats.

There may be complex interactions with other environmental factors, including some organic compounds. In waters of low pH the adverse effects of heavy metals on fish and other organisms can be enhanced, whereas at pH values nearer neutrality these substances would have been tolerated."

EFFECTS ON TERRESTRIAL ORGANISMS

Effects on terrestrial ecosystems generally have been less well documented than those on populations of fresh water fish and other aquatic organisms. Nevertheless, certain definite effects have been reported. The most striking of these effects was development of peat moss (Sphagnum sp.) as a submarine rather than a terrestrial plant in acidified lakes and streams in Sweden (Grahn et al., 1974). Dense mats of Sphagnum and an apparently parasitic aquatic fungus develop on the bottom of these lakes in water as deep as 18 meters. This growth induces oligotrophication (opposite of eutrophication) - a self-accelerating process that leads to a substantial nutrient impoverishment of lake waters.

Analyses of forest growth in southern Sweden from 1896 to 1965 showed a 2 to 7% decrease in growth between 1950 and 1965. Johnsson and Sundberg (1972) "found no good reason for attributing (this) reduction in growth to any cause other than acidification." Similar attempts to quantify possible effects on growth of forests in the United States have been inconclusive.

Other reports given at the meetings in Columbus, Ohio and Telemark, Norway indicated that: (1) forest canopies filter out sulfur and hydrogen ions from precipitation (Knabe and Günther, 1976; Mayer and Ulrich, 1976); (2) acidity of bark and development of cuticular features such as frequency and size of stomata can be used as biological indicators of sulfur pollution and acidification (Sharma, 1976); for example, pH values of 2.2 to 4.7 were reported in bark of several species of trees in Poland (Grodzinska, 1976); (3) vegetation developing in recently formed sandy soils or glacial outwash areas is more vulnerable to the effects of acid precipitation than vegetation growing in older, well-buffered soils of high clay content and consequently large base exchange capacity (Frink and Voigt, 1976; Tamm et al., 1976); (4) before reaching the soil, acidic substances in precipitation can induce changes in the physiology of foliar organs (Tamm and Cowling, 1976); (5) after reaching the soil, these substances also can induce changes in root function and the availability of essential cations (Frink and Voigt, 1976); (6) ammonia combines with sulfate ions in the atmosphere and tends to neutralize atmospheric acidity - when the ammonia is absorbed by plants, however, both the ammonia and the released sulfate ions contribute to the total acidification of agricultural and forest ecosystems (Oden, 1976); (7) acid rains increase the leaching of nutrient cations from the root zone of plants (Norton, 1976; Overrein, 1972): and (8) acid precipitation also increases the solubility (and therefore the toxicity) of metal in soil such as Al, Pb, and Hg (Malmer, 1976; McFee et al., 1976).

Simulated "rain" acidified with sulfuric acid has been reported to: (1) induce direct injury to foliage of pines, birch, and mosses (Shriner, 1976; Tamm et al., 1976; Wood and Bormann, 1974); (2) induce symptoms similar to those caused by certain other pollutants and biotic pathogens (Wood and Pennypacker, 1976); (3) induce poorer germination of spruce seeds (Abrahamsen et al., 1976); (4) accelerate leaching of nutrients from foliage (Abrahamsen et al., 1976; Wood and Bormann, 1974); (5) inhibit decomposition of leaf litter from the forest floor (Abrahamsen et al., 1976); (6) decrease erosion of epicuticular waxes on oak and bean leaves (Shriner, 1976); (7) decrease uptake of nitrogen by endomycorrhizae of sweetgum seedlings (Haines and Best, 1976); (8) inhibit reproduction of root-knot nematodes (Shriner, 1976); (9) inhibit development of bean rust and production of telia by the oak-leaf-rust fungus, Cronartium fusiforme (Shriner, 1976); (10) inhibit or stimulate development of halo blight in bean seedlings depending on the time in the disease cycle during which the simulated "rain" was applied (Shriner, 1976); and (11) inhibit nodulation and fixation of nitrogen by Rhizobium in bean and soybean seedlings (Shriner, 1976). As yet unknown constituents of precipitation have been shown to induce "russetting" - brown blemishes that cause degrade in the marketing of Yellow Delicious apples (Kender, personal communication).

With the exception of this latter effect on apples, the possible economic consequences of the various biological effects listed above are not known. Nevertheless, the variety of biological influences that have been observed suggests that: (1) much more research is needed to evaluate the ecological and possible economic influences of changes in the acidity and other chemical properties of precipitation; and (2) a well coordinated network to measure long-term changes in the chemistry of air and precipitation is needed in rural and urban areas throughout North America.

Based on a general analysis of effects reported to date, Heck et al. (1977) offered the following generalizations about relative vulnerability of various organisms and ecosystems to acid precipitation: (1) fresh water fish are more vulnerable to acute acid shock than fresh water vegetation; (2) aquatic vegetation is more vulnerable than terrestrial vegetation; (3) herbaceous vegetation is more vulnerable than woody vegetation; (4) plants growing in recently formed sandy soils or glacial outwash areas are more vulnerable than plants growing in well-buffered soils of high clay content and consequently large base exchange capacity; (5) reproductive stages are more vulnerable than vegetative stages; (6) plants growing in natural ecosystems

are more vulnerable than plants growing in agroecosystems where liming and other fertilization practices are common; (7) coniferous forests are more vulnerable than hardwood forests; and (8) plants growing in regions of high rainfall are more vulnerable than desert plants.

DEVELOPMENT OF A NATIONAL ATMOSPHERIC DEPOSITION PROGRAM FOR THE UNITED STATES

In 1975, the National Academy of Sciences Committee on Atmospheric Sciences published its report on "Atmospheric Chemistry: Problems and Scope" (National Academy of Sciences, 1975). In May of 1975, a workshop to define future needs for research on acid precipitation was held immediately following the First International Symposium on Acid Precipitation and the Forest Ecosystem. The recommendations of this workshop were published by Dochinger and Seliga (1976). In May, 1977, the Director of the Norwegian Special Project on Acid Precipitation - Effects on Forests and Fish provided an updated analysis of research needs on acid precipitation as perceived from the standpoint of knowledge of acid precipitation effects in Scandinavia (SNSF, 1977). In September, 1978, the Federal Interagency Advisory Committee on Water Data completed its report on "Research and Monitoring of Chemistry in the United States: Present Status and Future Needs" (Interagency Advisory Committee on Water Data, 1978). In September, 1977, the congressionally mandated "Committee of Nine" responsible for regional research in the Agricultural Experiment Stations of the United States approved plans for establishment of a long-term interagency research project designated NC-141, "Chemical Changes in Atmospheric Deposition and Effects on Agricultural and Forested Land Surface Waters in the United States" (Cooperative State Research Service, 1976). In March of 1978, the Fish and Wildlife Service published their extensive analysis of impacts of cool-fired power plants (including acid precipitation) on fish, wildlife, and their habitats (Fish and Wildlife Service, 1978). In November 1978, the name of the NC-141 program was changed to the National Atmospheric Deposition Program (NADP) and in December, 1978, a subcommittee of NADP completed a report to the President's Council on Environmental Quality on "A National Program to Assess the Problem of Atmospheric Deposition (Acid Rain)" in the United States (Galloway et al., 1978).

In each of these major studies, needs for research on the changing chemistry of precipitation and its ecological effects were analyzed. A remarkable degree of similarity in research recommendations is evident in all of these reports: (1) establish a permanent network to continuously monitor changes in the chemistry of precipitation, and (2) coordinate this long-term

monitoring program with research to determine the sources, transport, and transformation of pollutants; the meteorology of their dispersal and deposition; and the nature and magnitude of their ecological effects on agricultural land, forested land, range land, urban lands, and aquatic ecosystems.

An adequate program of monitoring and research should ideally include the various State Agricultural Experiment Stations, the regional Forest Experiment Stations of the Forest Service and the Agricultural Research Division of the Department of Agriculture, pertinent units of the United States Geological Survey, the Department of Energy, the National Oceanic and Atmospheric Administration, the Environmental Protection Agency, the Tennessee Valley Authority, the National Center for Atmospheric Research, the National Science Foundation, and the National Aeronautics and Space Administration. An effective program will also require the interest and expertise of many scientists in a diverse array of publicly and privately supported colleges and universities in the United States and Canada.

The design for such an integrated interagency program of research and monitoring has been developed (Galloway et al., 1978). It should include each of the following elements: (1) chemical transformations and transport; (2) measurement of spatial and temporal trends in atmospheric deposition in various regions of the United States; (3) analysis of the effects of atmospheric deposition on water quality, vegetation, soils, fish, microorganisms, and simple synthetic ecosystems; (4) calibrated watershed studies to provide an understanding of the chemical linkages between the atmosphere and both terrestrial and aquatic natural ecosystems; and (5) economic assessment and criteria development.

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PAPER NO. 25

RESPONSE OF SELECTED PLANT AND INSECT SPECIES TO SOLID ROCKET FUEL EXHAUST GASES

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INTRODUCTION

Space shuttle launches from Kennedy Space Center (KSC), Merritt Island, Florida are scheduled to start in November, 1979. The area around the shuttle launch site is part of the Merritt Island National Wildlife Refuge and is a mosaic of different coastal ecological communities. There are over 1000 hectares of land planted in citrus, which is the major commercial crop on the Island. Honey production is also commercially important.

Mention of commercial products or equipment by name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, or North Carolina State University and does not imply their approval to the exclusion of other products that may be suitable.

This research was supported through an Interagency Agreement from the National Aeronautics and Space Administration (NASA) to Agricultural Research (AR) of SEA, USDA. It was cooperatively done through a Specific Cooperative Agreement (No. 12-14-7001-815) between SEA/AR and the North Carolina Agricultural Research Service.

The research reported in this paper was developed to determine possible biological effects of exhaust products from solid rocket fuel (SRF) associated with the Space Shuttle. The major exhaust components that might have adverse biological effects are hydrogen chloride (HCl) and aluminum oxide (Al_2O_3). It is possible that chlorine (Cl_2) and nitrogen dioxide (NO_2) may have an effect, but the concentrations of these gases in the SRF exhaust should be less than 10% of the HCl. Vegetation is sensitive to HCl and the gas has been a problem around various industrial processes and open burning of chlorine containing plastics. Lerman et al. (1976) surveyed the literature and documented exposures of several ornamental plants to HCl, Al_2O_3, and their mixtures. The Al_2O_3 should not be a direct toxicant but may induce injury by acting as an HCl carrier. There are no reports on effects of either HCl or Al_2O_3 on insect species, but they are sensitive to high concentrations of SO_2 and O_3.

The primary objective of this research was to determine dose-response curves for certain native and cultivated plants and selected insects exposed to SRF exhaust and to component chemicals of the exhaust. Specific objectives were to: (1) develop systems for dispensing and monitoring HCl, Al_2O_3, and SRF exhaust (controlled fuel burns); (2) determine dose-response curves for selected plant species to HCl, Al_2O_3, mixtures of the two, and to SRF exhaust; and (3) determine the effects of HCl on honeybee and the corn earworm, and of SRF exhaust on honeybee colonies (hives).

FACILITIES

The facilities developed for the exposures are briefly discussed in this section. A detailed discussion of facility construction and testing is presented in two theses (Sawyer, 1978; Tyson, 1978).

HYDROGEN CHLORIDE EXPOSURE SYSTEM

Hydrogen chloride was dispensed from a bottled HCl gas mixture (24.6% in N_2) through flowmeters into continuous stirred tank reaction (CSTR) exposure chambers (Heck et al., 1978). The chamber concentration of HCl was monitored at each chamber with a Geomet (Model 401B) HCl monitor.

Charcoal filtered ambient air was circulated through a four-chamber greenhouse exposure system and then through a scrubber that cleaned the chamber air before it reached the circulating blower. This high pressure blower was attached to the outlet manifold and maintained the chambers under a slight negative pressure. The flow rate through each chamber was kept at about 800 liters/minute. The chambers (Figure 1) were cylindrical with a central impeller to insure uniform mixing of gases within the chamber. The inlet duct was constructed of 4-inch diameter PVC pipe with three 60% baffles to insure uniform mixing of gases in the entering air stream. The outlet duct was constructed of 2-inch diameter PVC pipe. The HCl gas was dispensed through teflon tubing into the inlet duct below the baffling system.

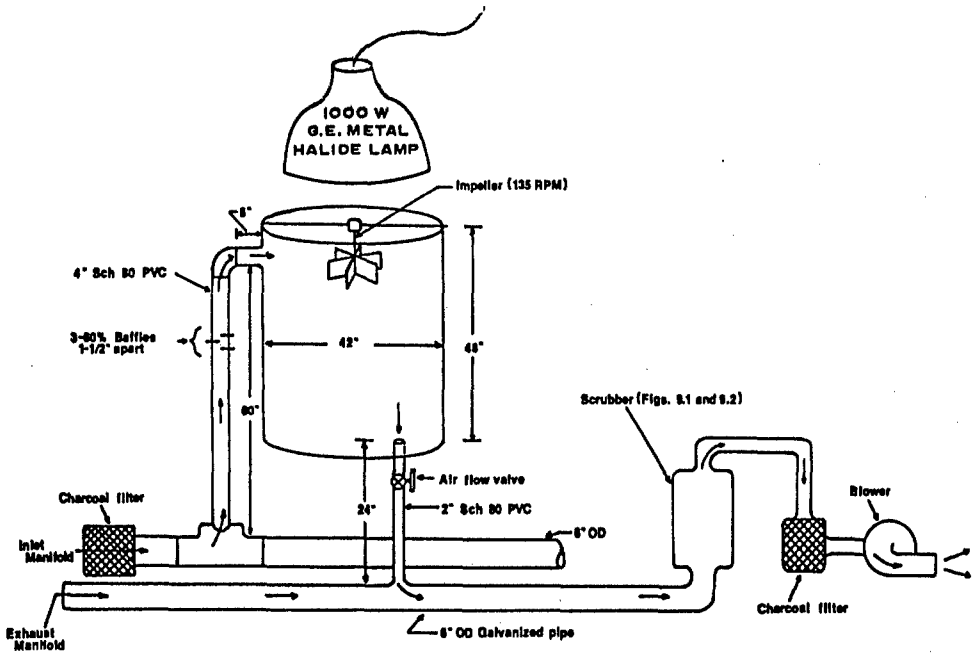

Figure 1. Schematic diagram of a single cylindrical exposure chamber (1000 liters in volume).

Each chamber was originally designed for both inlet and exhaust monitoring. However, in early tests, the HCl adsorption and desorption on the teflon sample tubing did not permit accurate measurement of HCl concentration within the chamber (through long tubes). Thus, the sampling tube for the Geomet was inserted directly through the wall of each chamber for all HCl monitoring. The Geomet values were generally comparable with bubbler samples except that the Geomet measurements were more uniform than the bubbler measurements.

Light and humidity were continuously recorded in the control chamber during exposure, and temperature was measured in all four chambers during both the HCl and Al_2O_3 exposures.

Al_2O_3 PARTICULATE EXPOSURE SYSTEM

A single CSTR chamber was modified to dispense and monitor Al_2O_3 (Figure 2). The dispensing equipment consisted of a motor-driven variable speed syringe pump that drove a teflon-tipped metal rod through a teflon cylinder that opened into the inlet duct of the chamber. The Al_2O_3 was put into a sectioned teflon tube within this cylinder. The pump drove the sectioned tube with the Al_2O_3 into the inlet duct at a designated speed that was dependent on the desired chamber concentration. The Al_2O_3 was carried by the air stream into the chamber and monitored by pulling air from the chamber at a specified flow rate. The air stream was monitored by pulling air samples through 10 μm, 2 μm, or 0.1 μm preweighed millipore filters. The filters were weighed and the Al_2O_3 concentration in the chamber was calculated. The Al_2O_3 was added as a 2-phase sample made up of gamma (<1 μm) and an alpha form (>1 μm). The gamma is a reactive phase and was used because we thought it might react with the HCl to produce a more toxic effect. The alpha form is considered inert. In practice, we found the gamma form was rarely by itself but was agglomerated on the alpha form.

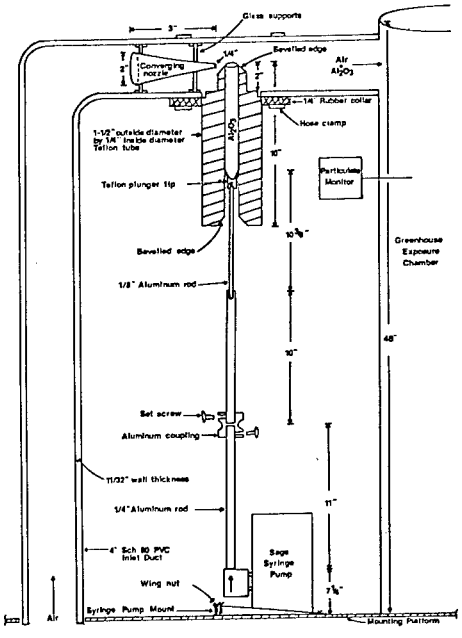

Figure 2. Schematic diagram of the Al_2O_3 particulate dispensing system.

SRF EXHAUST SYSTEMS - CONTROLLED BURN

The SRF exhaust exposure system is a field system that used four 3.1 m diameter 2.4 m high chambers with tops (Figure 3). This system used a constant flow blower (90 m^3/minute) that pushed air through a burn chamber (Figure 4) containing the fuel for the burn. The exhaust was carried into a tripartite plenum, through three separate ducts into three exposure chambers, out of each chamber by a duct to a second tripartite plenum, and into a single water-scrubber chamber. Variable-flow blowers were attached to the chamber inlet ducts so that dilution air could be added. By adjusting these blowers and adding obstruction baffles to the inlet ducts of two of the chambers, a different exhaust concentration could be dispensed to each of the three chambers. The chambers were monitored for HCl and Al_2O_3 in the same manner as the greenhouse chambers.

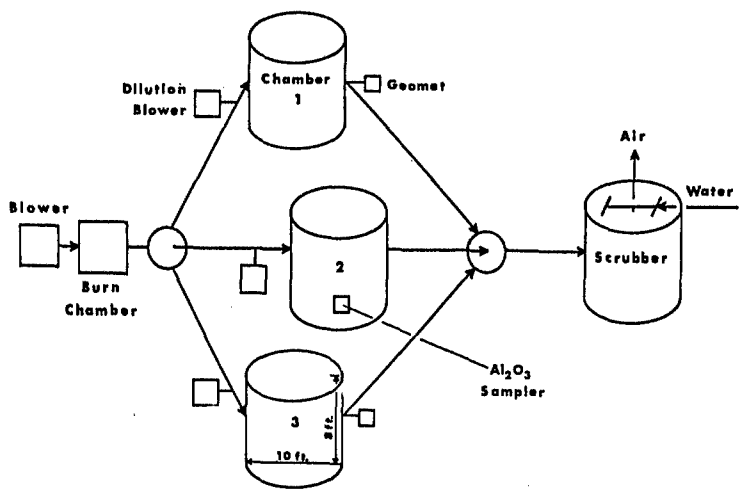

Figure 3. SRF controlled burn and exposure system.

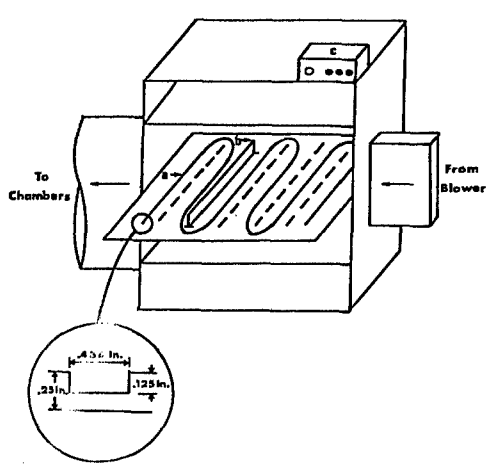

Figure 4. SRF burn chamber, a = groove for SAF strips, b = baffles, c = ignition box.

