LA--8948-PR

DE82 002955

LA-8948-PR
Progress Report

UC-48
Issued: September 1981

The Los Alamos Life Sciences Division's Biomedical and Environmental Research Programs

January—December 1980

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ABSTRACT

This report summarizes research and development activities of the Los Alamos Life Sciences Division's Biomedical and Environmental Research program for the calendar year 1980. The principal features of the program have been carried out by groups in the Life Sciences Division, supported by the United States Department of Energy. Previous annual reports in this series prepared under the auspices of the United States Atomic Energy Commission, the United States Energy Research and Development Administration, and the United States Department of Energy are LA-5227-PR (1972), LA-5633-PR (1973), LA-5883-PR (1974), LA-6313-PR (1975), LA-6898-PR (1976), LA-7254-PR (1977), LA-8023-PR (1978), and LA-8577-PR (1979).

Technical reports related to the current status of projects in the major program areas of each contributing group are presented in sufficient detail to permit the informed reader to assess their scope and significance and to obtain some specific information. Further details can be obtained from the publications listed in Appendix A. Summaries useful to the casual reader desiring general information have been prepared by the Group Leaders and appear at the beginning of each section of the report. Investigators on the staff of the Life Sciences Division will be pleased to provide further information.

OUTLINE OF PROGRAM

Last year's Annual Report of the Biomedical and Environmental Research Program at Los Alamos reported the formation of the Life Sciences Division from certain segments of the Health Division. This report covers the period January-December, 1980, and reflects the reorganization by reporting only the activities of the new Life Sciences Division. Health Division activities are to be reported separately. Further adjustments in organization took place during late 1980 and early 1981 and will be reported in detail next year. The principal change during this interim period was the naming of Dr. Mark W. Bitensky as Life Sciences Division Leader. Dr. Bitensky came to Los Alamos from Yale University where he was a Professor of Pathology.

The format of the report consists of a brief summary of the research accomplishments in each of the operating groups followed by short articles which include pertinent data on specific programs. Since the articles are brief we encourage the reader to contact the principal investigators for further detail or discussion.

Because the Division is organized along disciplinary lines, we are able to focus a multi-disciplinary approach on the problems facing the Department of Energy in the relevant energy technologies. Because these efforts cross group lines they create beneficial interactions between those interested in fundamental biology and those pursuing more applied problems. Major programs such

as nuclear energy and oil shale receive the benefits of capabilities in toxicology, biophysical measurement, genetics, pathology, chemistry, and environmental science in a way that provides broadly based information focused in hazard analysis. While not included here, there are continuing collaborative efforts with research activities in Health Division (specifically in industrial hygiene and occupational medicine) that further this synergy.

The emphasis on the immediate problems associated with developing technologies, while rewarding and important, has not caused us to overlook the generic questions which may be the most important in the long run. Generic research includes the effects of pollutant intrusion on biochemical and cellular mechanisms, pathological mechanisms within organ systems, use of specific labelled organic compounds as environmental tracers or diagnostic probes and the use of genetic manipulation to determine cellular protective phenomena. Basic information related to the intrusion and transport of potential pollutants is addressed in the environmental sciences effort. This multi-disciplinary strategy enables us to bring state-of-the-art techniques to the study of current problems and at the same time to investigate the interactions of toxic materials and fundamental biological processes in a way that will permit intelligent assessment of effects on the environment and on man.

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INTRODUCTION

The Toxicology Group is comprised of individuals who employ in their research programs a variety of analytical systems ranging in complexity from cells in culture to intact animals. This interdisciplinary approach generates information which is relevant to hazard assessment and prioritization of energy technology-associated materials. Parallel studies are designed to delineate the underlying biochemical mechanisms of pollutant-induced alterations in metabolism.

Intact animal studies employ a variety of rodent species commonly utilized in standard toxicology procedures. Various routes of exposure to test materials are used, although major emphasis is placed on inhalation studies, due in part to our close ties with the Aerosol Physics Group (H-5) in the Health Division. Both short term (acute) and lifetime exposure studies are carried out, and different dose levels are employed to define dose-response relationships and to identify, where applicable, threshold levels of activity.

There are three major ongoing intact animal studies at present. One program examines health effects associated with various materials generated during production of shale oil. Inhalation studies are concerned with effects of particulates such as raw or spent shale on lung tissue. Liquid products/by-products are analyzed by means of topical application of oils to the skin or ingestion of product waters by pregnant females; in the former case, the endpoints measured are acute toxicity and tumor formation, while in the latter, the teratogenic potential and toxicity are monitored. (For organizational purposes, a teratogenesis study involving shale process water is included in this section, even though the authors are not members of the Toxicology Group.) Upon completing construction of an experimental retort, studies of the effects of retort gases either alone or in combination with specific particulates will become feasible. As a result of ongoing and contemplated studies, the information obtained utilizing various

shale oil materials and reference standards will allow site- and process-specific health effects comparisons to be made among existing technologies.

The lung tumorigenesis program is designed to delineate the role of internally deposited radiation in carcinogenicity of the respiratory tract. Of special concern are factors which affect the tumorigenicity of inhaled radionuclides such as type of radiation (i.e., alpha- vs. beta-emitter), degree of localization and fraction of lung irradiated, composition of inhaled particles and role of tumor-enhancing agents. More recent studies have begun to study lung cell turnover resulting from exposure to radionuclide/tumor-enhancing agents as a means of identifying likely target cells and for examining the role of cell division during the early stages of carcinogenesis. Collectively, these studies provide information of relevance in evaluation of health protection standards for inhalation of the type of radionuclides of concern in the nuclear industry.

A third animal study is designed to study the effects of inhalation of various manmade fibers (e.g., microfibers, fiberglass, mineral wool, ceramic wool, etc.). This project is supported by the Thermal Insulation Manufacturers' Association, a consortium of companies which manufacture insulation products. The goal of this project is to provide information on both the relative toxicity of various commercially available materials and on the role of particle geometry in tumor induction. (Details of this project are not included in this package of DOE-funded programs.)

In view of the large number of tissue samples accruing from these studies, a computerized data-handling system has been evolved which provides a large and comprehensive repository of systemic pathology information. The repository includes not only data from the major projects described above but also extensive information on the tissues of normal animals.

Complementing these studies are three ancillary programs. The goal of the immunology program is to delineate the immunological events which are altered during conversion to the neoplastic state.

Initial studies have utilized chemical carcinogens. The information gathered with model systems currently is being utilized to design programs for monitoring immunological changes in animals enrolled in the lung tumorigenesis and oil shale programs.

The cultured cell project focuses on pollutant-induced alterations in metabolism at the cellular level. Pure compound pollutants known to be associated with oil shale related materials and particulates of complex composition are administered to cultured cell populations, and alterations in growth rate and cell survival are monitored to establish dose-response relationships. Mechanistic studies are directed toward elaboration of alterations in macromolecular synthesis and identification of interactions between pollutant and the cellular genome, with special emphasis on changes in the chemical composition/structure of chromosomal proteins which may play a role in altered gene expression.

Finally, the radiobiology and oncology project is concerned with the other end of the toxicology spectrum, treatment of tumors. Cultured cells, spheroids and intact animals are utilized for these studies. The two major thrusts of the program are directed toward increasing fundamental knowledge of the radiobiology of high LET particles (π mesons, heavy ions, plutonium alpha particles, etc.) and applying flow cytometric techniques in the design of more effective treatment schedules utilizing radiotherapy and/or chemotherapy.

When combined with the expertise available in the other groups within LS Division and the aerosol physics personnel in H-5, the Los Alamos toxicology effort is an extremely broad-based multidisciplinary program which has the capability of: 1) specifying the forms and levels of toxic materials that impinge on man, 2) assessing the relative hazards associated with a variety of technology-specific materials, 3) identifying toxic constituents in complex mixtures which are responsible for the observed toxicity, and 4) identifying cellular protective mechanisms which are elaborated to combat the effects of toxic substances.

Long-Term Epidermal Carcinogenicity Studies

[L. M. Holland and J. S. Wilson]

The repetitive application of test materials to intact mouse skin is a generally accepted method of determining comparative carcinogenic potential.¹ We have used the mouse-skin assay to evaluate two crude shale oils and two natural petroleum. Groups of C3Hf/He mice were exposed, 3 times a week, to either one of the oils or one of the control materials (Table I). One shale oil was a product of the Paraho vertical-kiln retort located near Rifle, Colorado (PCSO) and the other shale oil was a laboratory sample believed to have originated from early pilot *in situ* experiments (ICSO). The two natural crudes were obtained through the courtesy of the American Petroleum Institute and represent either a U. S. gulf coast petroleum (API #2) or a middle-eastern petroleum (API #1).

Both shale oils and one natural petroleum proved to be carcinogenic. The only positive effects were seen at the highest dose (5 mg/dose = 15 mg/week) with each test oil with the exception

TABLE I
RESEARCH PROTOCOL

Oil	No. of Animals	Dose/ Application
Kuwait (API #1)	40	10 µg
	40	50 µg
	40	0.5 mg
	40	5.0 mg
U. S. Gulf Coast (API #2)	40	10 µg
	40	50 µg
	40	0.5 mg
	40	5.0 mg
Shale Oil (Paraho Retort)	40	10 µg
	40	50 µg
	40	0.5 mg
	40	5.0 mg
Shale Oil (<i>In Situ</i> Retort)	40	10 µg
	40	50 µg
	40	0.5 mg
	40	5.0 mg
BaP	40	2 µg
	40	10 µg
Solvent	40	50 µl
Shaving Control	40	---
Untreated Control	40	---

of two tumors observed very late at the next highest level of PCSO. In the group treated with PCSO, the first tumor was observed at 78 days, the next two at 136 days and all of the surviving mice exhibited tumors by 453 days (mean latency = 261 days). Within the group treated with ICSO the first tumor occurred at 176 days, the second at 215 days and 64% of the mice had tumors by 472 days (mean latency = 380 days). Only two tumors were observed in the group treated with the middle-eastern oil while the U. S. gulf coast petroleum produced tumors in 43% of the treated mice after 526 days (mean latency = 410 days). A statistical evaluation of the three positive oils compared to two dose levels of benzo(a)pyrene (BaP) is shown in Fig. 1. In the figure, the data points represent the non-parametric Kaplan-Meier method² which censors animals alive without tumor or that have died from other causes. The curves are Weibull fits of the data. Most of the tumors were either papillomas or squamous carcinomas, however for the purpose of estimating latency no distinction was made between histological types.