A separate chamber identical to the three used in the exhaust exposure system was used as a control chamber. A variable speed blower was attached to the inlet duct of this chamber, and air was delivered to this chamber by the blower at a rate similar to that going through the exhaust exposure chambers.

The solid rocket fuel (SRF) was obtained from Thiokol Corporation, as 10 by 15 by 0.64 cm thick pieces. The pieces were hand cut into 1.25 by 0.64 by 15 cm strips, dipped into a burn restrictor solution (methyl-ethyl-ketone, ethyl cellulose, and tricresyl phosphate) and air dried. The restrictor was removed from the ends of the fuel strips which were then laid end to end in the grooves on the copper plates of the burn box. In 10 minutes of burn exposure, 1.52 m of fuel were used (15.2 cm/minute). Each copper plate held 3.4 m of fuel. The fuel was ignited by a nichrome wire attached to a switch on the outside of the box. The system was designed so that, when two or three plates of fuel were necessary, the second and third plates were ignited automatically as the prior plate burned out. The system permitted a 60-minute uninterrupted exposure.

Light and humidity were recorded in one of the three exposure chambers before and after each exposure. Temperature was measured continuously in all four chambers. Light and humidity were measured outside the chamber during the exposure.

VEGETATION STUDIES

Twenty-three native species, 9 horticultural species (16 cultivars), and 3 agronomic species (8 cultivars) were studied in at least one phase of this project (Table 1). We have chosen several aspects of the study and presented representative data for species that represent the susceptibility range for all species tested. We have reported only the foliar injury responses. We found a fairly good correlation between injury (%) and percent reduction in biomass.

TABLE 1. A LIST OF PLANT SPECIES AND CULTIVARS. THE PLANTS ARE ALPHABETIZED BY COMMON NAME UNDER EACH MAJOR CATEGORY.

Type	Common Name	Cultivar	Scientific Name
Native	Arrowhead	-	*Sagaitarria lancifolia* L.
	Marsh elder	-	*Iva frutescens* L.
	Pennywort	-	*Hydrocotyle umbellata* L.
	Slash pine	-	*Pinus elliottii* Engelm.
	Sunflower	-	*Helianthus debiles* Nutt.
	Wax Myrtle	-	*Myrica cerifera* L.
Horticultural	Citrus	Red Grapefruit	*Citrus maxima* Merr.
		valencia orange	*Citrus senensis* Osbeck
	Radish	'Comet'	*Raphanus sativus* L.
	Tomato	'Roma'	*Lycopersicon esculentum* Mill.
Agronomic	Corn	'Coker 16'	*Zea mays* L.
	Soybean	'Dare'	*Glycine max* (L.) Merr.

METHODS AND MATERIALS

Agronomic and horticultural plants were grown from seed in a greenhouse that was covered with two layers of plastic. The potting mixture consisted of sand:sandy loam soil:Metro Mix 200 A (1:1:1 ratio). Pots were over-planted, then thinned to the most uniform plant per pot, at a specific time after germination. Plants were watered as needed and were fertilized once a week with a liquid fertilizer (10 gms of VPHF per 3.8 liters of water) at the rate of 100 ml/pot. Generally, plants grown from seed were exposed at a specific chronological age (14, 21, or 28 days). During the cool winter months, plants were grown to a specific leaf number (physiological age) before exposure.

Native plants were collected from Merritt Island, shipped bare rooted or as cuttings to Raleigh, then potted (one plant per pot) in the same soil mixture used for the horticultural/ agronomic plants. Cuttings were treated with a commercial rooting powder before planting. The pots were 10, 12, or 15 cm in diameter depending on the size of the plant. Citrus trees (about 1 m tall) in 8 liter or 9 liter containers were

purchased in Florida and shipped to Raleigh. Slash pine seedlings were purchased in Florida and shipped bare rooted to Raleigh where they were potted individually in 20 cm diameter pots. The native plants in the smaller pots were watered and fertilized in the same way as the horticultural/ agronomic plants. The trees were watered as needed and fertilized with slow release fertilizer pellets in amounts prescribed by the manufacturer for the various pot sizes. They were also treated with 300 ml per pot of the liquid fertilizer solution approximately every other month. The native plants and the trees were exposed after they had become well established and had added substantial new growth.

The greenhouse used during this research is located on a research farm south of Raleigh, N. C. Temperature was controlled during the summer by two Alpine water coolers and during the winter by a LP gas heater. Sunlight was augmented on cloudy days by auxiliary 1000 w multivapor halide lamps. The lamps were also used during the shorter winter days to extend the day length to 14 hours.

Plants were exposed to HCl and Al_2O_3 singly and in mixtures in the four greenhouse CSTR chambers. Temperature and humidity varied with the greenhouse conditions. Each chamber was illuminated with one 1000 w multivapor halide lamp to assure a minimum light intensity of 25 klux for all exposures. The chamber was preset, and the plants were added to the chambers at specific times to get the desired exposure durations. Plants were exposed to the SRF exhaust in the four field exposure chambers. Field chamber conditions were dependent on ambient conditions.

Forty-eight to seventy-two hours after each exposure, foliar injury was estimated for individual leaves on a 0-99% basis (in 5% increments including 1 and 99%). When plants had too many leaves for individual evaluation, injury was estimated for the whole plant in 10% increments including 5 and 99%.

RESULTS AND DISCUSSIONS

Plants were exposed to HCl and SRF exhaust using a dose-response (time by concentration) design. The concentrations in the HCl exposures were determined by the results of the HCl screens (not reported in this paper). The following concentration series were used: 0, 5, 10, 20 ppm; 0, 8, 16, 32 ppm; and 0, 10, 20, 40 ppm. The range for citrus was 0, 20, 40, 80 ppm. Duration of exposure was 10, 20, 40, and 80

minutes. All SRF exposures used approximate concentrations (measured as HCl) of 0, 10, 20, and 30 ppm with exposure durations of 10, 20, and 40 minutes. Three replications over three consecutive days and three duplicate plants per replication were run in both experimental designs.

Typical foliar injury from HCl first developed as water soaked areas during or immediately after exposure which became large bifacial interveinal necrotic areas (within 48 hours) that were usually white to off-white in color. For exposures causing slight or moderate injury, necrosis usually occurred on the margins and tips of the leaves. For exposures causing severe injury, the area toward the base and center of the leaves was affected. After short exposures to low concentrations, many plants developed scattered chlorotic spots on the upper leaf surface which resembled oxidant injury. The tips of new pine needles turned distinctly tan; citrus showed yellowish necrotic spots, from a moderate exposure, on the lower leaf surface. Citrus symptoms were bifacial with prolonged exposures to high concentrations of HCl. Injury from the SRF exhaust was generally similar to that produced by HCl. However, some symptoms were suggestive of oxidant injury.

The results of the dose response designs are summarized in Tables 2-5. From these results, the threshold HCl concentrations that are necessary to cause 3-5% foliar injury are shown in Table 6. The data show that plants would not be injured if ground level concentrations were below 5 ppm HCl (in an exhaust cloud) for a 5-15 minute period.

Radish was exposed to Al_2O_3 alone and in combination with HCl. Exposures were also made when the plants were misted either before or just after exposure. Exposure of plants to 20, 40, and 80 mg/m^3 of Al_2O_3 for 60 minutes with or without misting caused no effects on radish and other plants that were tested (Table 7). In HCl + Al_2O_3 mixtures, injury was similar to what was found with HCl alone. A premist increased plant sensitivity to HCl but a postmist had no effect.

Soybean was exposed in a dose response design to HCl to determine if chloride uptake was related to dose (Engel, 1978). The design used 0, 4, 8, and 16 ppm of HCl for 15, 30, 60, and 120 minute exposures. The results (Figure 5) suggest a good correlation between dose and chloride accumulation. Uptake also correlated well with foliar injury. In Figure 5, the dotted lines connect equal doses of HCl and show that at equal doses, more chloride is taken up when the concentration is higher for a shorter time period. The data suggest that no effects should occur at concentrations predicted for the shuttle launch.

Radish was used in a simple experimental design to determine whether the method of applying HCl affected foliar injury. Plants were either exposed to HCl gas or to a submicron hydrochloric acid aerosol. The results (Table 8) suggest that if HCl occurs as an acid aerosol, plants will be less severely affected.

TABLE 2. EFFECT OF HCl DOSE ON FOLIAR INJURY TO SELECTED HORTICULTURAL AND AGRONOMIC CROPS*

Exposure Time (min.)	Foliar Injury (%) at 4 HCl Concentrations (ppm) 0	5	10	20
Radish				
10	0	+	6	49
20	0	3	36	66
40	0	5	49	91
80	0	16	89	98
(LSD - 0.05, 7.7%)				
Soybean				
10	0	0	+	9
20	0	0	1	70
40	0	+	14	70
80	0	6	69	94
(LSD - 0.05, 7.6%)				

Exposure Time (min.)	Foliar Injury (%) at 4 HCl Concentrations (ppm) 0	5	10	20
Tomato				
10	0	0	0	21
20	0	0	3	20
40	0	1	4	35
80	0	5	10	47
(LSD - 0.05, 4.8%)				
Corn				
10	0	0	+	29
20	0	0	2	35
40	0	+	3	31
80	0	+	5	67
(LSD - 0.05, 6.4%)				

Citrus	0	40	60	80
20	0	0	+	+
40	0	0	2	5
80	0	1	9	23
(LSD - 0.05, 11.7%)				

*All values are average foliar injury to the test plants. The injury covers a 0-100% injury range estimated in 5% increments and averaged over 9 test plants (3 duplicates and 3 replicates). Data were analyzed by an analysis of variance, and treatment means were separated by LSD (0.05). The + signifies less than 0.5% average injury.

TABLE 3. EFFECTS OF HCl DOSE ON FOLIAR INJURY TO SELECTED NATIVE SPECIES*

Exposure Time (min.)	Foliar Injury (%) at 4 HCl Concentrations (ppm) 0	8	16	32
Arrowhead				
10	0	+	1	7
20	0	+	1	31
40	0	+	2	55
80	0	1	26	85
(LSD - 0.05, 7.9%)				
Marsh Elder				
10	0	0	+	2
20	0	0	1	22
40	0	+	2	34
80	0	1	5	52
(LSD - 0.05, 4.2%)				

Exposure Time (min.)	Foliar Injury (%) at 4 HCl Concentrations (ppm) 0	10	20	40
Pennywort				
10	0	+	9	23
20	0	1	11	72
40	0	4	60	94
80	0	49	88	98
(LSD - 0.05, 8.2%)				
Wax Myrtle				
10	0	+	1	15
20	0	+	3	21
40	0	1	5	53
80	0	1	12	45
(LSD - 0.05, 10.7%)				

*All values are average foliar injury to test plants. The injury covers a 0-100% injury range estimated in 5% increments and averaged over 9 test plants (3 duplicates and 3 replicates). Data were analyzed by an analysis of variance and treatment means were separated by LSD (0.05). The + signifies less than 0.5% average injury.

TABLE 4. EFFECT OF SRF EXHAUST ON FOLIAR INJURY TO SELECTED HORTICULTURAL AND AGRONOMIC CROPS*

Exposure Time (min.)	Foliar Injury (%) at 4 HCl Concentrations (ppm)** 0	10	20	30
Radish				
10	0	17	54	62
20	0	13	42	64
40	0	34	57	74
(LSD - 0.05, 8.3%)				
Soybean				
10	0	4	21	44
20	0	3	25	39
40	0	23	41	54
(LSD - 0.05, 7.9%)				

Exposure Time (min.)	Foliar Injury (%) at 4 HCl Concentrations (ppm) 0	10	20	30
Corn				
10	0	0	1	5
20	0	1	2	4
40	0	2	4	11
(LSD - 0.05, 1.9%)				
Citrus				
10	0	0	0	0
20	0	0	0	1
40	0	0	0	3
(LSD - 0.05, 1.8%)				

*All values are average foliar injury to test plants. The injury covers a 0-100% injury range estimated in 5% increments and averaged over 9 test plants (3 duplicates and 3 replicates). Data were analyzed by analysis of variance, and treatment means were separated by LSD (0.05).

**Concentrations of SRF exhaust are measured in terms of HCl present in the exhaust and represent approximate concentrations obtained.

TABLE 5. EFFECTS OF SRF EXHAUST ON FOLIAR INJURY TO SELECTED NATIVE SPECIES*

Exposure Time (min.)	Foliar Injury (%) at 4 HCl Concentrations (ppm)** 0	10	20	30
Pennywort				
10	0	4	11	20
20	0	4	13	18
40	0	12	14	44
	(LSD - 0.05, 7.0%)			
Marsh Elder				
10	0	2	3	4
20	0	2	5	12
40	0	4	7	22
	(LSD - 0.05, 3.1%)			

Exposure Time (min.)	Foliar Injury (%) at 4 HCl Concentrations (ppm) 0	10	20	30
Arrowhead				
10	0	0	0	7
20	0	2	8	25
40	0	1	16	35
	(LSD - 0.05, 5.6%)			
Wax Myrtle				
10	0	0	0	1
20	0	0	3	7
40	0	1	6	9
	(LSD - 0.05, 1.2%)			

*All values are average foliar injury to test plants. The injury covers a 0-100% injury range estimated in 5% increments and averaged over 9 test plants (3 duplicates and 3 replicates). Data were analyzed by an analysis of variance, and treatment means were separated by LSD (0.05).

**Concentrations of SRF exhaust are measured in terms of HCl present in the exhaust and represent approximate concentrations obtained.

TABLE 6. CONCENTRATIONS OF HCl (PPM) THAT CAUSE 3-5% FOLIAR INJURY IN EXPOSURES OF DIFFERENT DURATION

Plant Species*	Concentrations of HCl (ppm) at 4 Times (min.) 10	20	40	80
Radish	8	5	4	3
Soybean	16	11	7	4
Tomato	15	10	9	4
Corn	14	11	10	8
Pennywort	15	13	9	5
Arrowhead	26	20	18	10
Wax Myrtle	30	19	16	12
Marsh Elder	35	22	19	14
Citrus	>80	>80	70	50

*The species are ranked in order of susceptibility (sensitive to tolerant) over the 4 exposure durations.

TABLE 7. EFFECT OF Al_2O_3, HCl AND WATER MIST ON FOLIAR INJURY TO RADISH*

Treatments Toxicant	Misting	Average Foliar Injury (%)
Control		0
HCl	No mist	13
	Premist	54
	Postmist	16
HCl plus Al_2O_3	No mist	11
	Premist	50
	Postmist	12
Al_2O_3	No mist	0
	Premist	0
	Postmist	0

*Exposures were 40 minutes in duration; the HCl was at 10 ppm and the Al_2O_3 was 15 mg/m^3.

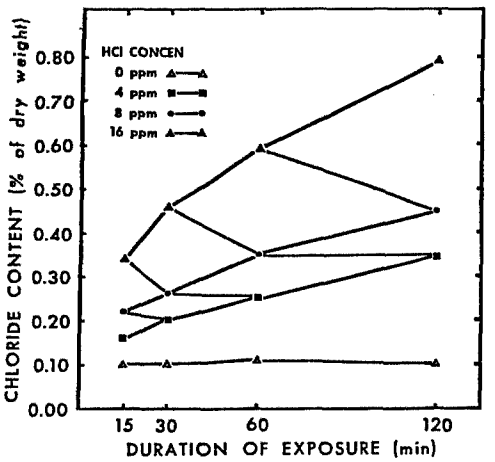

Figure 5. Relation between hydrogen chloride gas concentration and exposure duration on the chloride content of soybean shoots. Solid lines connect points of equal dose (concentration x duration). Each point is the mean for all 12 plants (6 plants per replication). LSD (P = 0.05) = 0.10. The figure was taken from Engel (1978).

TABLE 8. EFFECT OF THE METHOD OF HCl GENERATION ON FOLIAR INJURY (%) TO RADISH*

Method of Generation	Foliar Injury (%) at 4 HCl Concentrations (ppm)			
	0	5	10	20
HCl gas	0	1	30	87
Hydrochloric gas aerosol	0	1	16	49

(LSD - 0.05, 9%)

*Exposures were 60 minutes in duration.

INSECT STUDIES (Romanow, 1979)

Honeybees (Apis mellifera) are of considerable economic importance in Florida for pollination of citrus and vegetable crops, and the production of honey. During the last two decades, the effects of various insecticides and herbicides on bees have been investigated intensively. Atkins et al. (1970) developed many methods for this type of research. We used methods similar to theirs to determine the ED_{50} for HCl gas on honeybees. We also studied the effects of SRF exhaust on bee colony behavior. Using similar techniques, we determined the ED_{50} for HCl for several stages of the corn earworm (Heliothis zea).

MATERIALS AND METHODS

The HCl exposures for both insects were conducted in the greenhouse CSTR chambers used for the plant exposures. The SRF exhaust studies on honeybee were conducted in the field burn system on whole hives of bees.

Two bee colonies were used to collect bees for the greenhouse ED_{50} studies. In the last stages of development, the honeybee worker leaves the hive as a forager; thus, only foragers were exposed to HCl. Groups of 20 foragers were collected in polyester screen cages and exposed to HCl (0-120 ppm) for 15 to 360 minutes. The bees were then moved to observation cages where they were fed continuously and checked daily over a 96-hour period to determine mortality. All tests were duplicated and replicated.

Corn earworm was reared using standard rearing practices. All five larval stages, the egg, and the adults were exposed to HCl. The larva were raised on an artificial diet and transferred, at the appropriate stage, to five mesh cages in groups of 20. They were exposed to HCl at doses similar to those used for the bees and were then returned to their diet cups. Observations of mortality were made daily, and the percent adult emergence was determined.

Data were analyzed using a probit analysis and the ED_{50}'s were calculated.

For the bee colony, behavior studies to SRF exhaust, two colonies, each in a beehive, were used in each of four chambers. The colonies were exposed three times per week for about 3.5 weeks (May 19 to June 12) to a 0, low, medium, and high concentration of SRM exhaust (about 0, 10, 20, 30 ppm HCl). All exposures were for 60 minutes. The colonies were placed in the chambers on May 11 so the bees could adjust to the move. Chamber sides were removed except when exposures were being run. The colonies were left until July 6 to see how they recovered from the exhaust stress.

RESULTS AND DISCUSSION

The honeybee dose response design with HCl clearly shows that they are not sensitive to HCl near the concentration range expected in exhaust clouds. ED_{50} values for honeybee (Figure 6) suggest that over 250 ppm for 10 minutes or 25 ppm for 8 hours would be required to effectively kill 50% of the forager bees. These values suggest that the concentration factor is about 5 times as important as the time factor.

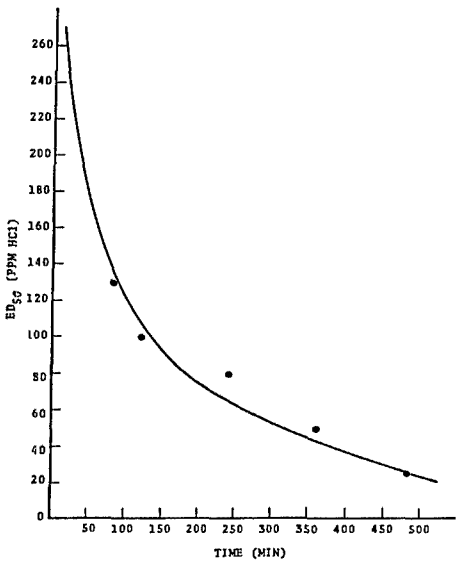

Figure 6. The ED_{50} for HCl effects on honeybee at increasing exposure durations.

The results for the corn earworm show that this species (in the adult stage) is about as tolerant as the honeybee. ED_{50} values are shown in Table 9 for several life stages at exposure durations of 120 minutes. The post ovipositional adult is the most sensitive, but the ED_{50} (55 ppm for 120 minutes) is well above any expected exhaust cloud concentration.

Honeybee behavior responses to SRF exhaust were documented in several ways. The most sensitive and easiest to quantify was brood production as a function of exposure behavior. Brood productivity was measured from May 11 to July 6 to include preexposure and postexposure time periods. Results (Figure 7) show effects at the high exhaust concentrations and suggest effects at all three SRF exhaust concentrations. Brood production in the control colonies shows essentially a linear increase, the low SRF caused a dip in production during exposure, the medium SRF caused a plateau, and the high SRF shows a loss of production. In all cases, recovery appears rapid after the last exposure. However, extended observations showed that the colonies in the high SRF never recovered and were eventually lost.

TABLE 9. THE ED_{50} FOR HCl EFFECTS ON CORN EARWORM AT SEVERAL LIFE STAGES*

Life Stage		ED_{50} in ppm**
Eggs		>200
Larvae:	1st Instar	130
	3rd Instar	100
	5th Instar	>200
Adult:	Preovipositional	70
	Ovipositional (60 minutes)	125
	Postovipositional	55

*All exposures were 120 minutes in duration.

**We were not able to obtain concentrations in excess of 200 ppm.

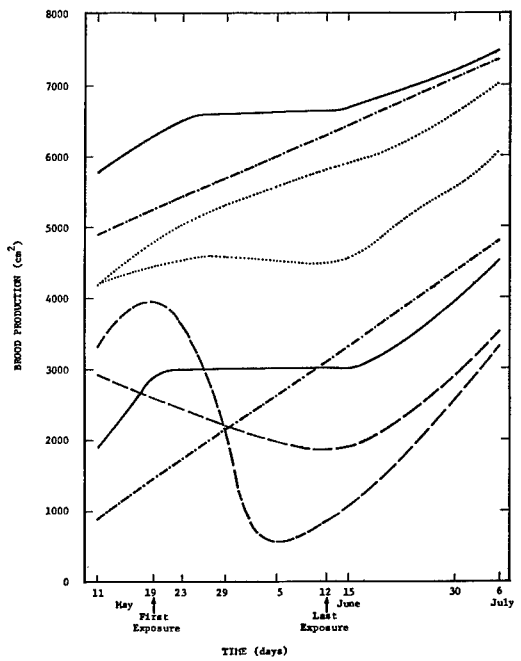

Figure 7. Effects of SRF exhaust on brood production in honeybee hives. The curves represent 2 replications of 4 concentrations of the exhaust (control —·—; low ·····; medium ———; high — —).

The results of these insect studies suggest that no adverse effects on insects will be found as a result of the shuttle programs.

ACKNOWLEDGMENT

The assistance of Mr. Hans Hamann in statistical analysis is gratefully acknowledged. We also thank Dr. Allen S. Heagle for his help in the plant sciences, Dr. Edward P. Stahel in engineering and design, and Dr. John Ambrose in the insect studies. Mr. James H. McCrimmon gave technical support to the entire project. Ms. Madeleine Engel, Ms. Louise A. Romanow, Mr. Alan G. Sawyer, and Mr. James D. Tyson completed their Master's Theses on this project, and parts of their research are used.

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PAPER NO. 26

PHYTOTOXIC EFFECTS OF GASEOUS HYDROGEN CHLORIDE ON CORN (ZEA MAYS, L.)

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INTRODUCTION

Hydrogen chloride (HCl) has been identified as a major exhaust component from the solid propellant rocket motors used to launch Space Shuttle. Gaseous hydrogen chloride is formed as a combustion byproduct of the ammonium perchlorate/aluminum/PBAN propellant. The total solid rocket exhaust constituent mass in the ground cloud formed during launch has been estimated at 250 tons, and HCl will account for approximately 21% by weight (Stephens and Stewart, 1977). One hundred and twenty-nine Space Shuttle launches from Vandenberg AFB, California are anticipated over a ten-year period making acid ground clouds a potential hazard to terrestrial vegetation (SAMSO, 1978; Coulombe and Cooper, 1976; Coulombe and Mahrdt, 1976).

In the past, gaseous hydrogen chloride has not been considered a major phytotoxicant except in isolated instances. Several authors including Heck et al. (1970), Lerman et al. (1976), and Stahl (1969) reviewed the literature concerning damage to plants due to HCl and reported that HCl exposure resulted in various damage to leaf tissue including acid type necrosis, tip burn, interveinal bronzing, rolling, and wilting. At high concentrations, wilting, severe necrosis, stem collapse, and plant death occur (Lind and London, 1971). Haselhoff and Lindau (1903) demonstrated a wide range of phytotoxic responses to HCl gas by various plant species. In two-day exposures,

Viburnum and larch seedings were killed by 5-20 ppm HCl (7.5-30 mg/m^3). Fir, beech, and oak leaves, however, showed only local bleached lesions after a one-hour exposure to 1000 ppm HCl (1500 mg/m^3). Means and Lacasse (1969) studied the relative sensitivity of twelve tree species to gaseous hydrogen chloride. They exposed seedlings to 3-43 ppm HCl for four hours and found Liriodendron tulipifera (tulip tree) to be the most sensitive broadleaf species while Pinus strobus (white pine) was the most sensitive coniferous species. Visible injury occurred at 3 ppm (4.5 mg/m^3) and 8 ppm (12.1 mg/m^3), respectively.

Shriner and Lacasse (1969) exposed 28-day old tomato plants (Lycopersicon esculentum "Bonny Best") to 5 ppm HCl (7.5 mg/m^3) gas for two hours. The test plants developed interveinal bronzing followed by necrosis within 72 hours after exposure. Thomas (1951) found the marking threshold for sugar beets was a few hours exposure at 10 ppm (15 mg/m^3), Godish (1970) exposed tomato seedlings (Bonny Best) to 8-10 ppm HCl (12.1-15.1 mg/m^3) for two hours and found that symptom expression and intensity were a function of relative humidity. As relative humidity increased from 40 to 75%, symptom expression ranged from no visible effects through undersurface glazing and interveinal bronzing to complete tissue collapse. In plants showing visible symptoms, photosynthesis per unit chlorophyll was reduced by twenty-five percent. Significantly, his study showed that plant respiration increased and transpiration decreased even at HCl concentrations causing no visible plant damage.

Studies by Lerman et al. (1976), Garnett and Taylor (1976, 1977), and Heck et al. (1978) considered the phytotoxic effects of hydrogen chloride on ornamental, agricultural, and native plants. They described dose response relationships related to short duration exposures expected from the transitory ground clouds generated by rocket launches. Endress et al. (1978a, 1978b) investigated the anatomical response of Pinto Bean leaf tissues to hydrogen chloride considering the mechanism of HCl damage in plants. In the present study, the effect of gaseous hydrogen chloride on the growth and yield of corn was investigated in order to assess the effects of predicted Space Shuttle exhausts on an agronomically important species.

METHODS

Two sweet corn hybrids, Early Sunglow and Golden Cross Bantam, were exposed to gaseous hydrogen chloride in this study. Seeds were obtained from the W. Atless Burpee Company and grown

under glasshouse conditions until 12 days old. Hydrogen chloride exposures were made at nominal concentrations of 10 ppm (1.5 mg HCl m^{-3} = ppm), 20 ppm, and 40 ppm HCl for periods of twenty minutes. Approximately 200 replicate seedlings were used in each treatment and control per hybrid variety. Exposures were accomplished in a Longley exposure chamber which had a volume of approximately 100 cubic feet. Chamber temperature and relative humidity during exposure periods ranged from 72-77 F and 65-75% RH, respectively. Air flow within the chamber was 32 cfm during the 10 and 20 ppm exposures and 46 cfm during the 40 ppm exposures. Actual mean chamber HCl concentrations are shown in Table 1. Contaminant hydrogen chloride was generated by diluting concentrated HCl cylinder gas (molecular weight, 36.46; technical grade, 99.0%) with chamber air. Continuous analysis of the HCl vapor concentration in the exposure chamber was provided utilizing specific ion electrodes. Known volumes of chamber atmosphere were mixed in a gas scrubber column with known amounts of an aqueous reagent absorber. The solution was then passed through a flow cell containing the electrodes which were calibrated prior to each exposure with standard solutions containing a known concentration of chloride ions.

TABLE 1. MEAN HCl CONCENTRATION (ppm) FOR 20 READINGS TAKEN AT ONE MINUTE INTERVALS DURING EXPOSURE PERIOD

Nominal HCl Concentration (ppm)	Early Sunglow Actual Concentration (ppm) Mean ± S.D.	Golden Cross Bantam Actual Concentration (ppm) Mean ± S.D.
10	8.39 ± 0.36	7.81 ± 0.18
20	19.72 ± 1.37	20.47 ± 1.54
40	48.32 ± 4.06	42.60 ± 4.58

Following the exposure period, plants were returned to the glasshouse where leaf tissue samples were taken for anatomical observation using the scanning electron microscope. Samples were taken immediately following and 48 hours postexposure. Both undamaged leaf tissues and tissues showing symptoms of hydrogen chloride damage were removed from plants in each exposure group. The samples were fixed in Karnovsky's solution and dehydrated in a graded series of ethanol. After transferring the tissues to amyl acetate, the samples were critical point dried. These specimens were then transferred to stubs, coated with gold-palladium alloy, and examined with the scanning electron microscope (Kessel and Shih, 1974). All remaining plants were held in the glasshouse for 10 days during which symptom development was recorded. When the seedlings were approximately three weeks old, each was transplanted to an outdoor agricultural plot. The plot had been fertilized to meet the recommendations of a soil analysis performed by the Ohio Agricultural Research and Development Center, and appropriate herbicides and insecticides had been applied.

At weekly intervals throughout the growing season, approximately 80 plants in each treatment and control were measured to determine plant height and growth rates. The corn was harvested at maturity, and the fresh weight yield of husked ears was determined for each treatment.

RESULTS

Following exposure to gaseous hydrogen chloride, plants were returned to the glasshouse benches where visible damage was observed 48 hours postexposure. Typical HCl damage included tip burn, wilting, marginal necrosis, and bifacial interveinal necrosis. Abaxial (lower surface) glazing reported as a typical symptom of HCl damage in many species (Granett and Taylor, 1976, 1977; Endress et al., 1978a) was not noted in these corn varieties. Damage recorded 48-hours postexposure is shown in Table 2. Both varieties showed similar symptoms; however, damage was more severe in Early Sunglow seedlings.

TABLE 2. HCl DAMAGE 48-HOURS POSTEXPOSURE

Variety	Treatment (20 Minute)	Number Plants Damaged	Percent Plants Damaged	Typical Symptoms
Early Sunglow	Control	0/202	0	None
	10 ppm HCl	176/193	91	Slight tip burn, marginal and interveinal necrosis
	20 ppm HCl	204/204	100	All leaves damaged, bifacial necorsis, wilting
	40 ppm HCl	209/209	100	Severe bifacial necrosis, wilting
Golden Cross Bantam	Control	0/218	0	None
	10 ppm HCl	37/207	18	Very slight tip burn, scattered necrosis
	20 ppm HCl	208/209	100	Bifacial necrosis, wilting
	40 ppm HCl	212/212	100	Severe necrosis over half of leaf surface, wilting

Five seedlings in each group were subsampled for quantitative measurement of HCl damage. All leaves were removed from these plants, and leaf surface area was determined with a Lambda Instruments LI-3000 Portable Area Meter equipped with a LI-3050A Transparent Belt Conveyer Accessory. Necrotic portions of the leaves were excised and their area determined. Results of this quantitative analysis are shown in Table 3. In general, damage area increased as HCl concentration increased. Damage was slight at 10 ppm; however, at 20 and 40 ppm exposure levels, significant percentages of the total leaf area became necrotic. The 40 ppm HCl treatment resulted in necrosis of nearly half of the leaf tissue (Figure 1).

TABLE 3. LEAF AREA DAMAGE 48-HOURS POSTEXPOSURE

Variety	Treatment	Mean Damage Area Per Plant (cm^2)	Percent Damage Per Plant
Early Sunglow	10 ppm	2.7	3.5
	20 ppm	19.6	34.1
	40 ppm	24.9	51.3
Golden Cross Bantam	10 ppm	0.2	0.4
	20 ppm	12.3	24.0
	40 ppm	18.0	46.9

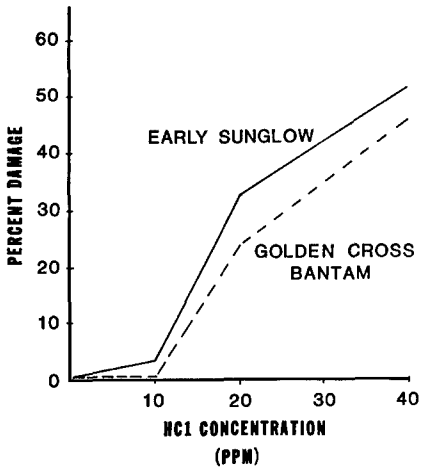
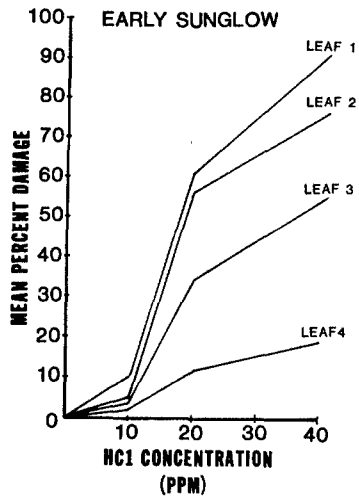


Figure 1. Percentage of plant damage.

Figure 2 shows the mean percent damage per leaf on plants exposed at the four leaf seedling stage. Figure 3 shows the mean area damaged per leaf. Leaf 1 is the primary or oldest leaf while leaf 4 was the uppermost, youngest leaf per plant. In all cases, the youngest leaf (leaf 4) was least expanded and had the least amount of visible damage both in actual area and percentage of leaf surface damaged. Leaf 3 was the most recently expanded leaf and was most sensitive to damage (Figure 3). Since it had the greatest surface area, the percentage of area damaged was less than smaller leaves.



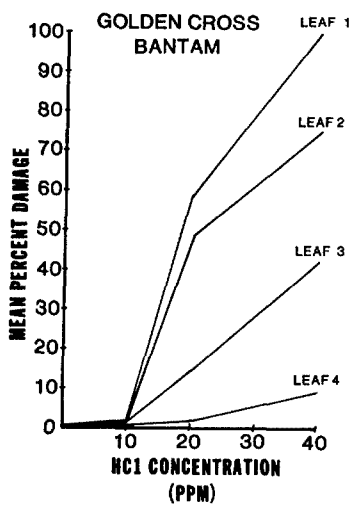
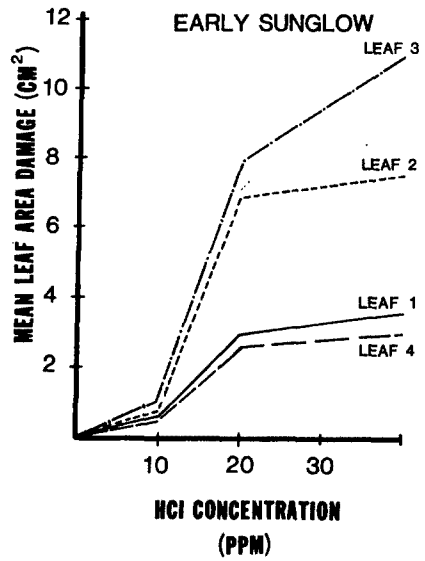


Figure 2. Percent damage per leaf.



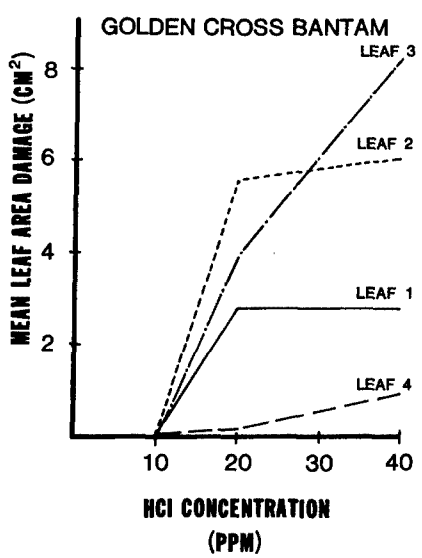


Figure 3. Area damage per leaf.

Figures 4-6 are scanning electron micrographs showing the adaxial surface of Golden Cross Bantam leaves. Figure 4 is a portion of a control leaf near the tip. Most of the surface shows intact, elongate epidermal cells (Ep). Stomates (ST) consist of narrow guard cells associated with subsidiary cells (SC). Along the vein are trichomes (Tr). Figure 5 is the adaxial surface in the region near the leaf tip. The micrograph shows a plant exposed to 10 ppm HCl, 48 hours after exposure. A general collapse of the epidermal and subsidiary cells is apparent. To the naked eye, this damage was evident as interveinal necrosis. Damage which appears as tip burn is shown in Figure 6. This leaf tip was exposed to 10 ppm HCl and shows collapsed epidermal cells although trichomes remain intact.

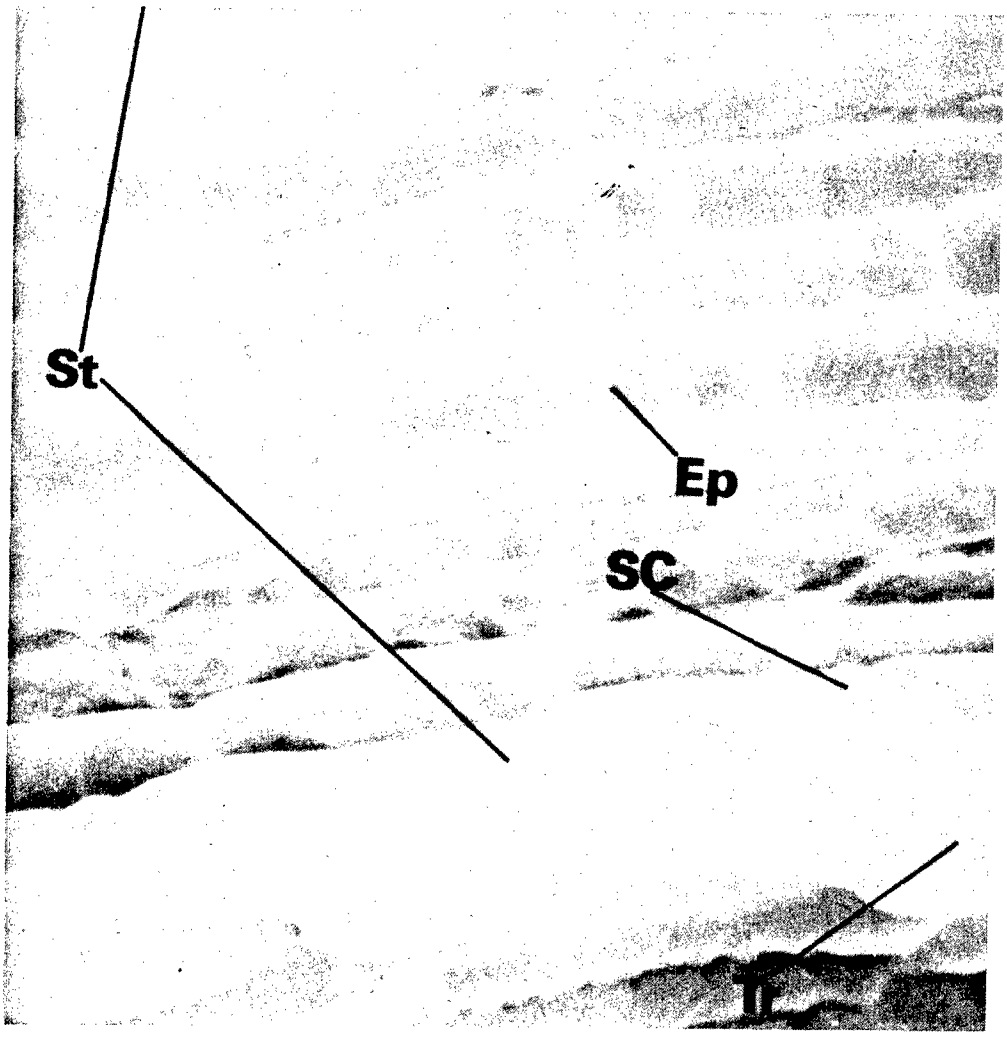


Figure 4. Golden Cross Bantam control leaf surface.