A second experiment, comparing a different sample of PCSO, its hydrotreated daughter product and a well-defined *in situ* oil from Occidental Oil Shale Company's Logan Wash, Colorado effort is still in progress. Interim results indicate a similar tumorigenic response for both crude oils but a diminished potency with the hydrotreated PCSO. In these studies our attempt to raise the

Fig. 1. Kaplan-Meier evaluation is represented by single data points (mice with tumors). The curves are Weibull fits of the data. Two levels of BaP (2 µg and 10 µg) are included for comparison.

dose ranges resulted in significant interference by overt cytotoxicity. Perhaps the most important observation to date in these new experiments is that while hydrotreating apparently reduces tumorigenicity the phlogistic qualities of the oil are affected very little. The reduction in carcinogenicity in the hydrotreated shale oil may be related to a reduction in total nitrogen content, however the reasons for the apparent connection between nitrogen levels and carcinogenic potency have yet to be defined. Furthermore, it is not clear whether the level of nitrogen is related to enrichment in the source shale or to technology-related process chemistry factors or both.

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1. J. H. Weisburger, "Bioassays and Tests for Chemical Carcinogens," in Chemical Carcinogens, Charles E. Searle, Ed., (American Chemical Society, Washington, D.C., 1976) ACS Monograph 173.
2. E. L. Kaplan and P. Meier, "Nonparametric Estimation from Incomplete Observation," *J. Amer. Statistics Assoc.* **53** (1958).

Inhalation and Intratracheal Exposures

[L. M. Holland, E. A. Vigil, M. Gonzales (H-5), D. Archuleta, and J. S. Wilson]

Inhalation studies comparing the effects of raw shale and two spent shales have been completed. Groups of 64 Syrian hamsters were exposed, 4 hours/day - 4 days/week, for 16 months to either Anvil Points raw shale, Paraho spent shale or TOSCO II spent shale. Single level (50 mg/m^3) dust concentrations were used for each material to determine if any differences in response by the pulmonary tissues could be identified. We chose the hamster as a species utilized frequently in chemical carcinogenesis studies. Animals in each group were allowed to complete their lifespans and histological examination of all relevant tissues has recently been completed. Two hamsters in the group exposed to raw shale and one animal from the group exposed to Paraho spent shale exhibited a proliferation of reticulo-endothelial cells within the lung. A generalized hyperplasia of this tissue is not uncommon in rodents as a response to toxins and may even be associated with tissue necrosis and cell death. In one of the raw shale exposed

animals the cellular characteristics were more typical of neoplasia than an inflammatory response. The significance of these changes is problematical at best. Indications of fibrotic tissue response, ranging from slight interalveolar thickening to extensive fibrotic scarring, were observed in most animals completing the exposure period, and both focal and generalized pneumonitis were common.

Both Syrian hamsters and Sprague-Dawley rats have been exposed by intratracheal instillation (Table I) to the same materials used in the inhalation study described above. While not as physiological as inhalation, intratracheal exposure offers more precision in dose and was used here as a range-finding procedure for both species. Some animals are still alive and pathology studies are incomplete, but no tumors have been observed to date.

Studies currently in progress utilize Fischer-344 rats exposed in a nose-only mode to either Anvil Points raw shale, Paraho spent shale, Estonian raw shale or quartz. This particular strain of rat is useful as a subject for inhalation studies designed to determine pulmonary fibrotic response. High dust concentrations (150 mg/m^3) of the shale dusts (Estonian shale, 50 mg/m^3) were used in an attempt to elicit an early and possibly

TABLE I
INTRATRACHEAL STUDIES

Agent	Dose/Frequency*
Paraho Raw Shale	3 mg/week x 10 weeks 7 mg/week x 10 weeks
Paraho Spent Shale	3 mg/week x 10 weeks 7 mg/week x 10 weeks
TOSCO II Spent Shale	3 mg/week x 10 weeks 7 mg/week x 10 weeks
Quartz**	3 mg/week x 10 weeks 7 mg/week x 10 weeks
Saline Only	0.2 ml/week x 10 weeks
Untreated Controls	-----

* 64 Syrian hamsters and 64 S-D rats/dose level.

** Particles 90% less than 5- μm diam.

more profound effect. Concentrations of quartz were established at $14\text{--}16\text{ }\mu\text{g}/\text{m}^3$, a level which, in relationship to the shale exposures, simulates the approximate free silica content of Green River shales (10–20%). As an adjunct to these experiments, one group of rats has been used for neutron activation studies of raw shale deposition in the lung.

Effect of Shale Oil on Delayed Type Hypersensitivity in Mice

[P. A. Baron]

The effects of 5-mg gas combustion, surface retorted shale oil (PCSO II) topically applied to the backs of C3Hf/HeBD mice 3 times/week have been demonstrated by L. M. Holland and J. Wilson¹ to result in tumorigenesis in 100% of the experimental animals within 360 days. It is of value to the developing shale oil technology to examine the effects of shale oil and by-products on the immune system to determine whether there is a correlation between altered immune function and tumorigenesis. This report describes the effects of shale oil treatment on gross physical parameters and delayed type hypersensitivity (DTH) in acute and long-term experiments.

A comparison of gross physical characteristics was made at 4 time periods: 6 and 15 weeks after the start of shale oil treatment; at the time of initial appearance of the tumor; and at advanced stages of tumor development. This comparison indicated no significant variation between experimentals and controls. Body weight, spleen weight, spleen cellularity, spleen weight:body weight ratio, and cells/gram of spleen were compared for groups of 8 to 20 animals for each of the time periods.

The changes in immune function that occur following acute and chronic exposure to oils and ultraviolet light were also examined. DTH, which is a T-cell function and is dependent upon antigen presenting cells (macrophages or macrophage-like cells), is an effective response for assessing immune function. Sensitivity is established by applying 2,4-dinitro-1-fluorobenzene (DNFB), a potent contact sensitizer, to the shaved skin on the backs of mice. The extent of the reaction is

measured 4 days later by challenging a different site (ear) with the same sensitizer. Influx of mononuclear cells into the challenged area and clonal expansion of cells already present may be quantitated by measuring the extent of ear swelling with a micrometer to determine the degree of sensitivity in the animal.

Figure 1 indicates that when an animal has been subjected to as few as 3 applications of 5 mg of shale oil in 50 μl of cyclohexane:acetone and then sensitized at that site, the animal is capable of manifesting only 42% of the DTH response of normal animals. Cyclohexane:acetone, the shale oil vehicle, had no effect on the response and showed an ear thickening equal to the non-shale oil painted positive control.

Ultraviolet light (UVB, $280\text{ }\mu\text{m}\text{--}320\text{ }\mu\text{m}$) is known to be carcinogenic and is capable of altering the structure of biological molecules as well as their biosynthetic rates. UVB is also known to produce perturbations in the number and function of various types of cells present in the skin. When animals are subjected to UVB irradiation for 10 min a day on 5 consecutive days, the animals express only 12% of the DTH response of normal animals.

When animals were tested for DTH after 30 weeks of oil exposure (3 applications/week), all experimental animals demonstrated from 60–100% suppression (Fig. 2) when compared to normal controls. The DTH response in PCSO II-treated animals which had not yet developed tumors were suppressed

Fig. 1. Delayed hypersensitivity in mice as determined by measurement of ear swelling in response to DNFB sensitization and challenge. Cyclohexane-acetone shows no effect on the normal response while UVB and PCSO II demonstrate a suppression of 88% and 58% when compared to the positive control.

Fig. 2. Delayed hypersensitivity in mice treated for 30 weeks and in various stages of response to the oils. All experimental animals demonstrate a suppressed response ranging from 60 to 100% when compared to normal animals.

by 60% while animals treated with the same oil, but having well-developed tumors, were suppressed by 100%. These latter animals expressed an ear thickness equivalent to that of the unsensitized negative control population.

A modified *in situ* shale oil (OCSO) was also examined under the same regimen as above. Animals treated with OCSO but not having tumors were suppressed by 83%, while tumor bearing animals were suppressed by 89%.

It is evident that shale oils from both above ground and *in situ* retorts had profound effects on the immune system as assayed by the DTH response. Given the differences in magnitude of response between 3x painted, 30 week painted/tumor free animals and 30 week painted/tumor bearing animals, it appears that the degree of suppression of DTH can be crudely correlated with frequency of oil application and appearance of frank tumors.

REFERENCE

1. L. M. Holland and J. Wilson, "Long-Term Epidermal Carcinogenicity Studies," in The Los Alamos Integrated Oil Shale Health and Environmental Program: A Status Report, Los Alamos Scientific Laboratory, LA-8665-SR (1981), p. 45.

The Prenatal Toxicology of Oil Shale Retort Water in Mice

[C. T. Gregg (LS-5) and J. Y. Hutson (LS-5)]

Oil shale retort water is a by-product of the production of oil from shale that potentially amounts to tens of millions of gallons per year and must be treated or recycled with due regard for public health. In this experiment, ninety-eight

outbred female ICR mice were given either 0, 0.1, 0.3, or 1.0% Oil Shale Retort Water (OSRW) *ad lib.* and were caged together for 2 h once a week with one male for every five females. Fetuses were removed for examination by Caesarian section on day 18 of gestation.

There was no clearly significant evidence of maternal toxicity even when 1.0% OSRW was consumed for up to 203 days. Of 75 treated animals, however, 7 had adenomas, adenomatoid nodules, or adenocarcinoma; none of the 23 control animals showed such abnormalities (Table I). There was no apparent increase in adenoma-like lesions with longer treatment times. This suggestion of carcinogenicity may warrant further investigation.

The percentage of pregnancies, maternal weights, number of live fetuses per litter, and average fetal weight per litter were not significantly different in control and treated animals. A striking alteration of male to female fetuses was found by the Laramie group after administering Omega-9 water;¹ no such effect was observed by us (Table II). There was also no clear evidence for fetal toxicity except for a possibly significant increase in preimplantation losses in animals drinking 1.0% OSRW (Table III).

Fetal development was not generally retarded by drinking OSRW. This is indicated by the fetal weights and by a calculated paw development score (number of ossification centers) that was not significantly different in control and treated animals.

The average sternebral malformation score, however, was 3.10 in control animals and 3.54 in the offspring from animals drinking 1.0% OSRW. This difference is significant at $p = 0.05$ by the Mann-Whitney test.

Three types of palatal abnormalities were observed: single clefts, double (or multiple) clefts, and a deformity not previously reported in which a portion of the palate is entirely absent. Taking all palatal abnormalities together, there was a statistically significant and dose-related increase in the proportion of fetuses with abnormalities from the beginning of the experiment up to 110 days (when the mice were 38 weeks old).