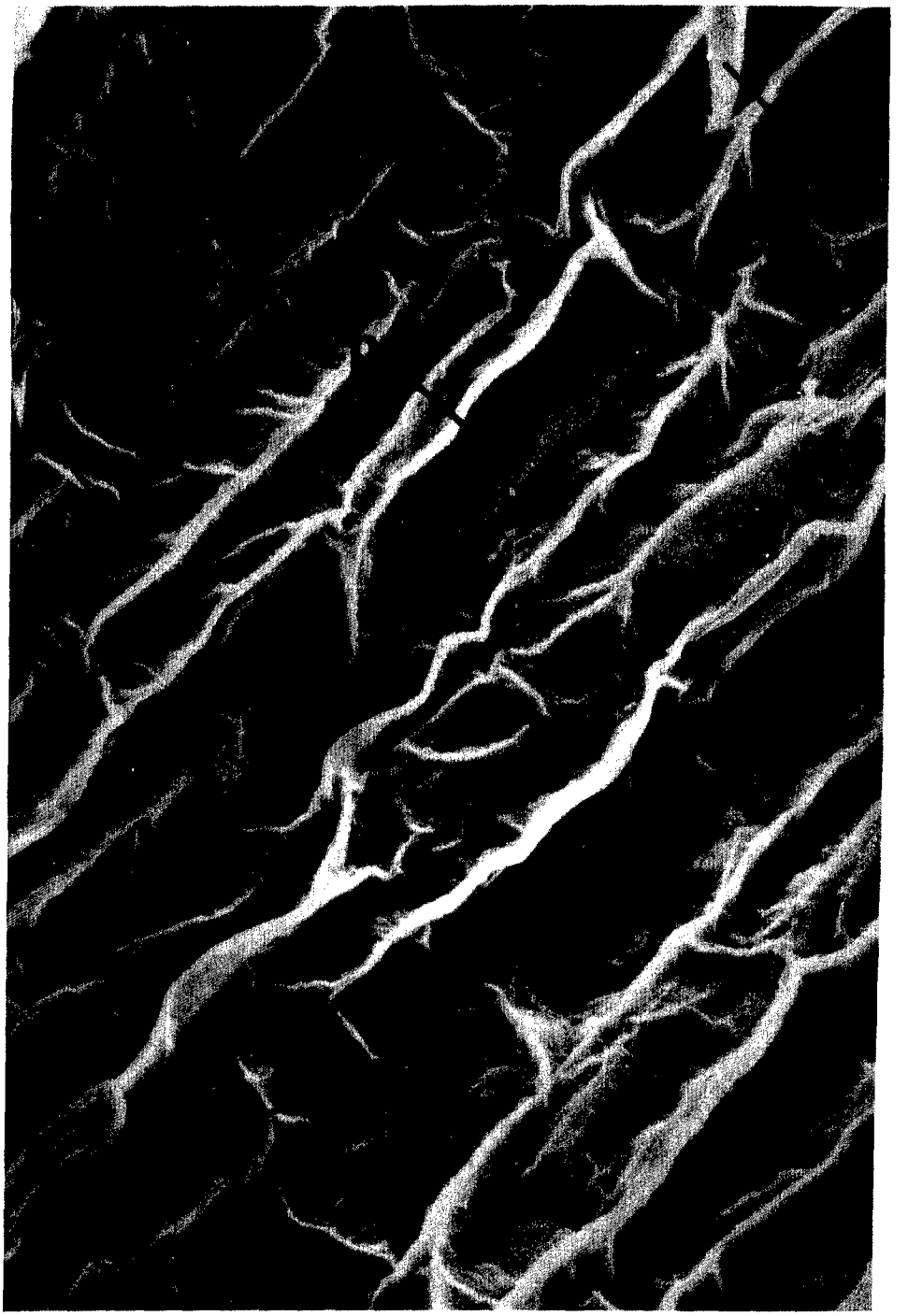


Figure 5. Golden Cross Bantam leaf surface: 10 ppm HCl, 48-hours post-exposure.

Figure 6. Golden Cross Bantam leaf tip: 10 ppm HCl, 48-hours postexposure.

After symptom development, the treated and control plants were transplanted to an outdoor test plot. Measurements of plant heights were taken each week. Table 4 gives the growth data for Early Sunglow. Analysis of variance was performed on the data from each measurement period, and F-ratios marked with asterisks indicate significant differences among treatments.

TABLE 4. EARLY SUNGLOW PLANT HEIGHT

Week	Treatment	Mean Height (cm) ± Standard Deviation	F-Ratio	LSD .05
1	Control	65.83 ± 12.12	**	
	10 ppm	61.38 ± 12.49	44.758	3.72
	20 ppm	55.67 ± 9.37		
	40 ppm	45.42 ± 11.10		
2	Control	64.58 ± 11.84	**	
	10 ppm	59.08 ± 11.95	28.755	3.66
	20 ppm	53.69 ± 9.08		
	40 ppm	48.54 ± 11.48		
3	Control	70.47 ± 13.25	**	
	10 ppm	66.37 ± 14.80	4.893	4.46
	20 ppm	63.44 ± 10.09		
	40 ppm	63.17 ± 15.03		
4	Control	101.17 ± 13.93		
	10 ppm	102.38 ± 13.74	2.017	4.55
	20 ppm	101.41 ± 12.87		
	40 ppm	106.11 ± 14.14		
5	Control	104.00 ± 13.23	**	
	10 ppm	107.46 ± 13.02	16.578	4.44
	20 ppm	108.40 ± ±3.75		
	40 ppm	118.79 ± 12.45		
6	Control	108.25 ± 12.22	**	
	10 ppm	111.00 ± 11.15	19.899	4.22
	20 ppm	112.26 ± 13.59		
	40 ppm	123.37 ± 12.37		
7	Control	107.64 ± 11.50	**	
	10 ppm	111.24 ± 11.67	21.190	4.24
	20 ppm	111.13 ± 12.42		
	40 ppm	122.93 ± 12.37		

**Significant at the 1% level.

Differences in treatment means were tested with the least significant difference test (LSD) at the .05 level of significance (Sokal and Rohlf, 1969). Table 4 shows that all treatment means are significantly different at one and two weeks after transplanting since they vary from each other by greater than the LSD value. Control plants showed the greatest growth while growth in HCl treated plants was inversely correlated with HCl concentration. By week 3, the controls remained significantly taller than the 20 and 40 ppm groups while the 10, 20, and 40 ppm HCl treatments did not vary significantly from each other. During the fourth through the seventh week, the control, 10, and 20 ppm groups were not significantly different, but the 40 ppm group showed a significant stimulation in plant growth. The growth curves are plotted in Figure 7.

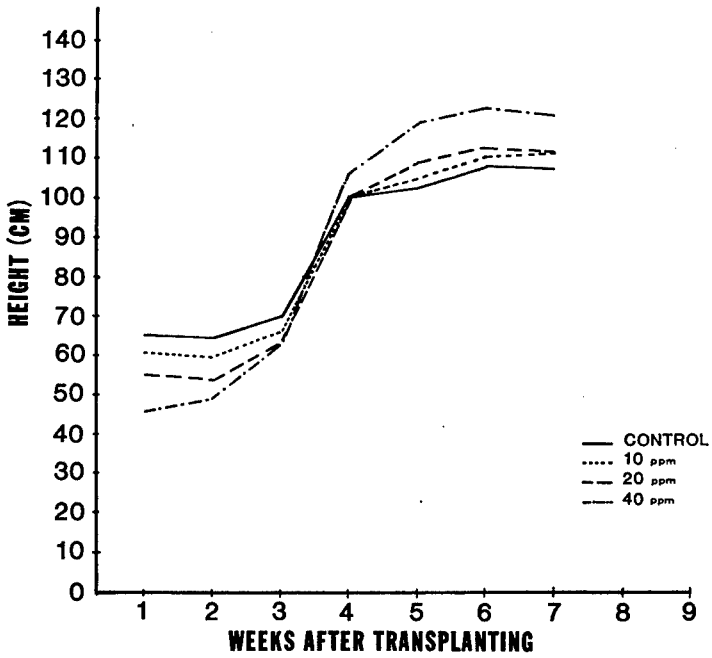


Figure 7. Early Sunglow plant height.

Table 5 presents the plant height data for Golden Cross Bantam. Analysis of the variance reveals significant differences among treatments throughout the growing season. At one and two weeks following transplanting, control plants were the tallest, and the 40 ppm group displayed reduced growth. Through the sixth week, all treatments were significantly smaller than controls, but in the ninth week, only the 10 ppm group was reduced when compared to control plants. This reduction in the 10 ppm group was narrowly significant at the .05 level. Figure 8 depicts the growth curves of each treatment.

TABLE 5. GOLDEN CROSS BANTAM PLANT HEIGHT

Week	Treatment	Mean Height (cm) ± Standard Deviation	F-Ratio	LSD .05
1	Control	62.36 ± 11.26	**	
	10 ppm	53.59 ± 14.19	31.408	4.27
	20 ppm	53.33 ± 12.67		
	40 ppm	43.01 ± 12.31		
2	Control	61.00 ± 12.91	**	
	10 ppm	53.73 ± 15.09	18.842	4.67
	20 ppm	50.07 ± 14.06		
	40 ppm	44.25 ± 12.63		
3	Control	78.07 ± 10.63	**	
	10 ppm	67.44 ± 15.62	13.366	4.80
	20 ppm	65.51 ± 15.41		
	40 ppm	65.83 ± 13.97		
4	Control	105.95 ± 11.55	**	
	10 ppm	92.19 ± 16.39	14.366	5.17
	20 ppm	91.48 ± 17.15		
	40 ppm	95.39 ± 15.16		
5	Control	136.93 ± 16.46	**	
	10 ppm	121.75 ± 20.13	10.304	6.35
	20 ppm	122.57 ± 20.07		
	40 ppm	127.00 ± 18.28		
6	Control	158.66 ± 18.01	**	
	10 ppm	146.43 ± 20.07	8.723	6.62
	20 ppm	143.31 ± 21.26		
	40 ppm	150.73 ± 16.73		
7	Control	164.05 ± 15.97	**	
	10 ppm	153.34 ± 17.19	5.532	5.62
	20 ppm	161.25 ± 16.94		
	40 ppm	160.43 ± 14.92		
8	Control	165.08 ± 13.93	*	
	10 ppm	158.23 ± 15.48	3.587	5.12
	20 ppm	163.14 ± 16.01		
	40 ppm	165.39 ± 13.39		
9	Control	163.81 ± 16.20	*	
	10 ppm	158.53 ± 11.75	3.764	4.96
	20 ppm	166.64 ± 12.35		
	40 ppm	162.80 ± 13.39		

*Significant at the 1% level.
**Significant at the 2.5% level.

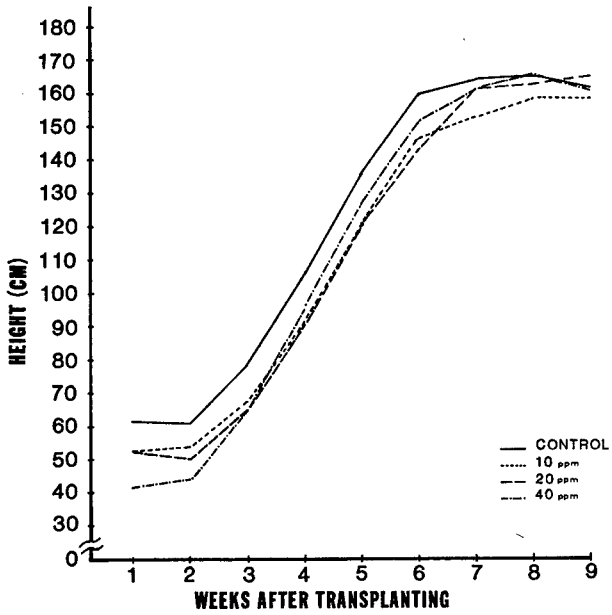


Figure 8. Golden Cross Bantam plant height.

At maturity, the prime ear on each corn plant was harvested and yield in fresh weight for each treatment was determined. Table 6 shows the data for each variety. In Early Sunglow, HCl treatment resulted in no detrimental effects on final yield. A stimulation in yield similar to that noted in plant height resulted from the 40 ppm exposure. Results in Golden Cross Bantam suggest that control plants and plants in the 10 ppm HCl treatment were similar in final yield, but the 20 and 40 ppm treatments were significantly reduced.

In both varieties, little difference resulted in the growing time required for plants to reach maturity among control, 10 ppm, and 20 ppm treatments. In the 40 ppm HCl treatment, however, the time to maturity was delayed by six to eight days.

TABLE 6. YIELD OF HUSKED EARS IN FRESH WEIGHT

Variety	Treatment	Number of Ears Sampled	Mean Weight (gm) + S.D.	F-Ratio	LSD .05
Early Sunglow	Control	161	109.84 ± 23.51	**	
	10 ppm	135	108.10 ± 23.49	18.982	6.98
	20 ppm	172	109.19 ± 29.34		
	40 ppm	172	128.73 ± 36.99		
Golden Cross Bantam	Control	146	160.89 ± 32.41	**	
	10 ppm	99	161.13 ± 37.48	4.102	9.71
	20 ppm	82	147.95 ± 29.10		
	40 ppm	37	147.95 ± 33.31		

**Significant at the 1% level.

DISCUSSION

In terms of visible symptom development, hydrogen chloride has a significant effect on plants. Tip burn and necrosis are apparent at 10 ppm HCl levels following 20-minute exposures. However, damage at 10 ppm is slight in the sweet corn hybrids reported in this study. At 20 ppm, visible damage was much more severe. This finding is in agreement with the results of Granett and Taylor (1977) who described HCl dose response relations in several ornamental and agricultural species. They used probit analysis techniques to calculate the HCl concentrations necessary to produce injury on 50% of exposed leaves (ED_{50}). For 20-minute exposures, their ED_{50} values were largely in the range of 16-20 ppm HCl (24-30 mg HCl m^{-3}). At 40 ppm, both corn varieties in the present study showed damage on all exposed leaves, and approximately 50% of the total leaf area was destroyed by the HCl treatment.

Electron micrographs of HCl damage revealed that the epidermal cells on both the adaxial and abaxial leaf surfaces were sensitive to gaseous hydrogen chloride. HCl damage appears to be a surface phenomenon resulting from the collapse of the epidermal cells. In these plants, the collapse of cells is distributed over large areas of leaf surfaces and appear to be unrelated to the location of stomates (Figure 5). Shriner (1969), however, indicated that the stomatal cells were injured first in tomato leaves as HCl gas entered through the stomates. Collapse of adjacent epidermal cells followed. The present findings are in agreement with Endress et al. (1978b) who showed the phytotoxic mechanism of HCl damage need not depend on HCl entry into the leaf interior through the stomates. They also found no relationship between the location of stomata and the distribution of collapsed epidermal cells. These findings suggest that hydrogen chloride gas may form an aqueous hydrochloric acid as a result of increased relative humidity at the leaf surface. The hydrochloric acid may penetrate the cell cuticle resulting in plasmolysis of the epidermal cells (Endress et al., 1978b).

The effect of HCl on plant growth is shown in Figures 7 and 8. HCl treated plants showed reduced growth following exposure; however, final plant height was not significantly reduced by HCl treatments. In Early Sunglow, a short season hybrid, both the final plant height and yield were significantly greater in the 40 ppm treatment than in control plants.

Crookston and Hicks (1978) have shown increased corn grain yields in short season hybrids affected by early defoliation. They suggest that early defoliation stimulates embryonic ear growth. Growth stimulation in the present study may have resulted from the defoliant effect of HCl treatment, but the extended time to maturity in the 40 ppm treatment may also have allowed greater yield by extending growth over a longer period. Similar enhancement did not occur in Golden Cross Bantam. In this 40 ppm HCl treatment, only 37 ears from a potential 200 ear harvest could be collected, and this reduced sample size is inadequate to explain the yield reduction noted (Table 6). Accurate comparison of yields in Golden Cross Bantam exposed to 20 and 40 ppm HCl cannot be made from this experiment, but no detrimental effect on yield was noted in the 10 ppm treatment.

Predictions of ground level HCl concentrations at Vandenberg Air Force Base, California have been made for a variety of meteorological cases using the NASA Multilayer Diffusion Model. Based on this model, the worst case prediction for maximum instantaneous ground level HCl concentration is 3.38 ppmv, and the approximate 10 minute mean ground level concentration is predicted to be 1.97 ppmv (SAMSO, 1978). The results presented in this paper indicate that these concentrations would have no significant effects on visible damage, plant growth, or yield in plants exposed early in their life cycle. However, other stages in the life cycle may be more sensitive to HCl damage, and further studies need to consider exposure effects during flower and grain formation.

Achievement of desired orbits from Vandenberg Air Force Base, California with payloads currently planned may require greater thrust than previously expected (SAMSO, personal communication). Thrust augmentation will require increased solid rocket propellants and may result in greater ground level HCl concentrations. No ground level HCl computer model predictions based on thrust augmentation data are available at this time, but it appears from this study that 20-minute mean HCl concentrations of 10 ppmv would not result in significant damage to the varieties tested.

CONCLUSION

Hydrogen chloride as a pollutant generated by the solid rocket motors used in the Space Shuttle is known to cause damage to plants. In 20-minute exposures to 10 ppm HCl, visible damage symptoms resulted in leaf necrosis. More severe damage resulted at 20 and 40 ppm HCl treatments where

approximately 25% and 50% of leaf surface area was destroyed, respectively. HCl treated plants showed reduced growth following exposure but final plant height was not significantly reduced by HCl treatments. In terms of plant yield, no detrimental effects were observed up to the 10 ppm HCl concentration. Predicted ground level HCl concentrations for Space Shuttle launches at Vandenberg Air Force Base, California are not likely to result in significant damage to plants.

Studies concerning the physiological effects of HCl on plants native to Vandenberg Air Force Base, California need to be accomplished. Additionally, the ecological effects of repeated HCl exposure on community structure, species diversity, and species composition in native plant communities should be considered over the ten-year period of Space Shuttle operations.

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PAPER NO. 27

EFFECT OF GASEOUS HYDROGEN CHLORIDE ON PLANT LEAF CELLS AND ORGANELLES

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INTRODUCTION

The effects of hydrogen chloride (HCl) gas on vegetation were first noted in Europe and Great Britain in the vicinity of alkali plants during the mid-nineteenth century. In the early days of the Le Blanc soda process, sodium chloride was treated with sulfuric acid, liberating HCl gas as a by-product. Extensive studies on the resultant injured vegetation were made at that time by Haselhoff and Lindau (1903) who reported that cells from fumigated rye, pea, rose and pine leaves were frequently plasmolyzed. In addition, numerous cells were occluded with tannins or contained chloroplasts which were bleached.