TABLE I
 LESIONS IN ADULT FEMALE ANIMALS CONSUMING OIL SHALE RETORT WATER (OSRW)
 FOR VARIOUS PERIODS (DAYS)

		Concentration of OSRW Consumed		
<u>Abnormalities</u>	<u>Control</u>	<u>0.1%</u>	<u>0.3%</u>	<u>1.0%</u>
<u>Adenoma-like lesions</u>				
Lung adenoma	---	61 days	---	---
		103 days	---	96 days
		203 days	203 days	---
Adenomatoid lung nodule	---	---	194 days	---
Rectal adenomacarcinoma	---	159 days	---	---
<u>Liver abnormalities</u>				
Many polyploid hepatocytes	---	---	---	103 days
Many mitotic figures	---	---	---	138 days

TABLE II
 THE EFFECT OF DRINKING OIL SHALE RETORT WATER (OSRW) ON
 ADULT AND FETAL PARAMETERS

Parameter	Concentration of OSRW Consumed			
	Control	0.1%	0.3%	1.0%
Pregnancies/No. of animals	20/23	19/25	22/25	24/25
Nonpregnant female weights ^a - g	33.7(0.31) ^b	33.9(0.33)	32.3(0.26)	34.2(0.51)
Maternal weight gain ^c - g	25.6(6.3)	29.3(4.5)	27.1(5.1)	24.8(4.9)
No. of live fetuses/litter	10.7(2.9)	12.3(2.5)	11.2(2.3)	10.0(3.2)
Ave. fetal weights/litter	1.33(0.16)	1.36(0.12)	1.33(0.10)	1.32(0.12)
Ave. males/total live fetuses/litter	0.52(0.11)	0.53(0.12)	0.45(0.11)	0.54(0.10)

^a Average of weekly weights from beginning of the experiment until 203 days, or until first day of gestation.

^b Standard deviations.

^c Average of difference between weight on day 18 and weight on first day of gestation.

After 110 days palatal defects began to appear spontaneously in the control animals (Table IV).

Significantly more treated than control animals had either large 14th ribs or rudimentary 14th ribs. The total proportion of defective fetuses for the controls, 0.1, 0.3, and 1.0% groups are 5.1, 8.9, 11.4, and 10.4% for the large 14th ribs and 21.1, 22.6, 28.9, and 30.8% for the small 14th ribs. Significant differences are detectable at the 5% level between the controls and the 0.3 and 1.0% groups, using a chi-square test with one degree of freedom.

On the basis of preliminary experiments, the Laramie group concluded that Omega-9 water does not pose a threat to human health.² They found, for example, only a 6% incidence of rudimentary 14th ribs when rats were given undiluted Omega-9 water between days 6 and 17 of pregnancy, and no other malformations. These results are very different from our own. The explanation probably lies in the vast dilution (22:1) of Omega-9 retort water by infiltrating ground water.³ The Paraho direct-mode process, on the other hand, yields a water-oil ratio of about 0.3 to 1.⁴ Omega-9 water was found

TABLE III
THE EFFECT OF DRINKING OIL SHALE RETORT WATER (OSRW)
ON PRENATAL MORTALITY

Parameter	Control	Concentration of OSRW Consumed		
		0.1%	0.3%	1.0%
Early deaths ^a - %	5.4	1.6	2.9	2.4
Late deaths ^b - %	2.2	1.4	0	1.9
Preimplantation losses ^c - %	13.3	8.9	7.7	20.6 ^d

^a Average percentage of implantations per litter with early deaths.

^b Same as for late deaths.

^c Average percentage of corpora lutea per litter that do not result in implants.

^d Significantly different from controls, Wilcoxon-Mann-Whitney rank-sum test, $p = 0.05$.

TABLE IV
EMBRYO DEFECTS FROM THE MATERNAL CONSUMPTION OF OIL SHALE RETORT WATER (OSRW)

Type of Defect	Basis of Calculation	Controls	Concentration of OSR Consumed		
			0.1%	0.3%	1.0%
Abnormal palate (19-110 days)	average fraction ^a litter	0.00(0) ^c	0.05(0.10)	0.11(0.17)	0.17 ^b (0.21)
Abnormal palate (19-110 days)	affected litters total litters	0/13=0.00	6/17=0.35	7/19=0.37 ^d	10/16=0.62 ^d
Abnormal palate (131-201 days)	average fraction ^a litter	0.28(19)	0.52(0.42)	0.29(0.25)	0.49(0.26)
Abnormal palate (131-201 days)	affected litters total litters	6/7=0.86	2/2=1.0	2/3=0.67	8/8=1.0

^a Fraction of fetuses per litter affected averaged over all litters.

^b Significantly different from controls, at the 1% level using the Mann-Whitney test on data Y, where $Y = \arcsin X/N+1 + \arcsin X+1/N+1$. X is the number of defective fetuses in each litter; N is the total number of fetuses in each litter.

^c Standard deviation of untransformed data throughout.

^d Significantly different from controls, chi-squared test with one degree of freedom, 5% level.

by the Laramie investigators to have an oral LD₅₀ in rats of 33 mL/kg.⁵ Our studies of Paraho retort water yield an oral LD₅₀ for this material of 3-4 mL/kg in two strains of mice.

Recently we have begun giving OSRW (at approximately 1.5 mL/kg by stomach tube to ICR mice at various points in gestation. Such treatment appears to cause a diminution of fetal weight (possibly due to the trauma of stomach tubing), and a sharp decline in fetal number that is more pronounced when animals are treated at day 7. In neither case, however, were external abnormalities

apparent; skeletal and visceral examination is not yet complete.

Another oil shale related study concerns the acute adult female and fetal toxicity of nickel arsenide (Ni₃As₂), one of five possible nickel arsenides that may be formed in oil shale retorting, and a compound that has been extensively studied by Gurley *et al.*⁶ Mice were given 0.5 mL of F-10 medium saturated with Ni₃As₂ (final concentration approximately 0.1 mM) on three consecutive days and then watched carefully and weighed at intervals over the next two weeks. The only evidence

of toxicity was a transient weight loss. More recently, this experiment has been repeated with pregnant animals to which Ni_3As_2 has been given at intervals throughout the period of organogenesis (days 5-13). Neither skeletal nor visceral abnormalities have been seen in the offspring from these animals.

Our results make it clear that under some circumstances oil shale retort water is a potent teratogen. More precise definition of this effect and of the fetal toxicity of Ni_3As_2 are under current study.

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Tumorigenesis in the Lung

[D. M. Smith, G. A. Drake, J. E. London, J. R. Prine, P. A. Baron, J. A. Carlson, and J. J. Cunningham]

This program began with the primary goal of studying carcinogenesis as a function of localized irradiations in the lung. The Syrian hamster was the species of choice because of its low incidence

of infectious respiratory disease and because of its wide use as a subject for studying the effects of various chemical carcinogens. As a localized radiation source, it was decided to use 10- μm -diameter spherical ZrO_2 particles that had been uniformly impregnated with plutonium during manufacture. The particles were injected into the jugular vein and lodged quantitatively in the capillary bed of the lung. The number of particles injected and the "specific activity" (pCi per sphere) could be controlled. Thus, the Pu- ZrO_2 spheres served as a quantitatively localized, focal source of irradiation of various fractions of total lung tissue. Regardless of the number of microspheres, their specific activities or resultant total lung burdens, plutonium administered to the lung in this manner is an extremely poor carcinogen unless greater than 20% of the lung is irradiated.

During FY 1980, the results of several experiments were tabulated (Tables I and II) and other new experiments initiated as shown in Table III. The effects of materials given in conjunction with intravenously introduced or intratracheally introduced microspheres have been compiled and listed in Table I. Hematite (Fe_2O_3) suspended in 0.15 N saline solution or saline alone both dramatically enhanced the development and growth of primary lung tumors following the administration of Pu-laden ZrO_2 microspheres. Multiple intratracheal (IT) instillations of Fe_2O_3 -saline or saline alone both were more effective in supplementing tumor formation than a single instillation of Fe_2O_3 -saline or saline alone following intravenous (IV) administration of spheres. Tumor enhancement also was seen when the microspheres were given IT, but the increased number of tumors with multiple Fe_2O_3 -saline or saline instillations versus a single instillation was not as clear-cut as in the IV microsphere case.

In those animals receiving 10 saline instillations IT after IV microspheres (Experiment Nos. 12-109, 12-92, 12-93, and 12-96), the tumor frequencies were directly correlated with the lung burdens resulting from the microsphere injections as noted in Table I. Ten instillations of 0.3-mg ZrO_2 IT also enhanced tumor formation significantly, but more so in those animals that were administered microspheres IT rather than IV. While the

TABLE I
MICROSPHERES PLUS SUPPLEMENTAL INSULTS SUMMARY

Microspheres Given IV				
Exp#	# Microspheres	Lung Burden (nc)	Supplement	Tumor Incidence (%)
12-108	30K	54	-	2 ± 2
10-10	30K	33	-	2 ± 2
<hr/>				
12-114	30K	54	ThO ₂ -Inhaled	1 ± 1
<hr/>				
12-97	30K	48	30mg Fe ₂ O ₃ IT x 1	0 ± 2
12-101	55K	140	30mg Fe ₂ O ₃ IT x 1	0 ± 4
12-94	150K	159	30mg Fe ₂ O ₃ IT x 1	12 ± 7
10-12	30K	33	3.0mg Fe ₂ O ₃ IT x 1	0 ± 3
12-95	150K	159	3.0mg Fe ₂ O ₃ IT x 1	12 ± 7
<hr/>				
12-110	30K	54	0.3mg ZrO ₂ IT x 10	11 ± 5
<hr/>				
12-109	30K	54	0.2ml Saline IT x 10	11 ± 5
12-92	35K	48	0.2ml Saline IT x 10	19 ± 6
12-93	80K	85	0.2ml Saline IT x 10	33 ± 8
12-96	300K	318	0.2ml Saline IT x 10	71 ± 10
<hr/>				
10-14	30K	33	0.3mg Fe ₂ O ₃ IT x 10	17 ± 6
10-16	30K	33	3.0mg Fe ₂ O ₃ IT x 10	4 ± 4
<hr/>				
Microspheres Given IT				
10-11	30K	33	--	5 ± 3
12-111	30K	54	--	0 ± 2
<hr/>				
12-98	30K	54	30mg Fe ₂ O ₃ IT x 1	7 ± 5
10-13	30K	33	3.0mg Fe ₂ O ₃ IT x 1	38 ± 11
12-103	100K	35	3.0mg Fe ₂ O ₃ IT x 1	20 ± 7
<hr/>				
12-113	30K	54	0.3mg ZrO IT x 10	30 ± 7
<hr/>				
12-100	30K	54	0.2ml Saline IT x 10	34 ± 8
12-112	30K	54	0.2ml Saline IT x 10	56 ± 11
<hr/>				
10-17	30K	33	3.0mg Fe ₂ O ₃ IT x 10	51 ± 8
10-15	30K	33	0.3mg Fe ₂ O ₃ IT x 10	39 ± 9

TABLE II
PULMONARY EFFECTS OF $^{239}\text{PuO}_2$ IN THE SYRIAN HAMSTER

GROUP IDENTIFICATION	LTLD ¹	MEDIAN LUNG DOSE (RAD)	MEDIAN DOSE RATE (RAD/DAY)	NON-NEOPLASTIC PULMONARY LESIONS (NON TUMOR-BEARING HAMSTERS)			NUMBER TUMOR-BEARING NUMBER ANIMALS	NEOPLASTIC PULMONARY LESIONS ²	
				FIBROSIS	BAL ²	PNEUMONITIS ³		*ADENOMA	ADENO-CARCINOMA
00-30	--	0	0	0/35 (0%)	0/35 (0%)	1/35 (3%)	0/35 (0%)	0/35 (0%)	0/35 (0%)
13-06	96	8880	24	46/58 (79%)	2/58 (3%)	7/58 (12%)	7/58 (12%)	7/58 (12%)	0/58 (0%)
13-07	110	8530	27	46/50 (92%)	1/50 (2%)	1/50 (2%)	3/50 (6%)	1/50 (2%)	2/50 (4%)
13-08	114	12080	38	53/56 (95%)	15/56 (27%)	12/56 (21%)	8/56 (14%)	7/56 (13%)	1/56 (2%)
13-09	40	3860	7	28/51 (55%)	1/51 (2%)	3/51 (6%)	2/51 (4%)	1/51 (2%)	1/51 (2%)

¹ Long Term Lung Burden (nCi).

² BAL, bronchiolar adenomatoid lesion.

³ Characterized by infiltration of mononuclear inflammatory cells into alveolar walls and increased numbers of alveolar macrophages in alveolar spaces.

magnitude of tumor enhancement resulting from IT saline instillations was similar to that from IT Fe_2O_3 -saline instillations, a higher proportion of the tumors in the Fe_2O_3 -saline treated hamsters were malignant (data not presented here). Nonetheless, the dramatic increase in tumor production following IT instillation of 0.2-ml physiologic saline is an extremely interesting finding with important potential implications regarding tumor-risk assessment.

In another set of experiments (Table II), hamsters were exposed to aerosols of $^{239}\text{PuO}_2$ alone with long-term lung burdens ranging from 40-114 nCi. Tumor incidences varied from 4% in those animals receiving median lung doses of 3,860 RAD to 14% in animals receiving 12,080 RAD. These results are generally consistent with those of other laboratories using plutonium oxide aerosols, but they represent considerably lower lung tumor incidences than previously observed in this laboratory using aerosols of $^{238}\text{PuO}_2$ - ZrO_2 particles.

To assess the potential effects of IV PuO_2 - ZrO_2 microspheres on immune function, Wistar-Lewis rats were given 1.7 million spheres (mean total lung burdens of 120 nCi). The following parameters were monitored at one week, 6 months, and 12 months post exposure: body weight; spleen weights; hemolytic plaque formation (T-cell-dependent B-cell function); hemagglutination and hemolysin titers (humoral antibody response); phytohemagglutinin (PHA) delayed skin hypersensitivity (T-cell function); complete blood counts; lymphocyte mitogenesis-PHA; concanavalin A and Pokeweed mitogen stimulation (T- and B-cell function); ^3H -uridine uptake

(T-cell enumeration); erythrocyte-antibody-complement rosette formation 9B-cell enumeration); and direct rosette formation. There were no significant biological differences in any of these parameters at either 1 week, 6 months, or 12 months post injection in those animals receiving the PuO_2 - ZrO_2 microspheres compared to animals receiving non-radioactive ZrO_2 microspheres or unmanipulated caged controls. These data suggest either that this mode of internally deposited alpha-emitting radionuclides had little effect on immune function determined by monitoring CBC's or splenic lymphocyte function, or that possibly the resolution of our assays was insufficient to detect the changes, or that alpha-emitting nuclides internally deposited in the lung affect immune function parameters other than those monitored in this experiment. The two latter possibilities are highly unlikely.

For the coming year, the lung tumorigenesis part of the program will be carried out as in the past and will concentrate on combined alpha-beta emitter inhalation exposures. The projected exposure schedule for 1981 is in Table III. Results from the studies with graded mixtures of alpha-emitters should contribute sizably to the current knowledge of the relative biological effectiveness of various emissions in the pulmonary system. Additive or synergistic implications should prove very useful in assessing potential hazards in the practical world of nuclear power generation.

Mechanisms of radiation-induced pulmonary carcinogenesis that are being studied include:
1) monitoring of cellular kinetic changes in lung

TABLE III

PROJECTED AEROSOL EXPOSURE SCHEDULE FOR 1981^a

1981 Date	Particle ^b Matrix	²³⁸ PuILB (nCi)	¹⁴⁷ PuILB ^c (nCi)	⁵⁷ Co ^d
6 Jan	ZrO ₂	15	--	x10
27 Jan	ZrO ₂	--	--	x10
17 Feb ^e	--	--	--	---
10 Mar	UO ₂	70	--	---
31 Mar	UO ₂	70	--	---
21 Apr	ZrO ₂	--	7,000	---
12 May	ZrO ₂	15	7,000	---
2 Jun	ZrO ₂	30	7,000	---
23 Jun	ZrO ₂	30	1,000	---
14 Jul ^e	--	--	--	---
4 Aug	ZrO ₂	15	1,000	---
25 Aug	ZrO ₂	30	1,700	---
15 Sep	ZrO ₂	60	1,700	---
6 Oct	ZrO ₂	90	1,700	---
27 Oct	PuO ₂ + ZrO ₂	100	--	---
17 Nov	ThU ₂ + ZrO ₂	100	--	---
15 Dec ^e	--	--	--	---

^a Approximately 70 Syrian hamsters per exposure.^b ⁵⁷Co label will be used in each run, except in non-exposed controls.^c ILB + initial long-term lung burden.^d ⁵⁷Cobalt content increased by factor of 10 to allow long-term counting and retention studies.^e Control animals, no exposure.

tissues from Syrian hamsters exposed to aerosols of alpha-emitting radionuclides, and 2) culturing of hamster alveolar macrophages containing PuO₂ particles which have a low tumorigenicity when inhaled and also macrophages containing the highly tumorigenic PuO₂-ZrO₂ particles. Possibly, the macrophages will be able to discriminate between the two particles, providing an illuminating insight into radionuclide-induced lung cancer.

Modification of Software Used in Processing Data for the Aerosol Inhalation Studies

[D. E. Jackson (Eastern New Mexico University), G. A. Drake, and J. E. London]

Raw count data from the aerosol experiments are used to calculate retention for each animal over time. These curves (lung burden vs time) are

first fit (least squares) to an analytic function which is then integrated over time to give cumulative radiation dose.

In the past these count data have been stored in MUMPS (MUMPS-11 is a single-language, interactive, multi-user operating system for the PDP-11 developed jointly by Digital Equipment Corporation and Mediatech Corporation). The language is a dialect of MUMPS (Massachusetts General Hospital Utility Multi-Programming System) which was developed at the Laboratory of Computer Science at Massachusetts General Hospital and is supported by Grant HS-00240 from the National Center for Health Services Research and Development. As MUMPS has essentially no arithmetic capabilities, the data were transcribed to magnetic tape and hand carried to the Central Computer Facility (CCF) for processing on Network Operating System (NOS). This system is now somewhat unwieldy for a number of reasons including the following.

- 1) NOS is very slow (because of heavy use).
- 2) MUMPS storage capacity is being reached.
- 3) It is difficult to read magtapes at the CCF.

It is now possible to store the data, calculate retention, curve fit and plot the data on the PDP-11/40 using an RT-11 operating system. To operate this RTW (Retention, Time, Weight) software package demands only a fraction of the time required by the previous system. A brief description of the input, output and operational requirements of the RTW software follows.

Hamsters are counted by a crystal counter and the raw count data are printed and punched onto paper tape by a small computer (PDP-8). The paper tape is then read into the PDP-11 so the RTW programs can process the data. Soon we plan to switch from paper tape to floppy disk as the transfer medium. It is necessary to transfer to the PDP-11 since the PDP-8 does not have the peripherals (line printer, RK06 disk and graphics terminal) used by the RTW software.

Once the data are entered, the user must correct any errors in the data using an editor in the RT-11 system. When the data from a tape have been corrected, they are appended to a file which will eventually contain all data from one experiment.

The program which verifies the correctness and appends the data onto a file also gives a printout of biological and effective retention for each animal counted (see Table I for day 0 and Table II for a later day).

After all data from an experiment have been entered, the object is to fit a function (sum of exponentials) to the retention versus time curve for each animal. The program which chooses this best fit needs some initial guess for the parameters which determine the function. To obtain reasonable guesses, the user runs a program which assists in choosing parameters by displaying graphs of the new data and various fitting functions at the terminal.

Finally, the fit program is executed and the printout and plots are displayed in Table III and Fig. 1. The printout includes the fit parameters and the retention data plus information which serves to identify the experiment; the animal plots allow visual verification that the fitting function is close to the retention data.