HCl gas has not been considered a major air pollutant in the U.S., but elevated levels of the gas and related injury to vegetation have been noted in localized regions near industry utilizing chlorine and in areas where refuse, paper, and plastics containing chlorine or chloride are incinerated (Hindawi, 1968; Lacasse, 1968; Wood, 1968). Previous studies of vegetation injury produced by gaseous HCl have largely emphasized chronic or acute effects (Hindawi, 1968; Means and Lacasse, 1969; Shriner and Lacasse, 1972; Guderian, 1977). Responses to extremely high HCl concentrations of very short duration have not been well examined. Such supra-acute conditions would follow spills, equipment failure during transport, and exhaust from certain solid propellant rockets where massive quantities of HCl would be rapidly released at ground level. Our interest is primarily the responses of plant cells to short-term, high concentration exposures.

Our primary research interest is to elucidate the mechanism with which plants respond to HCl gas. If it is assumed that a plant species responds in a consistent manner when challenged to a particular air contaminant, then supra-acute, that is short-term, high concentration exposures are appropriate vehicles for mechanism studies. We have elected this approach because plants are exposed in nature to supra-acute HCl contamination and because plant responses are intensified and thus are likely to be more readily detected. It is apparent that there are two immediate candidates for the mechanism of gaseous HCl phytotoxicity: (1) acidity with consequent loss of cellular buffering capacity and (2) salinity with accumulation of toxic quantities of chloride ion. We have engaged in a broad approach consisting of structural, metabolic, physiologic and ultracytochemical components to characterize cellular responses to stress from gaseous HCl and to distinguish between the alternative mechanistic possibilities.

OBSERVATIONS

MACROSCOPIC SYMPTOMS

The first visible symptom of injury to unifoliate bean leaves was glazing of the lower leaf surface. Injury increased and interveinal necrosis occurred as [HCl] was increased. These symptoms were similar to those reported by Lerman et al. (1976) for other species. Approximately 15% of the unifoliates from 8-day old plants were injured following exposure to 6.0 mg HCl m^{-3} for 20 minutes, and the percentage increased rapidly until all unifoliates were injured at 41.3 mg m^{-3} (Figure 1). Necrotic lesions were not present on leaves exposed to less than 17.9 mg m^{-3}. Only at concentrations greater than 25.0 mg m^{-3} did more than 10% of the unifoliates exhibit necrotic lesions. Not only were more of the unifoliate leaves adversely affected by increased HCl concentrations but, on the average, more of each leaf's surface area was necrotized with increased [HCl].

In subsequent studies, a distinct relationship between leaf age and incidence of injury to the unifoliate leaves of exposed plants was evident following exposure to anhydrous HCl (Figure 2). Unifoliate leaves on 6-day old plants were completely resistant to injury. The number of leaves injured, expressed as a percent of the total exposed, increased with maturity until a maximum of 83% exhibited injury of 11 and 12 day old plants. The percentage of leaves with only necrotic lesions also increased with maturity to a maximum of 37% at day 12. Although the functional relationship between injury and leaf chronological age was significant when fit to a linear regression, the spatial distribution of the data suggested that

that relationship could be sigmoidal. This possibility was examined utilizing probit analysis (Finney, 1971). The data conformed to the required binomial distribution necessary for probit analysis and an estimated stage of maturity where 50% of the unifoliate leaves were injured (EM_{50}) was calculated to be 9 days of age.

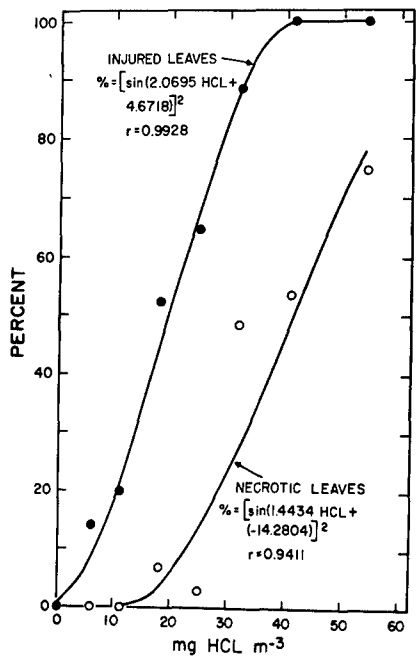


Figure 1. Regression of HCl concentration on the percent of injured 8-day old bean unifoliate leaves and on the percent of leaves with necrotic lesions. Percent leaves injured and percent necrotic leaves were transposed to arcsine degrees. Each percent injured leaves point is the average of a minimum of 32 leaves (from Endress, Swiecki, and Taylor, 1978).

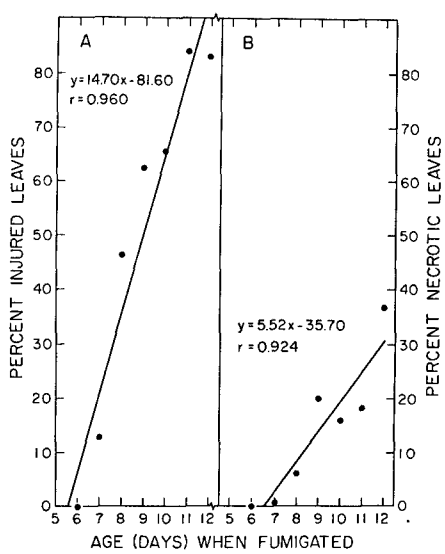


Figure 2. Relationship of plant age and (A) the percent of unfoliate leaves expressing visible injury (abaxial glazing + necrosis) and (B) the percent of unifoliate leaves with necrotic lesions following exposure to HCl gas. Each represented point is based on 120 leaves (from Endress, Oshima, and Taylor, 1979).

The observed age-HCl sensitivity relationship was subsequently related to alterations of leaf expansion after treatment (Figure 3). Leaf expansion, measured in terms of leaf area, was found to be linear with the natural logarithm of leaf age. Expansion was progressively retarded by increased leaf age at treatment with gaseous HCl up to 12 days of age. The older, more susceptible leaves (11 and 12-day old) were affected most whereas only minor differences were detected between control leaves and the youngest leaves (6 and 7-day old) exposed to HCl. The rate of leaf expansion of both control and HCl-treated plants was statistically indistinguishable, even though the unifoliates of plants treated at 11 or 12-days of age were smaller than the controls. Age-dependent differences in leaf expansion resulted from delayed expansion and not from alterations in the rate of expansion. This relationship was observed for all leaf ages except for plants fumigated at age 9 days, which expanded at a significantly greater rate. Despite this statistical inference, the biological response illustrated by exposures at the other maturity stages indicated that leaf expansion of the 9-day old plants was probably not representative. Leaf expansion was delayed by a single HCl treatment and then proceeded at the same rate as control leaves. The extent of the delay appeared to be directly related to leaf maturity. Older, more sensitive leaves were delayed longer than younger leaves before expansion was resumed.

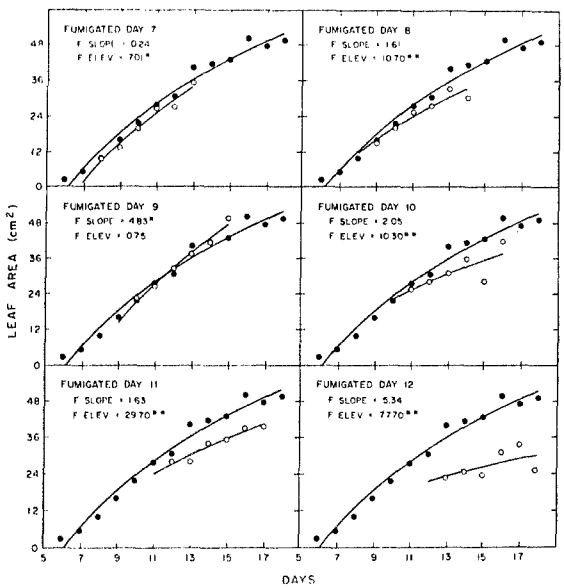


Figure 3. Area increase of control (o) and HCl-treated unifoliate leaves (●) following exposure of different aged plants; for control leaves, area (cm^2) = 48.31 (in days) - 87.66. All comparisons are of control and treated leaves and all fumigation expansion functions, except for 9-day leaves, were significant at the 0.05 level or better. Each represented point is the mean of 20 leaves. * and ** is significant at the 5% and 1% levels, respectively (from Endress, Oshima, and Taylor, 1979).

The difference in leaf area expansion was not explained by the severity of injury or by the reduction of viable leaf tissue due to HCl treatment. The preponderance of injury was glazing of the lower leaf surface. No significant relationship was observed when the percent injured leaves or percent necrotic leaves were compared to leaf area expressed as percent of control leaf area. Moreover, the ratio of fresh weight to dry matter, an estimate of water content, remained stable and unaltered. In all instances, the weights of treated leaves were colinear with those of control. As anticipated, statistical comparisons of slope and elevation of treated and control leaves demonstrated no significant fresh or dry weight differences. Thus, there was no change in the relationship between fresh and dry weight following HCl exposure.

MICROSCOPIC SYMPTOMS

Numerous injury symptoms were evident in sectioned bean leaves exposed to different concentrations of HCl gas. A given symptom appeared in most samples throughout the concentration spectrum. Scattered or random cells were injured with lower HCl concentrations and increased with increasing concentration. Plasmolysis, cytoplasmic vesiculation, cell wall deformation, and accumulation of particulate materials in vacuoles were noted in treated tissue.

After treatment with 6.0 or 11.3 mg m^{-3}, glazing of the lower leaf surface only became apparent 24 hours after HCl exposure. In sectioned tissue, the glazing symptom appeared to result from the collapse of epidermal cells (Plates II E; III A,C.D). Collapse resulted from deformation of both the inner and outer (periclinal) cell walls. The anticlinal walls were commonly distorted and folded (Plate I C,D) and collapse of the outer periclinal wall occurred toward the cell lumen. The net consequence of cell wall deformation was that, with time, the collapsed epidermal cells appeared crushed. In the 24-hour postfumigation samples, collapsed regions appeared as unusually thick, single cell walls on both the upper and lower leaf surfaces (Plates II E; III A,C,D). Collapsed areas of epidermis were distributed independent of stomata and their subsidiary cells, of vascular bundles in the adjacent mesophyll, and of each other. Guard cells were usually less affected than the surrounding epidermis (Plate II B).

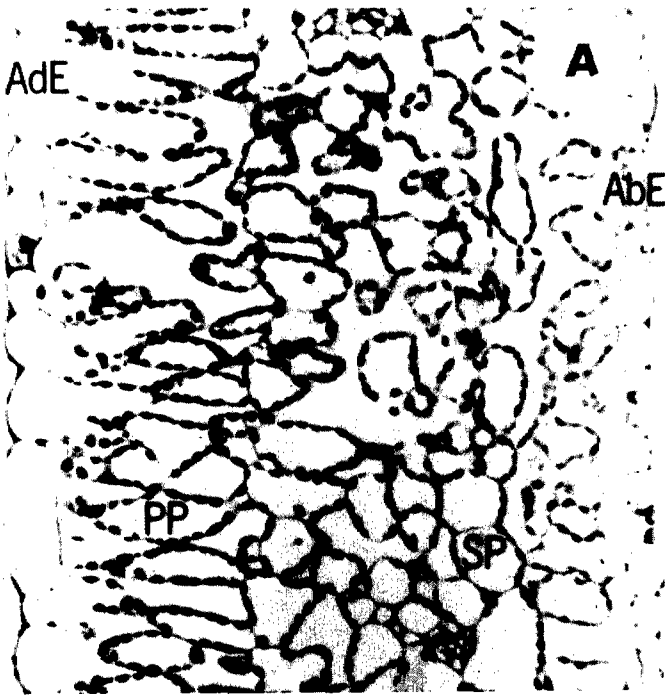


Plate I (from Endress, Swiecki, and Taylor, 1978).

Plate I A. Section of an 8-day old pinto bean leaf exposed entirely to filtered glasshouse air. AdE = adaxial epidermis, AbE = abaxial epidermis, PP = palisade parenchyma, SP = spongy parenchyma. 205x.

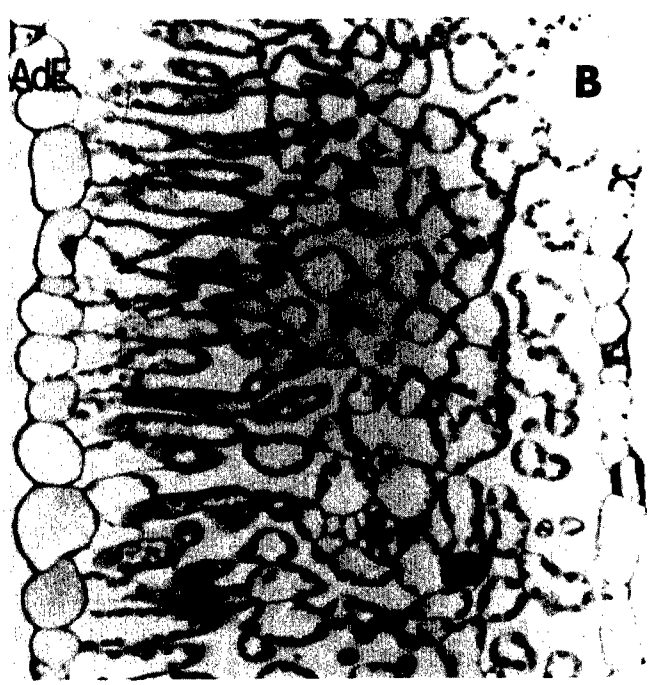


Plate I B. Section of an 8-day old pinto bean leaf sampled immediately after 20 minute exposure to filtered glasshouse air in the fumigation chamber. Note that adaxial epidermal cells (AdE) retain stain. 205x.

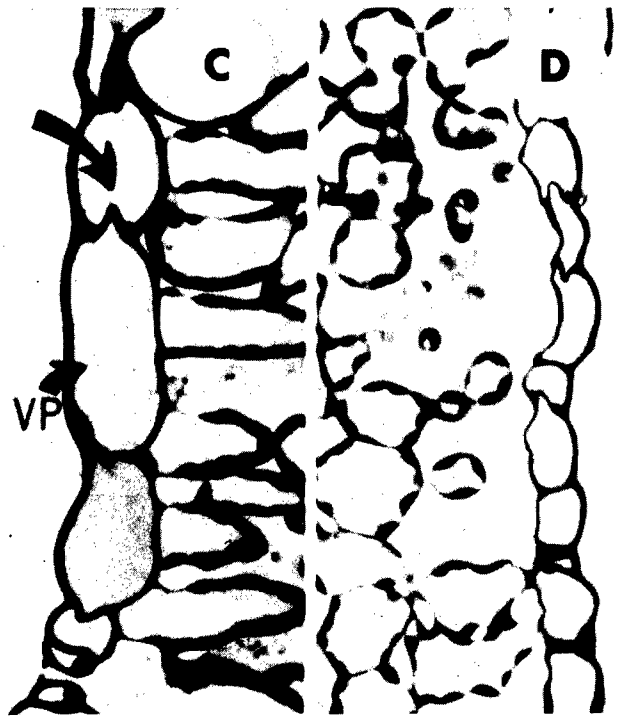


Plate I C. Partial collapse of the adaxial epidermis and staining of epidermal vacuoles is due to presence of a granular material (VP). The anticlinal cell walls of the epidermal cells are distorted. Tissue obtained immediately after exposure to 6.0 mg HCl m^{-3} for 20 minutes. 320x.

Plate I D. Partial collapse of the abaxial epidermis. Vacuolar precipitates are apparently absent from these cells. Tissue sampled immediately after exposure to 6.0 mg HCl m^{-3} for 20 minutes. 320x.

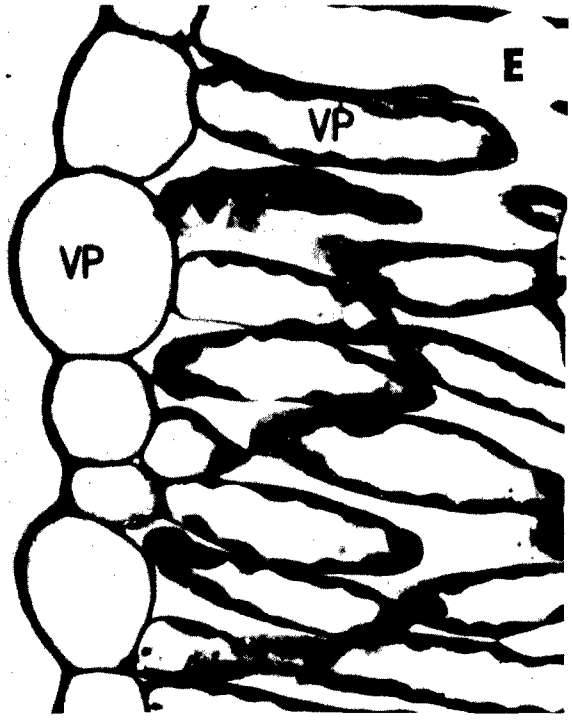


Plate I E. Primary bean leaf sampled immediately after exposure to 6.0 mg HCl m^{-3} for 20 minutes. Palisade parenchyma cells as well as adaxial epidermal cells contain granular precipitates in vacuoles. 485x.

Plate II (from Endress, Swiecki, and Taylor, 1978).

Plate II A. A leaf exposed for 20 minutes to 41.3 mg HCl m^{-3} and sampled immediately. Note cytoplasmic vesiculation in the adaxial epidermis (Ve) and presence of vacuolar particulates (Vp). Raphide-like crystals (Cr) are apparent in chloroplasts of the palisade parenchyma. 550x.

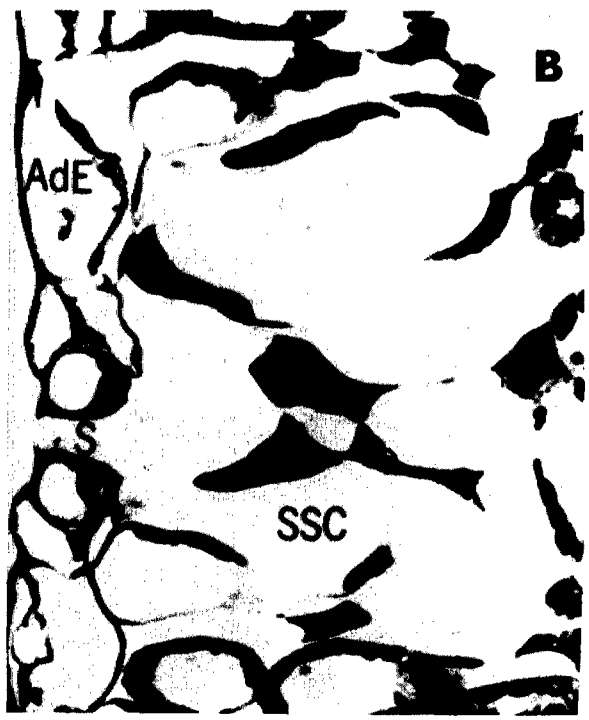


Plate II B. Plasmolysis of the adaxial epidermis (AdE) and palisade parenchyma bordering a substomatal chamber (SSC) are illustrated in this section taken from a leaf immediately after exposure to 25.7 mg HCl m^{-3} for 20 minutes. Most parenchyma cells are not plasmolyzed under these conditions. 530x.

Plate II C. Treatment as in A except that protoplasts of the adaxial epidermis are completely collapsed and in palisade parenchyma, there is considerable vesiculation of the central vacuoles. 530x.