TABLE I

OUTPUT RETENTION DATA FOR DAY 0

Experiment Number: 523
Day of Counts: 11/20/77
Uncorrected Standard: 94.08
Corrected Standard: 94.08

---Day Zero---

<u>Serial</u>	<u>An</u>	<u>Wgt</u>	<u>Count Rate</u>
20838	1	106	275.58
20839	2	101	175.03
20840	3	151	372.41
20841	4	112	352.25
20842	5	11	313.16
20843	6	103	547.78
20844	7	121	255.41
20851	8	121	181.21
20852	9	132	617.25
20853	10	126	324.36

Average Count: 341.44
Average Weight: 118.8

TABLE II

OUTPUT RETENTION DATA FOR LATER DAY

Experiment Number: 523
Date of Counts: 3/1/78
Uncorrected Standard: 59.96
Elapsed Days: 91

Corrected Standard: 93.69

Ratio: 1.0041

<u>Serial</u>	<u>An</u>	<u>Wgt</u>	<u>Corrected Rate</u>	<u>Bio Ret</u>	<u>Eff Ret</u>
18022	1	144	22.33	0.10229	0.08105
18023	2	141	8.42	0.06074	0.04812
18025	4	126	12.77	0.04578	0.03628
18026	5	141	24.20	0.09754	0.07728
18027	6	135	19.52	0.04499	0.03565
18028	7	120	17.83	0.08813	0.06983
18029	8	156	10.59	0.07378	0.05846

Average Weight: 96.3

Average Count: 17.84

Average Bio ret: 0.06208

Average Eff ret: 0.04918

TABLE III
RETENTION, TIME, WEIGHTING FACTOR OUTPUT

Experiment: 523	Q	0.1101E+03
Animal Number: 1	Fit from Day 2.0	-0.1473E-01
Number of Points: 11	ITER = 5	0.1174E+03
Number of Parameters: 4	CHI2 = 0.3027E+02	-0.2310E+00

Days	Bio Retention	Curve Value	Weighting Factor +
0.0	0.2756E+03	*****	0.2116E+00 +
2.0	0.1850E+03	0.1808E+03	0.7642E-01 +
5.0	0.1326E+03	0.1392E+03	0.1349E+00 +
12.0	0.1063E+03	0.9957E+02	0.1917E+00 +
19.0	0.8584E+02	0.8465E+02	0.2264E+00 +
29.0	0.6885E+02	0.7294E+02	0.3758E+00 +
36.0	0.6679E+02	0.6479E+02	0.3175E+00 +
50.0	0.5231E+02	0.5269E+02	0.5291E+00 +
64.0	0.4059E+02	0.4287E+02	0.7140E+00 +
91.0	0.2839E+02	0.2880E+02	0.1024E+01 +
111.0	0.2358E+02	0.2145E+02	0.1123E+01 +
203.0	0.5471E+01	0.5529E+01	0.2378E+01 +

Fig. 1. Retention data from a hamster that received an aerosol of ^{238}Pu in a Zr_2O_3 matrix. The illustrated data are derived from gamma counting of ^{57}Co which was included in the matrix.

Preliminary Lung Proliferation Studies

[G. A. Drake, J. E. London, and L. R. Gurley]

One of the principal objectives of the lung tumorigenesis program is to explore the possible mechanisms of tumor development in the pulmonary milieu following exposure of animals to aerosols containing α - and β -particles. Cytological and autoradiographic methodologies have been developed

for examining the lung tissue of Syrian hamsters following particle exposure in order to determine what specific locations and cell types are involved in the tumorigenesis process. However, these methodologies are very labor intensive and the time period of tumor development is quite long (on the order of several months). This makes the use of only these methods to explore the time course of tumor development formidable. Thus, more rapid biochemical methods are being developed to determine when lung cell proliferation begins following particle exposure. The use of these methods should identify more efficiently the time period following particle exposure when cytological and autoradiographic studies will be most effective and revealing.

The onset of cell proliferation is most efficiently detected by the incorporation of [^3H]thymidine into DNA. Thus, this method was examined in detail to determine how to best apply it to lung tumorigenesis studies. Previous studies had been reported in which the [^3H]thymidine incorporated into Chinese hamster lung cells was measured by liquid scintillation counting of the combustion products of whole lung samples subjected to oxidation in a tissue oxidizer.^{1,2} Since this method is very simple and rapid, it was

investigated to determine if it could be applied to our problem. The method of Orlov and Orlova³ was also investigated. This procedure differs from the tissue oxidation method in that DNA is first isolated from whole lung tissue. The radioactivity of the DNA is then measured by liquid scintillation counting and the mass of DNA is determined by the Burton diphenylamine assay⁴ so that a specific activity of DNA synthesis can be obtained.

In these investigations, 18 ninety-day old Syrian hamsters weighing 108-140 gm were each administered 1 μ Ci [³H]thymidine/gm body weight (thymidine sp.act. 6.7 μ Ci/mM by intra-peritoneal (IP) injection. A control group of 6 animals was not injected. The [³H]thymidine was administered on a schedule so that all animals were sacrificed at 10 A.M. in order to minimize problems caused by the diurnal mitotic activity demonstrated to occur in rodents between 4-10 A.M.⁵ Hamsters were sacrificed under ether sedation by severing the axillary vessels for exsanguination. Lungs were removed in toto and dissected into 5 lobes: apical, caudal, middle, left, and medial plus accessory combined.⁶ Wet lobe weights were recorded. The apical, caudal, and middle lobes were subjected to tissue oxidation and radioassay using a Packard Tri-Carb Tissue Oxidizer[®] which automatically collects the ³H₂O and ash from the burned sample and dilutes it with scintillation fluid for

counting radioactivity. The left and medial plus accessory lobes were used for the isolation of DNA by the Orlov and Orlova procedure.

Table I shows the data from these lung lobes reported as cpm [³H]thymidine per mg lung. When the tissue oxidizer was used on the apical, middle, and caudal lobes, a decrease in ³H was observed at longer times after [³H]thymidine injection. However, using the DNA isolation method incorporated [³H]thymidine did not appear to decrease so markedly. It is concluded that the tissue oxidizer measures unincorporated [³H]thymidine as well as that incorporated into DNA, and that the decrease in the ³H value measured with the tissue oxidizer probably results from the degradation of unincorporated [³H]thymidine circulating in the blood supply.

Table II shows the data from the DNA isolated by the Orlov and Orlova procedure from the medial plus accessory lobes and the left lobe which is reported as the specific activity of [³H]thymidine incorporated into DNA (cpm ³H/ μ g DNA). It is concluded from these data that: 1) a maximum incorporation of [³H]thymidine into DNA has occurred 2 hr after injection; and 2) no increase in incorporated ³H occurs after 2 hr, indicating that the injected [³H]thymidine was no longer available for incorporation after 2 hr.

TABLE I
TIME COURSE OF [³H]THYMIDINE INCORPORATION INTO LUNG LOBES
MEASURED BY TWO DIFFERENT METHODS

Time After [³ H]thymidine Injection	cpm ³ H/MG Lung*				
	Tissue Oxidizer Method			DNA Isolation Method**	
	Apical	Middle	Caudal	Medial plus Accessory	Left
0 Hours (control)	2.5 (0.5)	4.6 (2.3)	1.0 (0.3)	0.38 (0.07)	0.14 (0.03)
2 Hours	180 (72)	113 (39)	56 (21)	68 (23)	71 (28)
6 Hours	113 (38)	49 (22)	50 (16)	55 (28)	77 (31)
18 Hours	116 (34)	27 (12)	36 (16)	60 (14)	57 (7)

* Average of 6 hamsters with standard deviations ().

** DNA isolated by the Orlov and Orlova procedure.³

TABLE II
SPECIFIC ACTIVITY OF ISOLATED LUNG DNA
FOLLOWING INJECTION OF [³H]THYMIDINE INTO
SYRIAN HAMSTERS

Time After [³ H]thymidine Injection	cpm ³ H/microgram DNA*	
	Medial plus Accessory Lobes	Left Lobe
0 Hours (control)	0.09 (0.2)	0.05 (0.01)
2 Hours	14.2 (5.4)	18.1 (8.5)
6 Hours	11.9 (5.9)	18.3 (7.3)
18 Hours	12.2 (2.3)	18.4 (4.6)

* Average of 6 hamsters with standard deviations ().

DNA was isolated by the Orlov and Orlova procedure.³

These results demonstrate that the rapid method of estimating cell proliferation by measuring [³H]thymidine in whole lung with a tissue oxidizer is unsatisfactory. Thus, a longer method involving the isolation of DNA prior to measuring [³H]thymidine incorporation will be necessary. The results also demonstrate that DNA synthesis occurring over a long time period (greater than 2 hr) cannot be measured following a single injection of [³H]thymidine. Thus, it will be necessary to

pulse-label hamsters with [³H]thymidine at various times after administering labeled particulates in order to follow the time course of cell proliferation response.

To determine if DNA synthesis occurred at different rates in different lobes of the lung, a second experiment was performed. Seven 94 day old Syrian hamsters weighing 105-145 g were each injected with 1-μCi [³H]thymidine/g body weight and sacrificed 2 h post-injection as in the previous experiment. Two uninjected hamsters were also sacrificed as controls. The lung lobes were excised, DNA was isolated by the Orlov and Orlova procedure, and the specific activity of the incorporated [³H]thymidine was determined for each lobe.

Table III shows the data for each hamster. It is concluded that the DNA synthesis rate is essentially the same in each lobe of the lung in an individual animal. However, there does appear to be a two- to three-fold variation in this rate among individual animals.

The Orlov and Orlova procedure is a short procedure for isolating DNA from whole tissue.³ A longer but more commonly used procedure for isolating DNA for radioassay is the modified procedure⁷ of Schmidt and Thannhauser.⁸

TABLE III
SPECIFIC ACTIVITY OF DNA ISOLATED FROM THE VARIOUS LOBES OF THE LUNGS OF
SEVEN SYRIAN HAMSTERS TWO HOURS AFTER
INJECTION OF [³H]THYMIDINE

Animal Number	cpm ³ H/microgram DNA*						% C.V.
	Apical	Middle	Caudal	Left	Median plus Accessory	Mean	
1	15.13	16.91	16.55	17.37	16.63	16.52	4.1
2	13.40	15.12	15.63	19.56	14.81	15.70	14.6
3	11.64	12.58	11.73	10.47	10.75	11.43	7.3
4	19.36	19.81	20.37	20.16	18.32	19.60	4.1
5	23.44	17.37	25.68	26.00	22.38	22.97	15.1
6	20.23	25.68	19.77	23.83	23.00	22.50	11.1
7	23.52	24.53	29.61	25.97	26.48	26.02	8.9
Control	0.001	0.004	0.004	0.001	0.008	0.004	
Control	0.008	0.001	0.003	0.000	0.004	0.003	

* DNA was isolated by the Orlov and Orlova procedure.³

This method was also investigated to determine if it offered any advantages over the shorter procedures, and to determine if the variations in [³H]thymidine incorporation observed between individual animals in Table III is due to variations in the assay.

First, to test assay variability, each lobe from the lung of one animal was treated with cold 10% trichloroacetic acid and 95% ethanol/2% potassium acetate and then dissolved in 1 ml of 0.3 M KOH for 18 h at 37°C.⁷ Six equal aliquots of each dissolved lobe were then subjected to the remainder of the Schmidt/Thannhauser procedure⁷ and the DNA in each sample was determined by the Burton diphenylamine assay.⁴ The results, shown in Table IV, indicate that the reproducibility of the Schmidt/Thannhauser procedure and Burton DNA assay is very good (compare the values in the vertical columns). This reproducibility of DNA isolation and assay indicates that any significant variations in [³H]thymidine incorporation observed among different animals must reflect differences in [³H]thymidine uptake, [³H]thymidine breakdown, DNA synthesis rate, and cell proliferation in individual animals.

To determine if any differences in the mass of DNA/mass of tissue could be detected between the various lobes of the lung or between individual animals using the Schmidt/Thannhauser procedure, 6 young adult hamsters (90 days old) were sacrificed. The DNA content of their lung lobes measured by

this procedure is shown in Table V. Only small variations between animals or between the various lung lobes were observed. This reproducibility supports the conclusion from the previous experiment that variations in [³H]thymidine incorporation observed between animals must reflect individual animal variations involving [³H]thymidine uptake, [³H]thymidine breakdown, DNA synthesis rate, and cell proliferation.

If the measurements of [³H]thymidine incorporation into lung DNA are to be used to determine which lung samples will be subjected to cytological and autoradiographic analysis, it is necessary that the preparation of the tissues be compatible with both types of analysis. To assess this compatibility, the Schmidt/Thannhauser DNA isolation procedure was performed on lungs prepared for autoradiography. Five young adult (93 day old) hamsters were injected with 1-μCi [³H]thymidine/g body weight. Two hours after injection the hamsters were sacrificed and their lungs were perfused intratracheally with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer and then submerged in this solution for 24 h at 4°C. Tissue blocks, 3 x 3 mm, were dissected from each lung; the remaining lung tissue was used for the DNA isolation procedure. The tissue blocks were dehydrated through a series of alcohol washes and embedded in JB-4 plastic. One micron sections of tissue were coated with Kodak NTB photographic emulsion and exposed for

TABLE IV
REPRODUCIBILITY OF THE DNA ANALYSIS OF ONE HAMSTER LUNG
BY THE SCHMIDT/THANNHAUSER PROCEDURE

Aliquot Number	Micrograms DNA/MG Wet Lung Weight					Mean	S.D.
	Apical	Middle	Caudal	Medial plus Accessory	Left		
1	—	3.8	4.5	4.5	3.5	4.08	0.48
2	3.3	3.7	4.1	4.4	3.5	3.79	0.43
3	4.0	4.1	4.4	4.4	3.4	4.05	0.38
4	4.0	4.2	4.4	4.4	3.6	4.11	0.36
5	3.9	3.7	4.5	4.7	3.6	4.09	0.48
6	4.2	4.2	4.6	4.4	—	4.33	0.1
Mean	3.9	3.9	4.4	4.4	3.5		
S.D.	0.34	0.24	0.17	0.13	0.08		

S.D. = Standard deviation.

TABLE V

DNA ANALYSIS OF THE VARIOUS LOBES OF THE LUNGS OF SIX SYRIAN HAMSTERS MEASURED
BY THE SCHMIDT/THANNHAUSER PROCEDURE

Animal Number	Micrograms of DNA/mg Wet Lung Weight						S.D.
	Apical	Middle	Caudal	Medial Plus Accessory	Left	Mean	
1	4.5	3.9	3.8	3.7	3.6	3.9	0.36
2	3.6	3.6	3.1	3.6	3.9	3.6	0.26
3	4.6	4.6	4.4	4.1	4.7	4.5	0.25
4	4.8	4.5	4.1	3.7	4.6	4.4	0.45
5	4.5	4.1	3.9	3.7	4.6	4.2	0.37
6	4.1	4.1	3.7	3.4	3.8	3.8	0.31
Mean	4.4	4.1	3.8	3.7	4.2		
S.D.	0.43	0.12	0.15	0.05	0.21		

2 weeks in darkness at 4°C. The autoradiographs were developed, stained with Lee's methylene blue, and examined by oil immersion microscopy. This procedure produced tissue samples of excellent quality for cytological and autoradiographic analysis.

The glutaraldehyde-fixed lung lobes were subjected to the Schmidt/Thannhauser DNA isolation procedure,⁷ the Burton diphenylamine DNA assay,⁴ and liquid scintillation radioanalysis. The results of this analysis are shown in Table VI. Very little variability in [³H]thymidine incorporation was observed between the various lobes of an individual lung. However, there was a two- to three-fold variability in ³H incorporation between various individual animals. This is the same animal-to-animal variability observed using the Orlov and Orlova procedure in Table III. Thus, this variability does not result from the analytical procedures, but rather, it is the natural background differences between individual animals which we can expect during the course of our studies.

The values of the [³H]thymidine incorporation in the glutaraldehyde-fixed lung tissue (Table VI) were approximately the same as those obtained from unfixed tissue (Table III). Thus, it is concluded that tissue fixation for cytological and autoradiographic analysis does not interfere with the DNA analytical procedure, and we will be able to perform both DNA analysis and autoradiography on the same lung. Comparison of the standard deviations

for the Orlov and Orlova procedure³ (Table III) with those for the Schmidt/Thannhauser procedure⁷ (Table VI) indicates that the Schmidt/Thannhauser procedure is more precise. It is concluded that this increase in precision justifies the use of the longer Schmidt/Thannhauser procedure in these studies.

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TABLE VI

SPECIFIC ACTIVITY OF DNA ISOLATED BY THE SCHMIDT/THANNHAUSER PROCEDURE FROM THE VARIOUS LOBES OF THE LUNGS OF FIVE SYRIAN HAMSTERS TWO HOURS AFTER INJECTION OF [3 H]THYMIDINE

Animal Number	cpm 3 H/microgram DNA						
	Lung Lobe						
	Apical	Middle	Caudal	Median plus Accessory	Left	Mean	C.V.
1	12.2	11.4	15.4	12.9	12.0	12.8	12.4
2	28.9	29.3	--	30.3	28.5	29.3	2.6
3	20.4	21.2	21.5	23.2	20.3	21.3	5.4
4	11.1	10.2	10.8	11.7	10.4	10.8	5.4
5	13.9	12.3	13.9	13.1	12.6	13.1	5.5

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The Toxic Effects of Particulate Nickel Arsenide (Ni_5As_2) on Cell Proliferation

[L. R. Gurley, R. A. Tobey, J. G. Valdez, J. J. Miglio (CMB-1), S. S. Barham (Mayo Clinic), and the J. Roy Gordon Research Laboratory (Inco Metals Co., Toronto, Canada)]

Oil shale, its crude oil product, and the retort water, product gases, and spent shale by-products from the oil production process are all excessively enriched in arsenic.^{1,2,3} Investigations by the Toxicology Group (LS-1) at the Los Alamos National Laboratory^{4,5} have demonstrated that very low concentrations (10 μM) of As (III) produced adverse effects on chromosomal DNA synthesis and histone phosphorylation when the arsenic was administered in the form of sodium arsenite (NaAsO_2). Also Deaven and Mock⁶ observed that NaAsO_2 induced a high frequency of endoreduplicated chromosomes in Chinese hamster CHO cells. These observations have caused some concern since Fruchter *et al.*³ have reported that 50% of the arsenic in retort water at the Paraho Semiworks Retort is in the form of As (III).

As a result of this concern we have considered what other inorganic arsenicals might be produced during oil shale retorting.⁷ Mass balance studies have shown that seven elements (Ni, S, Se, As, Co, Hg, and N) are released from the oil shale in significant quantity during the retorting process.³ This suggested that nickel arsenides may have an opportunity to form at the elevated temperatures occurring in the retort.⁷ The effects of nickel arsenides on biological systems have not been studied. Therefore, we have initiated an investigation to determine if these inorganic arsenicals pose a biological hazard. Five different stable nickel arsenides (Ni_5As_2 , Ni_2As , $\text{Ni}_{11}\text{As}_8$, NiAs , and NiAs_2)⁸ and a stable ternary nickel arsenic sulfide (ranging from $\text{NiAs}_{1.77}\text{S}_{0.23}$ to $\text{NiAs}_{0.77}\text{S}_{1.23}$)⁹ can be formed at high temperatures. In this report we examine the effects of Ni_5As_2 on the proliferation of cultured Chinese hamster cells.

Nickel arsenide (Ni_5As_2) was synthesized at the J. Roy Gordon Research Laboratory of Inco Metals Company near Toronto, Ontario. The material was ground to a powder in which 94% of the particles were 0.18-1.40- μm diameter with a modal size of 0.3 μm .

Chinese hamster (CHO) cells were exposed to the Ni_5As_2 by suspending the cells and powder together in a spinner culture vessel. Although the larger Ni_5As_2 particles settled out into the bottom of the vessel, powder concentrations as low as 10- μM Ni_5As_2 produced toxic effects on cell proliferation (Fig. 1). Culture growth was slowed

Fig. 1. Growth of CHO cells in the presence of Ni_5As_2 powder. Ni_5As_2 powder was added to the growth medium producing suspension concentrations of 0, 10, 25, 50, and 100 μM Ni_5As_2 .

from a 16.5-hr doubling time to a 40-hr doubling time by 10- μM Ni_5As_2 . Powder concentrations of 100- μM Ni_5As_2 produced almost complete inhibition of cell division after 5 hr of treatment.

Flow cytometry measurements of the DNA content of individual cells in the cultures were made after a 24-hr treatment with various amounts of the Ni_5As_2 powder. These data demonstrated that Ni_5As_2 concentrations of 50-100 μM arrested the proliferation of G_1 and G_2 cells, but did not completely arrest cells in S phase (Fig. 2). Computer analysis of the sub-compartments of S phase revealed that the number of cells in the first quarter of S phase was greatly reduced after a 24-hr treatment (Fig. 3) and the number of cells in G_2 was increased by this same amount (Fig. 2). This indicated that the cells were able to slowly traverse S phase during the treatment, but they were arrested somewhere in G_2 .

The effect of Ni_5As_2 on cell survival was also studied. Cells treated with various amounts of the Ni_5As_2 powder for 24 hr were removed from the presence of the Ni_5As_2 , resuspended in fresh untreated growth medium, and plated in a monolayer.

Fig. 2. Cell cycle distribution of CHO cells following 24 hr exposure of cultures to various concentrations of Ni_5As_2 powder. The percent of cells in each cell cycle phase was computed from flow cytometry DNA histograms as previously described.⁵

Fig. 3. Distributions of cells within four sub-compartments of S phase following exposure of CHO cultures to various concentrations of Ni_5As_2 powder for 24 hr. The number of cells in each quarter of DNA histograms using a procedure which integrates a broadened second degree polynomial over each quarter of S phase.⁵

Cell survival was determined from the number of colonies formed after one week. It was found that cells were able to survive a 24-hr treatment of Ni_5As_2 powder at concentrations of $25\ \mu\text{M}$, or less (Fig. 4).