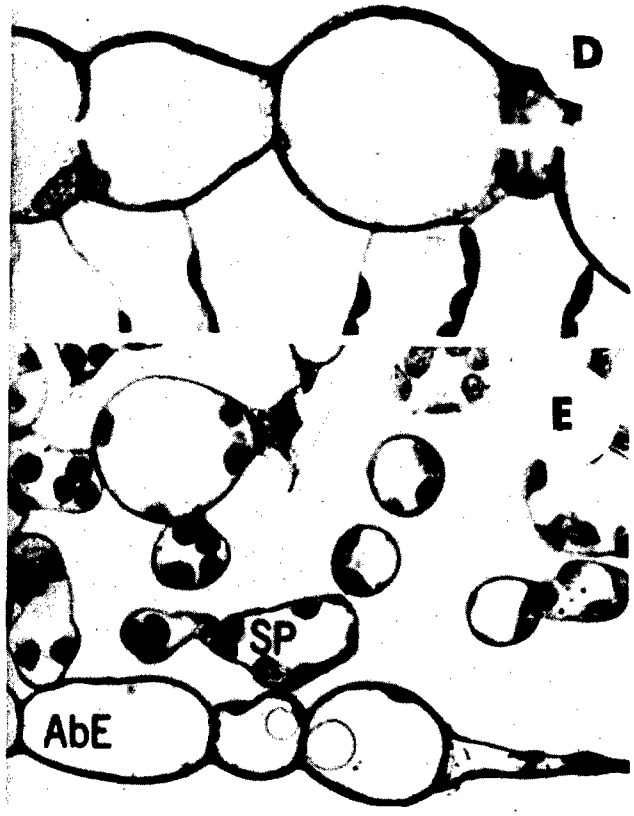


Plate II D. Rupture of the anticlinal cell walls of adaxial epidermal cells is shown in this section taken 24 hours after exposure to 6.0 mg HCl m^{-3} for 20 minutes. Note that vacuoles of the epidermal cells contain a particulate material. 575x.

Plate II E. Segment from a leaf 24 hours after being fumigated 20 minutes with 54.2 mg HCl m^{-3} showing intact and collapsed regions of abaxial epidermis (AbE). Although one of the spongy parenchyma cells is plasmolyzed (arrow), most appear unaltered. 485x.

Plate III (from Endress, Swiecki, and Taylor, 1978).

Plate III A. Montage from a rib area of a leaf sampled 24 hours after exposure to 54.2 mg HCl m^{-3} for 20 minutes. Extending laterally from the rib, most parenchyma cells are plasmolyzed and epidermal cells at both surfaces have collapsed. Cells at the rib appear to be relative unaffected. 175x.

Plate III B. Section of a primary leaf taken immediately after exposure for 20 minutes to 25.7 mg HCl m^{-3}. Some cells in both epidermal layers are plasmolyzed and/or partially collapsed at Ab and Ad. Other epidermal cells are of normal appearance save for adaxial epidermal cells with vacuolar staining. Note that spongy parenchyma bordering substomatal chambers (arrows) are not altered. 200x.

Plate III C. Portion of a leaf sampled 24 hours after fumigation with 41.3 mg HCl m^{-3} for 20 minutes showing extensive collapse of the abaxial and adaxial epidermis without any apparent adverse affects on the parenchyma cells. 180x.

Plate III D. Same as C except that treatment was with 54.2 mg HCl m^{-3}. Note that spongy parenchyma bordering a substomatal chamber (arrow) exhibit a normal appearance. 200x.

A frequently observed symptom related to cellular collapse was plasmolysis of the epidermal protoplast. The protoplast of collapsed cells was always plasmolyzed and numerous other epidermal cells were partially plasmolyzed. In instances of more severe HCl stress, irreversible plasmolysis occurred with the protoplast clumping in the center or at one side of the cell (Plates II B; III B,D). Further observations suggested that with treatments evoking maximum stress, cell wall collapse occurred subsequent to total plasmolysis of the protoplast.

Mesophyll tissue was usually affected to a lesser extent than the epidermis. The most common injury symptom in mesophyll tissue was plasmolysis. Plasmolysis of the parenchyma cells bordering substomatal chambers did not occur regularly, but in spongy parenchyma, plasmolyzed cells occurred near collapsed epidermal cells (Plate I D; II E). Membranous bubbles were also seen in vacuoles of palisade and spongy parenchyma and of epidermal cells (Plate II A,C) of all leaves treated with 6.0 mg HCl m^{-3} or more and sampled immediately or 24 hours after treatment. These were interpreted as vesicles or small vacuoles. In addition, epidermal cell vacuoles tended to accumulate a particulate substance (Plates I B,C,E; II D; III B).

Raphide-shaped crystalline arrays (Plate II A,C) were noted in mesophyll cells of leaves fumigated with 11.3 mg m^{-3} and above, and sampled within 30 minutes after completion of the fumigation. Except for the samples obtained from plants exposed to 41.3 or 54.2 mg HCl m^{-3}, these crystals were not present in cells one hour or more after the end of the treatment. These crystalline associations appeared to be contained within the chloroplasts and not cytoplasmic or vacuolar in location.

The absence of numerous plasmolyzed mesophyll cells and the apparent absence of crystals in chloroplasts from tissues sampled 24 hours after HCl exposure suggested that some cells, at least, were reversibly stressed and indicated that a more detailed characterization of cellular responses to gaseous HCl was needed. Since injured areas were dispersed throughout the lamina of leaves, it was possible to sample injured and apparently healthy tissue to approximate injury severity at the fine structural level. Twenty-four hours after HCl treatment, most leaf cells exhibited an ultrastructure which distinguished them from cells in control plants. Some leaf cells contained no conspicuous abnormal features while others were severely disrupted. It was apparent that cellular injury was variable, and it was possible to characterize the degree of injury as none, slight, moderate, severe, or total.

Slight Disruption

The general organization of cells remained unaffected by exposure to HCl gas, i.e., the cytoplasm was restricted to the cell periphery surrounding a central vacuole and the usual organelle complement was distributed throughout the cytoplasm in a normal matter. Chloroplasts, however, tended to form elongate protrusions with cytoplasmic in-pockets (Plate IV A), and the chloroplast stroma was more dense than that observed in control chloroplasts (Plate IV E). Mitochondria were also consistently misshapen with transparent matrices (Plate IV A-D).

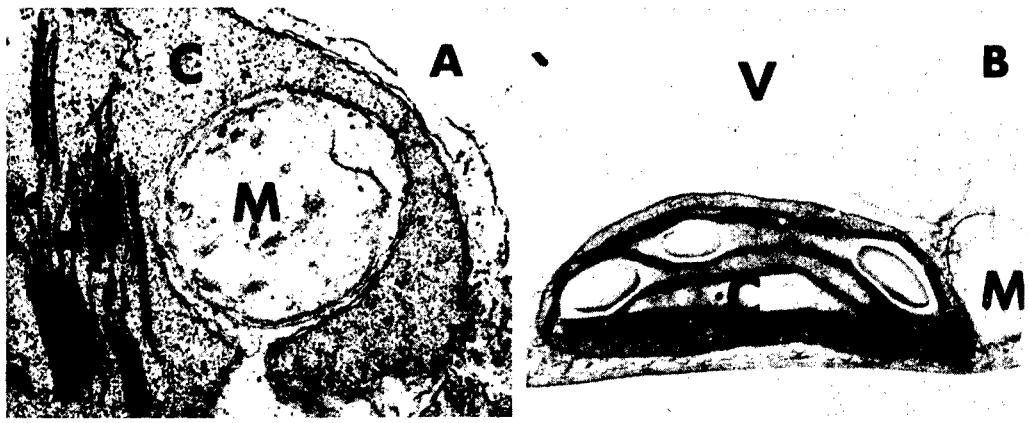


Plate IV (from Endress, Kitasako, and Taylor, 1978). (A-D, Slight disruption.)

Plate IV A. Segment of a spongy parenchyma cell showing a misshapen mitochondrion (M) with a transparent matrix and an elongate protrusion of a chloroplast (C) encircling it. 35,000x.

Plate IV B. Portion of a palisade parenchyma cell illustrating an abnormal mitochondrion (M). V = vacuole. 11,250x.

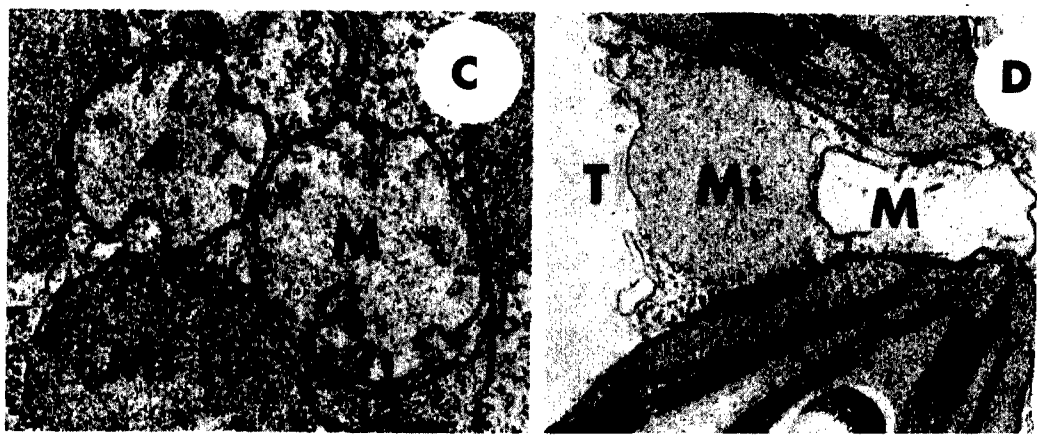
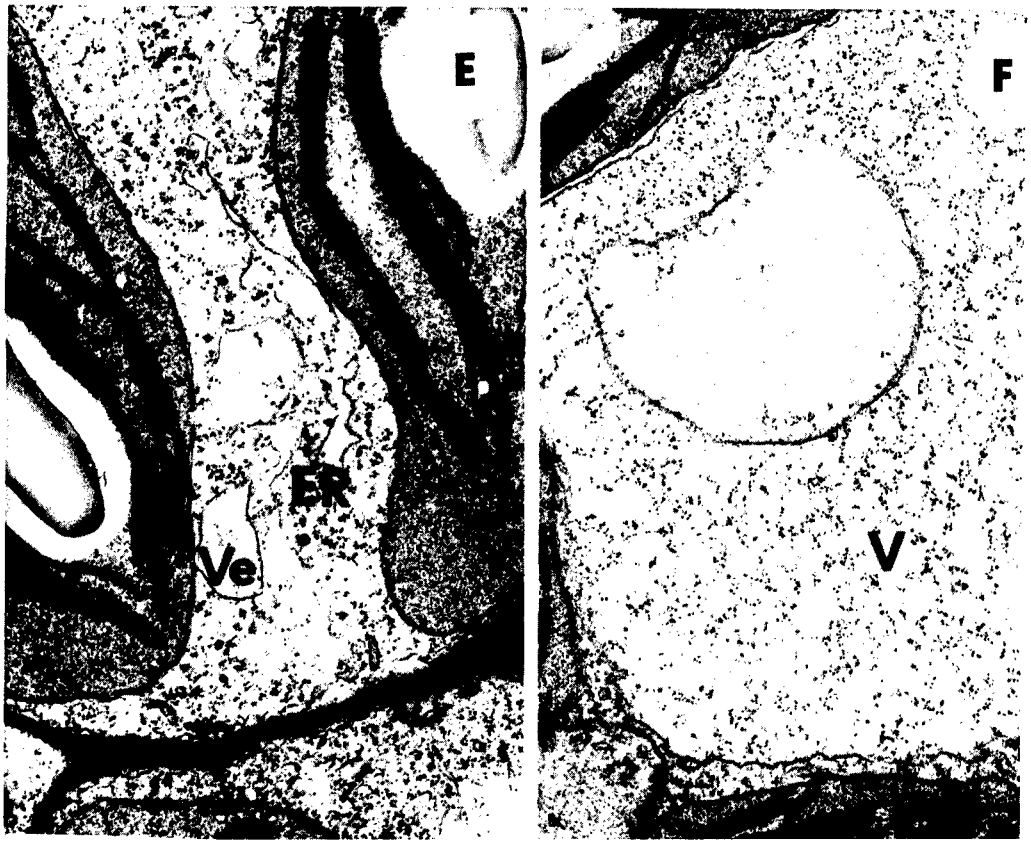


Plate IV C. Portion of a spongy parenchyma cell in which the microbodies (Mi) are normal in appearance, but mitochondria (M) are misshapen and transparent. 35,000x.

Plate IV D. Segment of a spongy parenchyma cell with disruption of the tonoplast (T) and an abnormal mitochondrion (M). 28,000x.

Moderate Disruption

In addition to chloroplasts with increased stromal density and misshapen, transparent mitochondria, moderately disrupted cells were characterized by increased injury to cytoplasmic membranes. The cytoplasm was slightly vesiculate (Plate IV E), containing vesicles of variable size and randomly located. Most vesicles were apparently devoid of content, but in some there were electron-dense particulates (Plate IV E) or membrane fragments. Central vacuoles of parenchyma cells also contained occasional membrane fragments and particulate deposits (Plate IV F). In a few instances, the tonoplast was disrupted (Plate IV D).



(E-F, Moderate Disruption).

Plate IV E. Portion of a palisade parenchyma cell illustrating the inflation of endoplasmic reticulum (ER) cisternae to form small cytoplasmic vesicles (Ve). 23,000x.

Plate IV F. Segment of a palisade parenchyma cell showing the accumulation of a particulate material and membrane fragments in the central vacuole (V). 23,000x.

Severe Disruption

With severe disruption, cytoplasmic vesiculation was very prominent (Plates V A,E; VI B). Vesicle size varied, but most were large and the majority contained a considerable quantity of particulate materials (Plate V B,D). Central vacuoles and cytoplasmic vesicles in both parenchyma and epidermal cells often contained membrane fragments. These fragments were seen as scattered, individual entitites (Plate V D,E) or as myelin-like associations and multivesicular arrangements (Plate V C). Electron-dense globules which

lacked a bounding membrane were distributed throughout the cytoplasm of severely disrupted cells (Plate VI A). These were particularly apparent at the cytoplasm periphery where they concentrated adjacent to the plasmalemma (Plate VI B).

Total Disruption

In the most severely affected cells, the contents were clumped into a single large mass at the center or periphery of the cell (Plate VI C). Degeneration of the plasmalemma, tonoplast, and organelle membranes was total or nearly so. Completely disrupted cells were noted in the palisade and spongy parenchyma tissue, but the preponderance of such cells occurred in both epidermal layers where cell wall deformation (Plate VI C) accompanied cellular disruption.

In contrast to the nearly normal chloroplasts in cells sampled one day after HCl treatment, the chloroplasts in cells sampled immediately after exposure to HCl gas exhibited several alterations. A distinctive feature of chloroplast appearance was the presence of crystalline structures primarily located in peripheral regions of the stroma adjacent to the chloroplast envelope (Plate VII A-D). These crystalline arrays were often massive, causing distortion of chloroplast shape (Plate VII B). The crystals most frequently consisted of linear, electron-dense elements separated by an electron-transparent space (Plate VII A,C), but some had a cross-hatched appearance (Plate VII B,D). Crystals were frequently observed at sites where alterations of the envelope occurred. These alterations consisted of localized distention and/or disruption of the envelope and accumulation of an electron-dense material between the two membranes of the envelope (Plate VIII A,B).

Alterations of the grana-fretwork membrane system were also noted (Plate VII B,D). Frets were occasionally undulated in appearance and slightly swollen. Terminal granal compartments also showed some swelling. Enlargement of terminal granal compartments and interconnecting frets was more pronounced in chloroplasts which were located in severely plasmolyzed cells. Additional fine structural modifications of chloroplasts included increased granulation and density of the stroma and staining of the envelope (Plate VIII C,D).

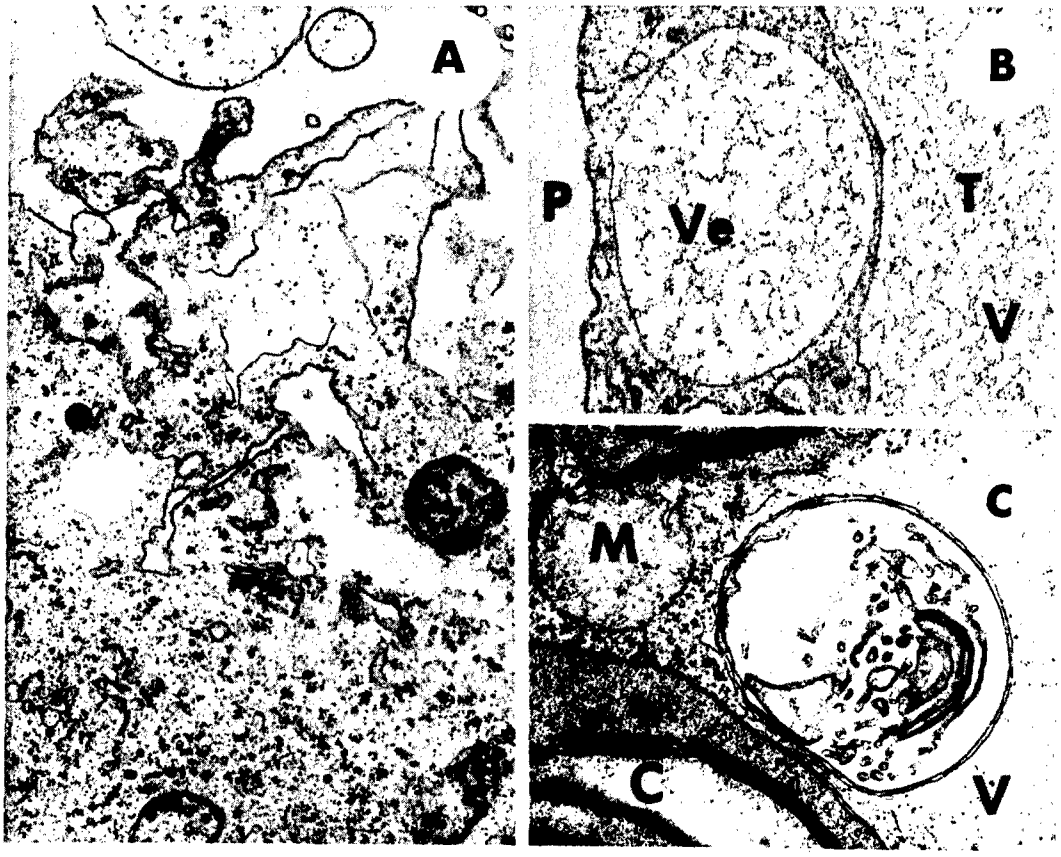


Plate V (from Endress, Kitasako, and Taylor, 1978). (A-E, Severe Disruption).

Plate V A. Part of a spongy parenchyma cell showing severe disruption of the cytoplasm. 19,000x.

Plate V B. Portion of an upper epidermal cell in which a particulate material has accumulated in the central vacuole (V) and smaller cytoplasmic vesicles (Ve). 39,500 x.

Plate V C. Segment of a spongy parenchyma cell illustrating a multi-membrane association with the cell vacuole (V). 28,000x.