Previous work in this laboratory has shown that nickel subsulfide (Ni_3S_2) toxicity in CHO cells is associated with the uptake of the Ni_3S_2 particles to the inside of the cell. To determine if this phenomenon also occurred with Ni_5As_2 particles, cells treated with various amounts of Ni_5As_2 powder for 8 hr were subjected to electron microscopy as previously described.¹⁰ No Ni_5As_2 particles could be seen in cells treated with 10–100- μM Ni_5As_2 . Particles were observed in cells treated with 250–500- μM Ni_5As_2 . However, x-ray spectrum analysis indicated that these intracellular particles contained only Ni; no As was present.

Fig. 4. Survival of CHO cells in Ni_5As_2 -free growth medium after treatment with various concentrations of Ni_5As_2 powder for 24 hr. After treatment of the cells with the Ni_5As_2 powder, the cells were separated from the Ni_5As_2 particles by centrifuging the culture sample through two layers of medium: an upper layer containing LSM/F10 (1:1) overlaying a lower layer containing undiluted LSM. The cells formed a band on top of the undiluted LSM layer, while the Ni_5As_2 particles formed a pellet on the bottom of the tube. The layer of cells was removed and plated for colony formation to measure cell survival.⁹

These observations suggest the intracellular particles are not nickel arsenide, but rather they are probably aggregates of metallic nickel or a nickel salt which has formed from solution at nucleating centers in the cell. This may account for the uniform size of these particles compared to the less uniform distribution of the original Ni_5As_2 particles added to the cultures. These observations suggest that nickel is being solubilized to some extent in the growth medium.

To determine the extent of solubility of Ni_5As_2 powder, F10 culture growth medium was treated with various amounts of Ni_5As_2 powder for 24 hr at 37°C . The Ni_5As_2 powder was then removed from the medium by centrifugation. Atomic absorption analysis of this powder-free medium indicated that Ni_5As_2 powder was approximately 40% soluble when suspended in growth medium at low concentrations (Table I). The analysis also indicated that both Ni and As existed in solution in the stoichiometric ratio of 5 to 2 as it does in the powder. Therefore, no disproportional dissolution of Ni and As occurred during these experiments.

These studies demonstrate that Ni_5As_2 is toxic to cells at very low concentrations ($10\ \mu\text{M}$, or 4.4 ppm). The active toxic agent is suspected to be a soluble product of the powder which is toxic at even lower concentrations ($3.6\ \mu\text{M}$, or 1.6 ppm) as seen in Table I when cultures were treated with $10\ \mu\text{M}$ Ni_5As_2 powder. The highly toxic nature of this substance suggests that analytical analysis of the oil shale retort process streams should include an analysis for Ni_5As_2 .

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TABLE I
ATOMIC ABSORPTION ANALYSIS OF Ni_5As_2 TREATED F10 GROWTH
MEDIUM FOLLOWING REMOVAL OF Ni_5As_2 PARTICLES

Sample No.	Ni_5As_2 Pretreatment		In Solution			Ni_5As_2 In Solution		Ni_5As_2 Solubilized
	μM	ppm	Ni ppm	As ppm	Atomic Ni/As	μM	ppm	%
1	0	0	0	0	—	0	0	—
2	10	4.4	1.05	0.5	2.68	3.58	1.59	35.8
3	25	11.1	3.09	1.6	2.46	10.53	4.67	42.1
4	50	22.2	3.15	1.6	2.51	10.73	4.76	21.5
5	75	33.3	4.43	2.1	2.69	15.09	6.69	20.1
6	100	44.3	5.09	2.1	2.41	17.34	7.69	17.3
Avg.					2.55*			

* The Ni/As atomic ratio of 2.55 indicates that the stoichiometry of Ni and As in solution is $\text{Ni}_{5.196}\text{As}_2$, essentially the same as the Ni_5As_2 powder.

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Lack of Effect of 60 Hertz Fields on Growth of Cultured Chinese Hamster Cells

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Proliferation of high voltage, alternating current transmission lines has generated interest in possible effects of extremely low frequency (ELF) electromagnetic fields (EMF) on biological systems. As part of a Department of Energy-initiated program involving a variety of biological systems of diverse complexity, a joint project by the Los Alamos National Laboratory, Lovelace Foundation, and Mission Research Corporation was established to investigate effects of 60-Hz fields on the growth and survival of cultured line CHO Chinese hamster cells. Information obtained with this system is indicative of effects at the cellular level, free of complicating (although unquestionably important) tissue-, organ-, and system-specific responses.

The initial exposure chamber utilized in these studies was designed and built at Sandia Laboratories in Albuquerque and consisted of a circular suspension culture flask equipped with a glass jacket through which heated water was circulated to maintain a constant temperature of 37°C (Fig. 1). The culture vessel was immersed in a Plexiglas-walled open water bath whose sides were cemented to a pair of aluminum plates spaced 10 cm apart. A Jefferson 721-111 high voltage transformer was utilized to apply voltage to the plates.

Fig. 1. ELF-EMF exposure chamber No. 1.

In a series of growth studies with this exposure vessel (designated chamber No. 1),¹ a variable degree slowdown in rate of cell division was observed at 12-16 h after application of an approximately 1 mV/m field (estimated value), the maximum field strength obtainable with this chamber. The magnitude of the decrease in culture doubling time was highly variable from experiment-to-experiment and ranged from 8 to 22%. During continued exposure to the field, however, the cells gradually returned to the rate of cell division observed in the non-field-exposed culture. At no time was there a decrease in cell survival (assayed by colony-forming ability).

One of two supposedly identical chambers constructed by Sandia Laboratories was plagued by extensive, readily detectable arcing and coronal discharge. The companion chamber used in the growth studies, though exhibiting no gross side effects, was destroyed by a fire of electrical origin; thus, the results obtained with this chamber design are suspect. That is, it seemed likely that the observed decrease in growth rate was attributable to arcing and coronal effects rather than the 60-Hz field.

Shortly thereafter, contact was made with Mission Research Corporation, and an agreement was established to provide an electrically safe exposure chamber (with negligible coronal effects and

arcing). An additional goal was construction of a device which would operate in the range of the initial test chamber (approximately 1 mV/m).

The unit which resulted from these directives (Fig. 2) was found to operate virtually free of corona/arcing over the range of 1.5 to 300 mV/m. The exposure vessel (chamber No. 3) consisted of a glass cylinder 3 inches in diameter by 2.5 inches in length. The ends of the cylinder were enclosed by 5-mil glass microsheets to which were epoxied 1/8 inch Kovar plates. These plates served as the external high voltage electrodes. The high voltage was supplied by the Jefferson transformer utilized in chamber No. 1. A sampling of results obtained with chamber No. 3 is presented in Fig. 3 and Table I. Identical growth rates were obtained in control and field-exposed cultures over the entire range of feasible field strengths. Of particular interest was the lack of effect observed at the lowest field strength (1.5 mV/m) which, in chamber No. 1, produced a reduction in growth rate. Replacement of the Jefferson neon sign transformer with a low distortion transformer yielded identical results (i.e., no effect on the rate of cell division).

While these results tended to verify the suspicion that the results obtained in chamber No. 1 did not result from the induced 60-Hz field, chamber No. 1 was reconstructed: 1) the internal field strength was monitored to ascertain that there was not some unexpected quirk in geometry

ELECTRICALLY IMPROVED TEST CELL

Fig. 2. ELF-EMF exposure chamber No. 3.

Fig. 3. Growth of CHO cells in 60 Hz electromagnetic fields in chamber No. 3. The apparatus was turned on at the start of the experiment and was on continuously except for brief periods (less than 1 min.) during which aliquots were removed for determination of cell number in a Coulter counter. Open figures represent the non-field-exposed culture control, while solid figures represent field-exposed cultures.

that produced an anomalously high field strength; 2) the chamber was examined for presence of arcing and coronal effects; and 3) the growth of cells was measured to see whether the results obtained initially with this type of exposure chamber could be duplicated.

The magnitude of field strength in the medium was that expected from theoretical calculations, but there was evidence for coronal discharge that was not apparent visually. Significantly, a slowdown in growth rate in the field-exposed cells was observed in the reconstructed chamber which was comparable to the results obtained with the initial chamber (Fig. 4).

The cause of the slowdown in growth rate observed in both the initial and re-constructed No. 1 model chambers is unknown. However, given the fact that 1) the culture doubling time for field-exposed cells grown in chamber No. 3 was identical to that of the control at field strengths comparable to those obtained with chamber No. 1 (in which an increase in doubling time was observed); and 2) chamber No. 3 was operating in an arcing/coronal discharge-free mode whereas chamber No. 1 was found to yield coronal effects, it is concluded that the reduction in growth rate observed in chamber No. 1 stems from an artifact in chamber design (coronal effects?) rather than an intrinsic field effect. Strengthening that conclusion is the observation

Fig. 4. Growth of CHO cells in a 140 kV/m (in air) electromagnetic field in chamber No. 1. The field was turned on at $t=0$ (indicated with an arrow) and maintained continuously thereafter except for brief periods of 1 min. or less when aliquots were removed for cell counts. Symbols as in Fig. 3.

that increasing the internal field strength in chamber No. 3 by a factor of two orders of magnitude greater than that achievable with chamber No. 1 fails to induce a reduction in growth rate (Table I).

While numerous *ad hoc* explanations for the corona-associated reduction in cell division observed in chamber No. 1 may be envisioned (e.g., production of ozone, radiation, etc.), the variability and low magnitude of the effect plus the artifactual nature of the phenomenon suggest that determination of the specific mechanism would be time consuming, costly, and most important, irrelevant to the problem at hand ... assessment of 60-Hz field effects. Future studies will utilize chambers producing far greater field strengths with negligible side effects.

Preliminary results obtained with a magnetic induction chamber capable of yielding fields of 2.5 V/m indicate that cell growth, determined by

TABLE I
RESULTS OBTAINED WITH THE MODEL NO. 3 ELF EXPOSURE CHAMBER

Applied Voltage (RMS Total)	E in Medium (mV/m Peak)	Current Density mA/m ²	Culture Doubling Time Control	+Field
50 V	1.5	2.75	15.0 h	15.0 h
100 V	3.1	5.5	16.0 h	16.1 h
300 V	9.3	16.6	16.5 h	16.5 h
600 V	18.5	33.3	18.5 h	18.5 h
1 kV	30.8	55.5	17.5 h	17.5 h
3 kV	92.5	166.5	18.0 h	18.4 h
4.5 kV	138.8	249.8	17.5 h	17.5 h
6 kV	185.0	333.1	16.8 h	16.8 h
10 kV	308.4	555.1	16.0 h	15.7 h

measurement of cell concentration over prolonged time periods, is not affected by fields of that magnitude; at that field strength, however, there is a pronounced reduction in the rate of potassium influx. These findings (which require confirmation) suggest that 60-Hz fields may affect cellular processes under conditions where the usually sensitive parameter, cell kinetics, is unaffected.