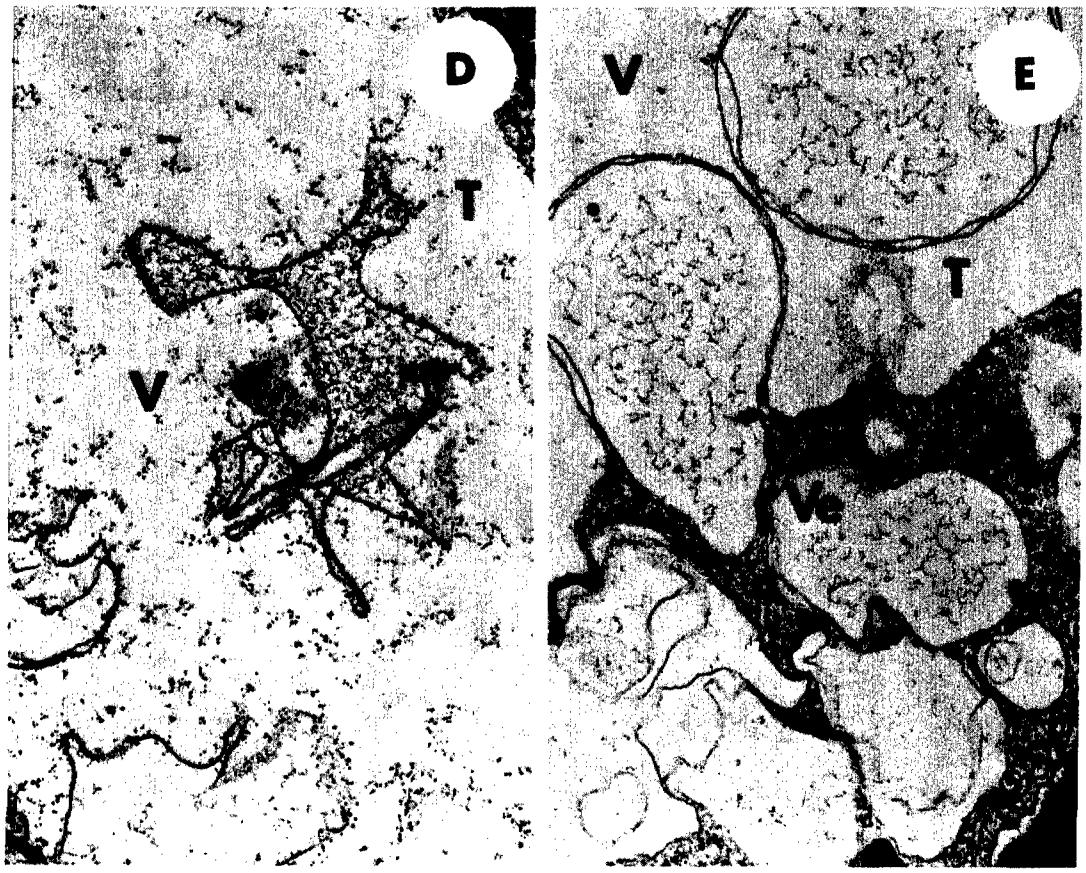


Plate V D. Part of palisade parenchyma cell in which membrane fragments and a particulate material are accumulating in the vacuole (V). 23,000x.

Plate V E. Portion of a spongy parenchyma cell in which vesiculation of the cytoplasm is prominent. 23,000x.

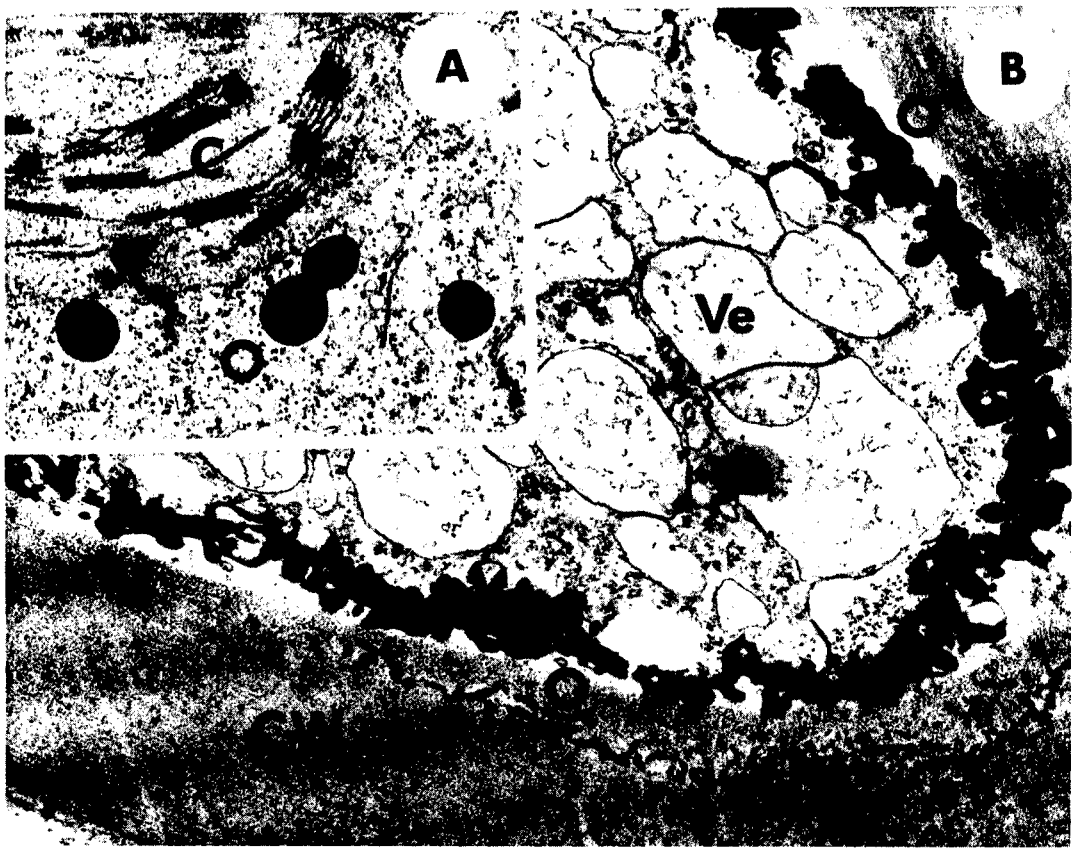


Plate VI (from Endress, Kitasako, and Taylor, 1978). (A-B, Severe Disruption. C, Total Disruption).

Plate VI A. Portion of a palisade parenchyma cell showing osmiophilic globules (O) in the cytoplasm. 28,000x.

Plate VI B. Part of a lower epidermal cell in which the cytoplasm has become highly vesiculate (Ve) and an osmiophilic material (O) has collected at the plasmalemma and in the cell wall (CW). 28,000x.

Plate VI C. Total disruption. Portion of the upper epidermis in which some epidermal cells are completely plasmolyzed. The anticlinal walls of these cells are greatly distorted as well. 8,500x.

Plate VII (from Endress, Kitasako, and Taylor, 1979).

Plate VII A. Chloroplast (C) from a leaf exposed to 21.1 mg HCl m^{-3} for 20 minutes. Crystals (cr) composed of linear elements are extensive and located at the stroma periphery. Between the membranes of the chloroplast envelope, there are deposits (arrows) of electron dense material. Mitochondria (m) are altered in conformation and have an electron transparent matrix. mb = microbodies. 18,500x.

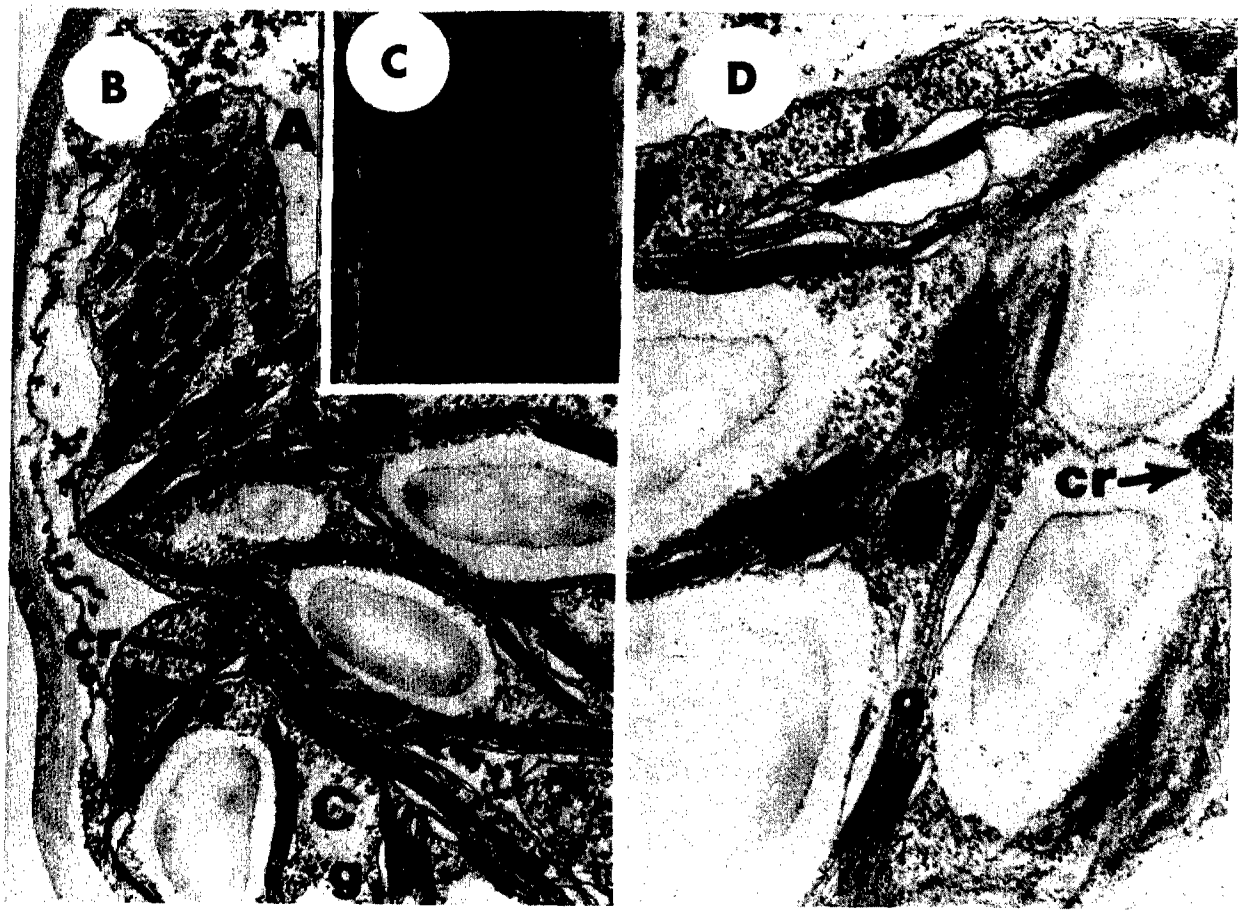


Plate VII B. Chloroplasts (C) containing crystals (cr) in periphery of stroma and adjacent to grana. Crystallization may promote chloroplast distortion as indicated by the protuberance at A. Swollen terminal granal compartments are evident (g). 27,500x.

Plate VII C. High magnification of a chloroplast stroma crystal taken from a leaf exposed to 32.9 mg HCl m^{-3} for 20 minutes. The 5.0 nm diameter electron-dense fibrils are separated by a space of 2.5 nm. 213,000x.

Plate VII D. Portion of a chloroplast showing swelling of terminal granal compartments (g) and cross-hatched crystal appearance (cr) when sectioned transversely. 39,500x.

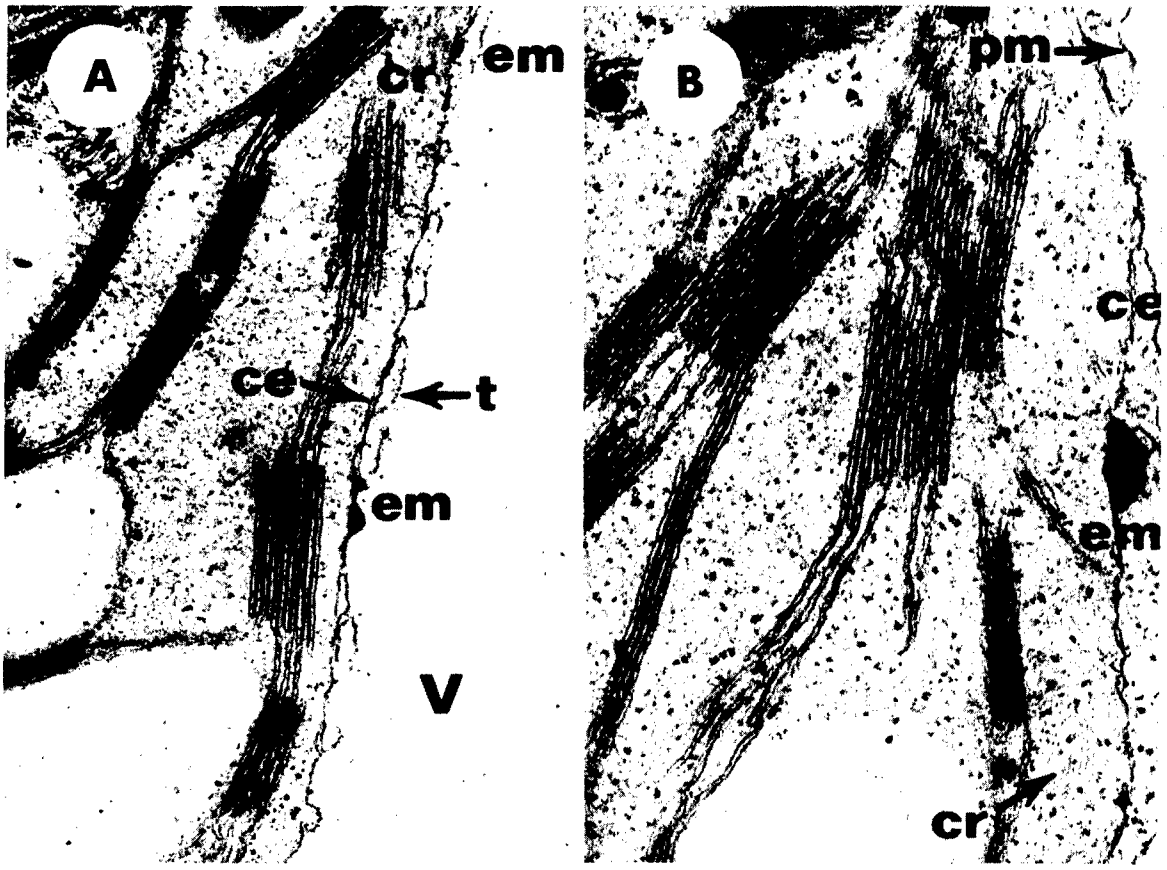


Plate VIII (from Endress, Kitasako, and Taylor, 1979). Chloroplasts in leaves sampled at various times after HCl gas treatments.

Plate VIII A. Chloroplasts from a leaf sampled 0.5 hour after end of exposure to 21.1 mg HCl m^{-3} for 20 minutes. Discontinuities of the tonoplast (t) and outer membrane of the chloroplast envelope (ce) are apparent. Crystals (cr) are associated with membrane rupture and accumulation of electron dense material (em) at the chloroplast envelope. V = vacuole. 44,500x.

Plate VIII B. Chloroplast from a leaf sampled 2 hours after end of exposure to 21.1 mg HCl m^{-3} for 20 minutes. Remnants of crystals (cr) in the stroma. Adjacent disruptions of the chloroplast envelope (ce) and the plasmalemma (pm) are illustrated. Larger deposits of electron dense material (em) appear to extend from the chloroplast envelope in the cytoplasm. 55,500x.

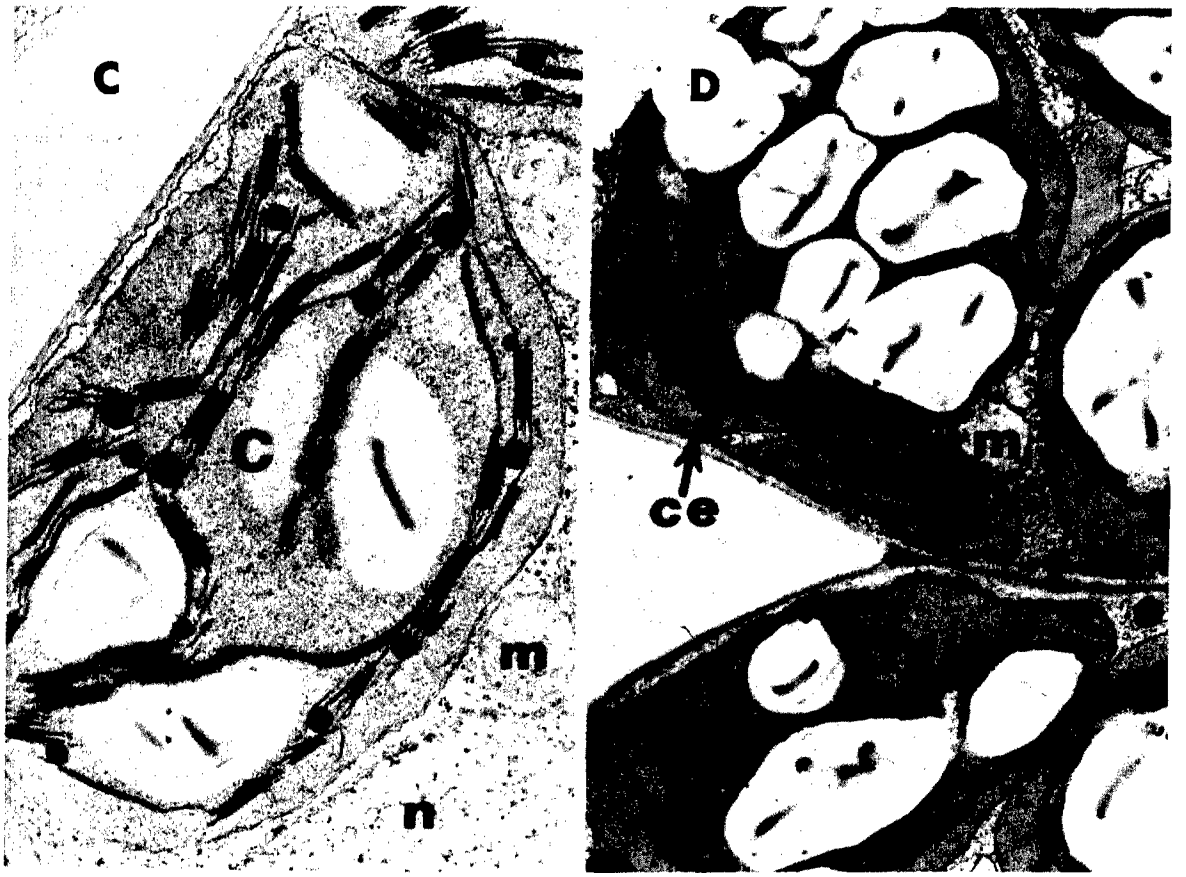


Plate VIII C. Chloroplast (C) from a leaf sampled 5 hours after end of exposure to 25.7 mg HCl m^{-3} for 20 minutes. Note that recovery from HCl stress has occurred. There are no crystals, swollen internal membranes, or major conformational changes. 19,750x.

Plate VIII D. Chloroplasts (C) from a leaf sampled immediately after a 20-minute exposure to 54.2 mg HCl m^{-3}. The stroma is dense and increased staining of the chloroplast envelopes (ce) is apparent. Of particular interest is the absence of stroma crystals. m = mitochondria. 8,800x.

The frequency of crystal formation in chloroplasts was related to both [HCl] during exposure and the time interval between exposure and sampling (Figure 4). The relationship between [HCl] and percent of chloroplasts with crystals taken right at the end of the fumigation period produced a sigmoidal curve (excluding the 54.2 mg m^{-3} treatment in which no crystals were observed). Reversibility of crystallization was evidenced by the lack of crystals in chloroplasts from samples taken 24 hours after HCl treatment. Only cells treated with 41.3 mg HCl m^{-3} contained chloroplast crystals at that time. Further

characterization of the recovery of chloroplasts was obtained from samples taken at 0, 0.5, 1, 2, and 4 hours after treatment with 21.1 mg HCl m^{-3} (Figure 5). Approximately 65% of the chloroplasts contained crystals immediately after HCl treatment and the percentage declined rapidly to 15% during the first 0.5 hour. Subsequent reduction was slower, and by 4 hours, no crystals were present.

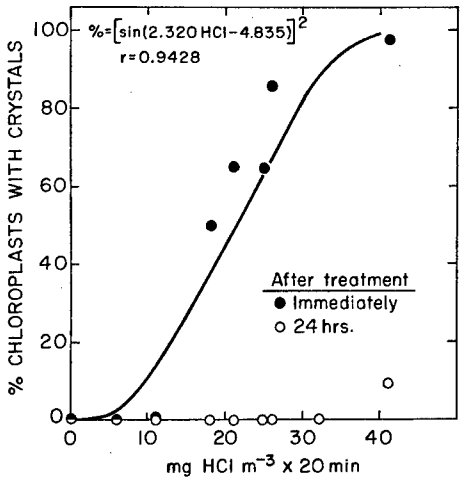


Figure 4. Dependence of the number of chloroplasts containing crystals to the concentration of gaseous HCl at fumigation and to the time of sample. Counts were taken from electron micrographs. Chloroplasts from control plants did not contain crystals. Percent chloroplasts with crystals was transposed to arcsine degrees (from Endress, Kitasako, and Taylor, 1979).

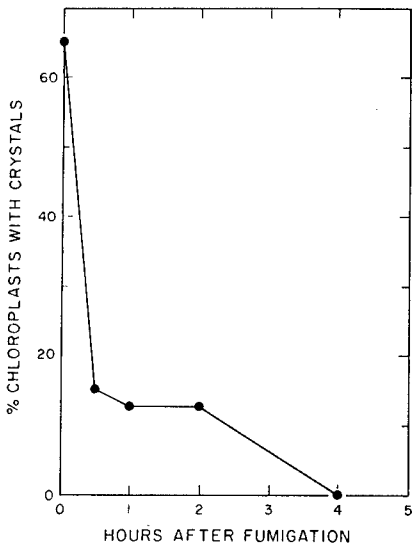


Figure 5. Dependence of the number of chloroplasts containing crystals following exposure to 21.1 mg HCl m^{-3} for 20 minutes to the time of sample. Counts were taken from electron micrographs. Chloroplasts from control plants did not contain crystals (from Endress, Kitasako, and Taylor, 1979).

PHYSIOLOGIC SYMPTOMS

The ultrastructural studies showed that mesophyll cells responded to the HCl treatments in a manner similar to the fine structural responses produced by other air pollutants, including O_3, NO_2, and SO_2. Further, development of visible injury depended greatly on leaf age. The similarities between ozone-induced alterations suggested that, as with ozone, changes of membrane permeability might also occur in the course of HCl injury. Using the method of Ting and co-workers (Perchorowicz and Ting, 1974; Sutton and Ting, 1977a,b), ^{14}C-deoxyglucose uptake by leaf discs from HCl-treated plants was measured.

In 12-day plants, both control and HCl-treated, the uptake of deoxyglucose was linear over the first two hours (Figure 6). This was consistent with the observed deoxyglucose uptake by leaf discs obtained from ozone-treated plants (Perchorowicz and Ting, 1974; Sutton and Ting, 1977b). The discs from treated and control plants sampled immediately after termination of HCl treatment showed no difference in uptake, while those from plants sampled 24 or 48 hours postfumigation exhibited a 1.5 to 5.1 fold increase in uptake rate. The elevated uptake rate was maintained for at least two days postfumigation.

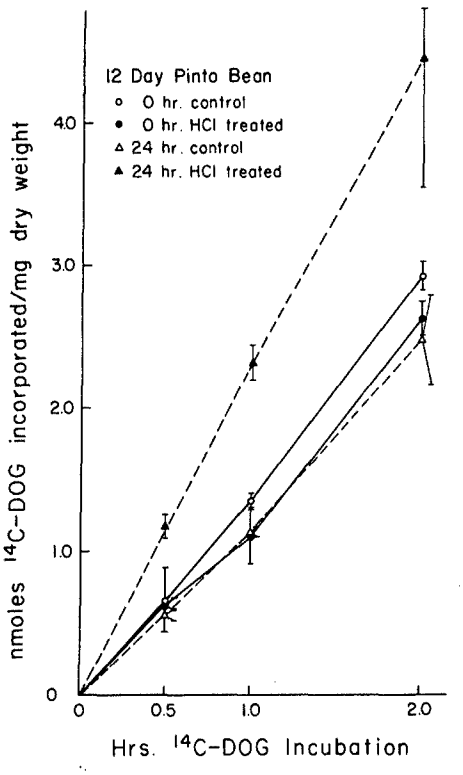


Figure 6. Uptake of the U-^{14}C-deoxyglucose by leaf discs from 12-day pinto bean unifoliate leaves exposed for 20 minutes to 22.7 ± 2.6 mg HCl m^{-3} gas (from Heath and Endress, 1979).

Unlike data for ozone-fumigated bean plants, no increase in permeability of discs obtained from 8-day plants (Figure 7) was noted following exposure to HCl at levels which produced injury or permeability increases in older plants.

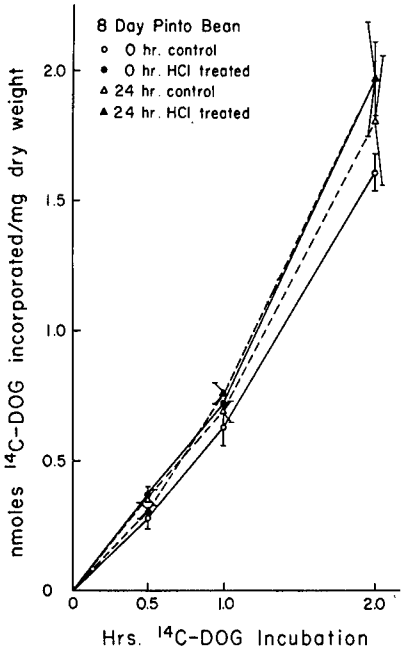


Figure 7. Uptake of U-^{14}C-deoxyglucose by leaf discs from 8-day pinto bean unifoliate leaves exposed for 20 minutes to 22.7 ± 2.6 mg HCl m^{-3} gas (from Heath and Endress, 1979).

We indicated previously that possible mechanisms of HCl phytotoxicity include (1) alterations of cellular pH and (2) accumulation of lethal quantities of chloride. The recognized reversible alterations of membrane integrity and associated changes in membrane permeability and of chloroplast fine structure do not conclusively allow either possibility to be excluded. Consequently, we attempted to determine if leaves accumulated chloride following supra-acute exposure to gaseous HCl.

Foliar chloride content of pinto bean unifoliates treated with HCl gas was increased over control levels and the increase of accumulated chloride was related to [HCl] (Figure 8). Eight-day leaves contained more chloride than 12-day leaves, confirming previous observations (Shriner and Lacasse, 1972) that immature leaves accumulate more chloride than mature leaves. Yet 12-day old pinto beans were more sensitive to macroscopic injury than 8-day plants (Figure 2), and leaf expansion was delayed (Figure 3). Thus, the leaf chloride content indicated

that age-dependent injury sensitivity was apparently unrelated to specific quantities of accumulated chloride since the 12-day leaves contained less chloride, but exhibited more frequent injury than did 8-day plants (Figure 9). Because the ability of the younger leaf tissues to more successfully avoid deleterious effects of accumulated foliar chloride may be explained by a differential ability to compartmentalize or transport chloride ion, ultrastructural localization of chloride ions by precipitation with silver salts was employed to examine sites of chloride accumulation.

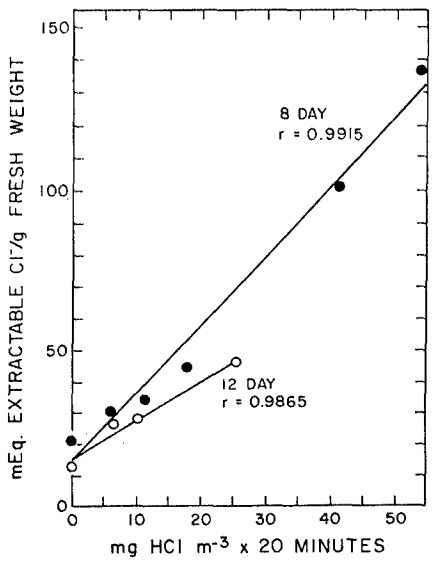


Figure 8. Relationships between the quantity of chloride ion in aqueous extracts of P. vulgaris unifoliate leaves and concentration of HCl gas employed. Plants were 8 or 12 days old at treatment and extracts were obtained 24 hours later (from Endress, Kitasako, and Taylor, submitted manuscript).

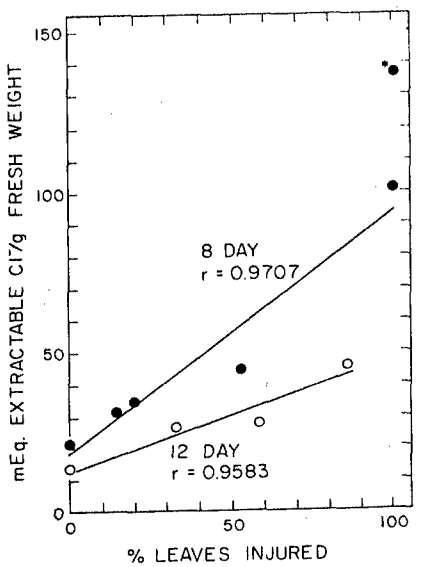


Figure 9. Relationship between the quantity of chloride ion in aqueous extracts of P. vulgaris unifoliate leaves and numbers of HCl-exposed leaves expressing injury symptoms (% of total). Data point * was not included in the regression calculation. Plants were 8 or 12 days old at treatment, and extracts were obtained 24 hours later (from Endress, Kitasako, and Taylor, submitted manuscript).

Deposition of silver chloride (AgCl) occurred within the cytoplasm and vacuoles of parenchyma and epidermal cells and in the chloroplasts of parenchyma cells from both HCl-stressed and control tissues. There were, however, apparent differences in the amounts of AgCl precipitated in various subcellular compartments, particularly in vacuoles and chloroplasts and throughout the cell walls. The distribution of AgCl deposits was further altered as the interval between termination of HCl exposure and sampling time lengthened. Subcellular compartments exhibiting increased AgCl precipitates were vacuoles and cell walls, while the groundplasm and chloroplasts contained fewer deposits. During the recovery period, chloroplasts and the groundplasm regained some of the lost chloride concurrent with disappearance of stroma-localized chloroplast crystals and formation of extensive AgCl clumps along the plasma and tonoplast membranes. With further recovery, cells associated with vascular traces bore abundant AgCl deposits.

CONCLUSIONS

Conventional understanding of plant injury by air pollutants usually requires that the pollutant enter the leaf interior through stomates with subsequent injury to the parenchyma cells bordering the substomatal spaces. This pattern of injury was not strictly observed in HCl-treated leaves. Epidermal cells were shown by light microscopy to be sensitive and readily injured by gaseous HCl. Location of the injured epidermal cells bore no relationship to placement on the leaf or position of leaf traces or stomates. These observations indicated that HCl is active both at the leaf surface and in the leaf interior.

Anhydrous HCl is very hygroscopic, but probably becomes solubilized at the leaf surface and on the internal cell walls bordering substomatal cavities because the humidity at both sites is quite high. The ultrastructural localization of chloride ions by precipitation with silver salts showed that the epidermal cell walls and the cell walls adjacent to the substomatal cavities contained considerable chloride. With delayed sampling, migration of chloride ion through the continuum of cell walls and uptake into vascular tissues was documented.

The parenchyma cells in leaves exposed to HCl gas sustained injury roughly according to [HCl]. The ultrastructural perturbations were varied, but the most interesting concerned the plasma and tonoplast membranes and the chloroplasts. By employing a range of HCl concentrations and sampling times, it was possible to acquire evidence for repair, particularly in

chloroplasts where crystals were prominent immediately after treatment, but disappeared shortly thereafter. These crystals are also present in bean chloroplasts from water-stressed plants suggesting that one of the consequences of HCl treatment is cellular water loss which presumably can lead to plasmolysis. The loss of chloride ion from treated chloroplasts indicates further that chloride ion efflux occurs with water loss and is reversible.

The cause of movement of water and associated ions out of chloroplasts and out of parenchyma cells is unknown. We have documented that general membrane permeability is increased by exposure to HCl. However, we do not yet know if this is due to a primary effect on cell membranes or a secondary effect dependent on changes in some other leaf or cell component. For example, it is possible that water loss from cells could be established by osmotic gradients due to the partial hydrolysis of hemicelluloses or pectins in the cell walls.

The prominence of visible injury symptoms was related to both [HCl] and plant age. Supra-acute exposures to HCl resulted in leaves accumulating chloride, but the older, more sensitive leaves accumulated less chloride, and were more severely injured. Furthermore, membrane permeability was enhanced when older leaves were fumigated, while no change was detected in the younger leaves. The relationship of susceptibility to plant age remains to be investigated. However, cells in younger leaves are more compact and have less exposed cell wall area.

ACKNOWLEDGEMENT

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COLONEL CARTER (6570 Aerospace Medical Research Laboratory): Major Lind, about a year and a half or two years ago, we made a tentative recommendation to SAMSO for a limit to HCl based on plant damage. Do you remember what that limit was?

MAJOR LIND (6570 Aerospace Medical Research Laboratory): That was a very conservative limit. At that time, we were looking at the data of Dr. Larriman and Dr. Taylor, primarily, and we had considered two factors. One was that at high humidities, plants had more HCl damage than under low humidity. Knowing that fog banks at Vandenburg Air Force Base occur and high humidity may be likely during a launch, we recommended 2 ppm HCl as a limit below the concentration at which you would expect damage in the most sensitive plants. That figure was based on the results that were found with cosmos and a few of the most sensitive plants. At that time, we made that recommendation for an environmental exposure limit.

COLONEL CARTER: Do you think now that we are ready to reevaluate that limit? I would like to address that to the panel.

MAJOR LIND: I believe we have sufficient new data to permit a reevaluation of the value we recommended as a limit two years ago. We can look at the results of Larriman and Taylor. They used probit analysis to evaluate the data collected in their studies of 1977 and 1978, and they calculated ED_{50} or the dose at which 50% of the leaves would be damaged. They showed the ED_{50} values in 15 to 20 different kinds of plants to be around 16 to 20 ppm HCl. In studies that I've conducted, I found the first sign of damage at 10 ppm which was minimal injury. I didn't find serious plant damage until I exposed them to 20 ppm. I now think that 2 ppm HCl is a much more conservative maximum exposure end than we need to impose as a control point for SAMSO. Keeping in mind that we're only looking at a small number of plant varieties and looking only at specific parts of their life cycle, I would be willing to say that 2 ppm is too low an estimate and that a 10 ppm exposure level may be tolerated.

COLONEL CARTER: Dr. Heck, do you know what position NASA is taking on HCl exposures at Cape Kennedy?

DR. HECK (U.S. Dept. of Agriculture): I don't know their position on that. I don't know if any of the NASA people would like to comment, but I would be very comfortable with 5-10 ppm.

DR. TAYLOR (University of California, Irvine): Dr. Heck, do you have any idea how much oxides of nitrogen were generated in the burning of fuel in your chambers?

DR. HECK: No, we did not measure oxide of nitrogen. It might have been as high as 10% of the HCl concentration, but I doubt if it would be quite that high.

DR. TAYLOR: Would you speculate that the oxides of nitrogen were NO by the time they reached the plant? Or were they present as NO_2?

DR. HECK: I think it was probably still NO by the time it reached the plant. The lag time was very short.

MR. VERNOT (University of California, Irvine): Major Lind, you mentioned that you measured the height of your corn plants, and you found in at least one strain that exposed plants were taller than the control plants at maturity. Do you think this might be true for the total mass of the plant as well or was the height of the plant a good measure for how much leaf mass there was?

MAJOR LIND: Yes, I think it was a good measure of what was happening to the corn. It's not practical to serially sample and get dry weights throughout the whole season because I would have run out of specimens before the study was complete. I was looking for a nondestructive measurement. We also attempted to use a portable leaf area meter which is produced by Lamb Instruments which was a very tedious process but the meter broke before we acquired enough data to report. We had originally wanted to look at plant height and leaf area throughout the growing season. It was just a matter of failure of the equipment.

MR. VERNOT: Dr. Dochinger, the title of your paper referred to beneficial as well as injurious effects of pollutants and you mentioned that NO or NO_2 pollution might have some beneficial effects on plants. Have any experimental or epidemiological studies been done that have shown beneficial effects from NO_2 or NO which actually have occurred?

DR. DOCHINGER (U.S. Forest Service): Yes, there are several studies that have been reported on the utilization of nitrogen from the atmosphere. One is published in Science. It was about corn plants. The authors were able to show that there was an increased yield resulting from the utilization of atmospheric nitrogen. I cannot identify the author. The paper was published 2 or 3 years ago. There was another study that was done which measured forest growth in the Hubbard Brook area where they found increases of nitrates but they weren't sure how the nitrates were being utilized in explaining the responses that were encountered there.

DR. HECK: We've done some research at Raleigh using $^{15}NO_2$ which was completed within the last 4 or 5 months. We found that using ambient concentrations of NO_2, the snap bean was able to take up NO_2 and a lot of it was found in proteins and amino acids and some was translocated to the roots. So, we feel that NO_2 can be used as a nitrogen source under certain circumstances.

DR. WEINSTEIN (Boyce Thompson Institute for Plant Research at Cornell University): There was research done in Germany by Fowler who was able to grow plants to maturity using atmospheric NO_2 as the only nitrogen source. He did a number of studies with sulfur oxide as well. And the beneficial effects of sulfur oxides have been known for about 100 years. Many soils in the United States and in many other parts of the world are low in sulfur, so the atmospheric input of sulfur oxide is probably very important in some areas. However, one could not ascribe a beneficial effect to those gases in all areas and at all concentrations.

DR. COLLINS (Ohio State University): Would someone care to comment on how HCl, which is quite polar, could penetrate directly into the leaf? It's a curious thing that a very polar compound would be able to penetrate through what essentially would be a hydrophobic layer on the surface of a leaf.

DR. ENDRESS (University of California, Riverside): I think the first important point that has to be recognized is that the cuticle which is indeed waxy on the leaf surface is not continuous. That is, that plants in nature have leaf surfaces continuously abraded by dust and other particles through rapid expansion of the leaves. There are frequent cracks and other breaks in the cuticle which appear to be commonplace and that the compounds then do not have a problem in directly diffusing or migrating through the cuticle but can penetrate into the polar cell wall.

DR. WEINSTEIN: I'd like to say one thing before we end and that is that I think that our research today is showing that foliar lesion or injury to leaves is not necessarily a good measure of injury or damage to the plant unless the plant is spinach or tobacco where the looks or the use of foliage is important. In terms of fruit or seed production, a plant that has susceptible leaves is not necessarily susceptible to that pollutant in terms of seed production. Conversely, plants that we call resistant based upon foliar injury are sometimes extremely sensitive in terms of the effect on fruit production. You can have a beautiful looking plant after exposure, such as snap beans and the bean production is reduced and then have a more susceptible plant such

as the tomato where the exposure has no effect on yield. You can reverse the sensitivity depending on what parameters you're measuring. I think that more concern has to be given to the intended use of the plant.

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