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Radiation Biology and Oncology

(N. Tokita, E. Bain, S. G. Carpenter, and M. R. Rajul)

Radiation responses of CHO cells cultured under prolonged hypoxic conditions: Radiosensitivity of cells depends on oxygen tension as well as their stage within the cell cycle. When the cells are kept under hypoxic conditions for prolonged periods, there may be increased cellular radiosensitivity due to hypoxia-induced cell cycle distribution changes, or decreased radiosensitivity if the cells accumulate in late S phase of the cell cycle. Chinese hamster cells (line CHO) were cultured under hypoxic conditions (oxygen tensions: 50 - 200,000 ppm) and their clonogenicity and cell cycle distributions were measured as a function of the gassing period. Cells cultured under 1000 ppm O₂ exhibited a biphasic reduction in cell survival. This reduction was more prominent for cells cultured under a lower O₂ tension environment.

Cell cycle analyses indicated that hypoxic cells traversed the cell cycle at a reduced rate depending on the level of oxygen tension.

The radiosensitivity of CHO cells cultured under hypoxic conditions was also measured at 24 hr intervals for 96 hr. The cells cultured under hypoxic conditions were split into two spinner flasks just before irradiation: one remained hypoxic, the other was gassed with an air-CO₂ mixture for approximately 10 min to provide aerated conditions. Radiation survival curves also indicated that the hypoxic cells traversed the cell cycle at slow rates, exhibiting a slightly increased radiosensitivity of the hypoxic cells at 24 hr, followed by decreased radiosensitivity at 72 hr. The latter was due to accumulation of hypoxic cells in late S phase. By 96 hr, the radiosensitivity of cells returned to that of time 0 hr. These results indicate that hypoxia induced a partial synchronization of cells which persisted over prolonged periods (i.e., up to six times the normal culture doubling time) (Fig. 1).

Progression delays after exposure to x rays under oxic or hypoxic conditions: In order to test whether the radiation-induced delay in cell progression is dependent on the level of cell survival, CHO cells were first exposed to nitrogen gas for a period of 1 hr followed by x-irradiation under the same nitrogen gassing or under aerated conditions. Radiation survival curves indicated that to achieve a 50% survival level, it required 220 rad for aerated cells, whereas it required 540 rad for hypoxic cells. At 5% survival levels, the

Fig. 1. CHO cells cultured under hypoxic conditions were irradiated under aerated (●) or hypoxic (▲) conditions. Increased radioresistance at 72 hr indicates that hypoxia induced partial synchronization.

dose required for aerated cells was 540 rad and for hypoxic cells 1420 rad. The results indicated that for a given dose, the cell progression delay was considerably less for hypoxic cells. However, if the level of survivals were matched, the magnitude of cell progression delay was similar for both aerated and hypoxic cells. These findings suggest that radiation-induced cell progression delay is cell-survival dependent.

Interactions between alpha particle radiation from plutonium and nitrogen mustard or actinomycin D: Although radiation cell survival curves for alpha particles from plutonium are simple exponential curves with no shoulder, it has been reported that interactions between x rays and high LET radiation exist.¹ In our study, interactions between x rays or alpha particles from plutonium and drugs [nitrogen mustard (NM), actinomycin D (AMD)] were measured using V79 cells. Cells were exposed to radiation first, followed by drug exposure for 1 hr.

"D₀" doses for NM administered following exposure to x rays at survival levels of 100%, 45%, and 6.6% were 0.74, 0.54, and 0.35 $\mu\text{g/ml}$, respectively. The "D₀" doses after alpha particle exposures at survival levels of 100%, 32%, and 8% were 0.74, 0.38, and 0.30 $\mu\text{g/ml}$, respectively. These data indicated that there seems to be no significant difference between x-ray-NM and alpha-NM interactions. Since x rays are known to produce sublethal damage, the data also suggest that a similar type of damage is also induced by alpha particles. A study using AMD instead of NM indicated that drug treatment after alpha irradiation resulted in an increased steepening of the survival curve, suggesting that there is an interaction between alpha particles and AMD (Fig. 2).

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Fig. 2. Interactions between: x rays or alpha particles and nitrogen mustard (left); alpha particles and actinomycin D (right).

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INTRODUCTION

The Biophysics Group, LS-2, has two sections: flow cytometer development and agricultural bioscience. The work of the flow cytometer section supported by the Department of Energy (DOE) will be reported here.

This work is directed toward developing better instruments and techniques to assist in solving biological problems of interest to DOE. Close collaboration with biologists in other groups is maintained to make the work relevant. Interest in analyzing and sorting individual chromosomes sparked the adaptation of the hybrid (Los Alamos/Becton-Dickinson FACS-II) flow cytometer to sort

chromosomes for biological studies. The addition of the time parameter in taking data was very helpful in demonstrating the leaching of strain from cells when they are removed from the staining solution. For best results, the cells must be run in the flow cytometer while still in the staining solution. The Los Alamos Cell Analysis (LACEL) computer data acquisition display and processing system is available for eight-parameter list mode data collection and subsequent data analysis for determining different correlations. Computer modeling has been helpful in determining the design parameters for chromosome analysis of the complete karyotype.

Chromosome Analysis and Sorting

(J. H. Jett, M. E. Wilder (LS-4) and L. S. Cram (LS-4))

Several programs at Los Alamos require either the analysis of mammalian chromosomes or pure populations of individual chromosome types. To provide an initial capability immediately, the LACEL (Los Alamos Cell Analysis) electronics and software system described in previous reports^{1,2} has been used in conjunction with an optical bench from a commercial cell sorter (Becton-Dickinson FACS-II) to analyze the DNA content of chromosomes and to sort sufficient quantities of chromosomes for recombinant DNA studies. The results obtained represent the resolution attainable in chromosome analysis with a standard flow cytometer which was not designed or specially optimized to obtain high resolution measurements of small particles.

The two distributions shown in Figs. 1 and 2 demonstrate the quality of results which have been achieved to date. The chromosomes analyzed were obtained from Chinese hamster line M3-1 cells and stained with either of the DNA specific fluorochromes propidium iodide or Hoechst 33258. The resolution attained is comparable for the two stains. The coefficient of variation is on the order of two percent for the larger chromosomes for both distributions.

With the resolution demonstrated it will be possible to sort chromosomes for multiple purposes. The chromosomes which generate individual peaks can be sorted in small number (less than 10^3) onto slides for subsequent cytogenetic analysis such as being done in the tumorigenesis studies (L. S. Cram, LS-4). Microgram quantities of specific chromosome types can be sorted for further biochemical studies. Specifically, libraries of DNA specific to given chromosomes will be built (J. Griffith and R. Walters, LS-3) with recombinant DNA techniques. The source material will be sorted chromosomes of greater than 95% purity. It is estimated that at the present sorting rates, microgram quantities of the larger Chinese hamster chromosomes can be obtained in less than a day.

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Fig. 1. Fluorescence intensity versus number of chromosomes. The chromosomes from the Chinese hamster cell line M3-1 were stained with Hoechst 33258, excited in the uv at 350 nm and analyzed on a standard FACS II flow cytometer.

Fig. 2. Fluorescence intensity versus number of chromosomes. The chromosomes from the Chinese hamster cell line M3-1 were stained with propidium iodide excited in the visible region at 488 nm and analyzed on a standard FACS II flow cytometer.

Kinetic Measurements of DNA Specific Fluorochromes Using Flow Cytometry

[J. C. Martin, D. E. Swartzendruber (LS-4), G. L. Travis (LS-4), T. S. Johnson (LS-1), and C. C. Stewart (LS-4)]

The kinetic interactions between the DNA-specific fluorochromes mithramycin and Hoechst 33342 and mammalian cells have been studied using a continuous time recording flow cytometric technique. Cell measurements were correlated with time using an analog signal derived from a linear voltage ramp generator. The details of this technique have been described.¹ Mithramycin uptake was studied in unfixed and ethanol-fixed Chinese hamster lung cells ($V_{279-171b}$ line). Fluorochrome leaching from fixed, mithramycin stained mouse spleen cells was measured. The kinetics of Hoechst 33342 binding to mouse bone marrow cells was also studied.

The Los Alamos computer-based multiparameter cell sorter system²⁻⁴ was used for these measurements. In this instrument, single cells flow through an enclosed chamber⁵ under conditions of laminar flow, sequentially passing through an electronic cell volume sensing orifice and then through the focused beam of an argon-ion laser. Mithramycin stained cells were analyzed with laser excitation at 457.9 nm. Sample flow rates were held at or below 10^3 cells/sec. For the present studies two analog signals, small angle light scatter, and fluorescence were measured. The light scattering signal was used to trigger data acquisition so that all cells passing through the laser beam were recorded even if they had zero or low fluorescence intensity. The third parameter recorded was the sampled output of a linear voltage ramp generator. A new linear voltage ramp generator was designed and fabricated. This ramp generator has a maximum time range of 99 min and is adjustable in 1-min increments.

During data acquisition the dc voltage level from the ramp generator was sampled and held by the signal processor each time a cell passed through the laser beam. This level was then digitized and stored along with the digitized cell volume, light scatter and mithramycin-DNA fluorescence signals. The time parameter was digitized into 256 channels

so that a 10-min time sweep corresponded to a time resolution of 2.3 sec per channel. Analog signals were processed by a four-parameter multiplexing circuit triggered by events meeting required logic conditions mentioned above. Peak-sense-and-hold levels were then digitized by 8-bit ADCs and transferred to a PDP-11/20 computer (Digital Equipment Corp., Maynard, MA) where the four-parameter data were stored on magnetic discs as correlated raw event files.³

For the dye binding studies there was an initial time delay of 15 to 40 seconds between the time the stain was added to the cell suspension and the start of data acquisition. In the early measurements, both the ramp generator and data acquisition were enabled when a stable sample flow was established. This necessitated using a stop watch to keep track of the initial start-up time. More recently we have started the ramp generator at the time of the dye mixing and then enabled data acquisition when stable flow was established. Thus, time zero corresponds to channel zero of the pulse height distribution.

The mithramycin cellular DNA-binding rate is determined to a large extent by the intracellular dye concentration. Previous results⁶ have shown that both ethanol-fixed and unfixed $V_{279-171b}$ Chinese hamster cells have essentially the same equilibrium values of fluorescence intensity after mithramycin staining. In the present study we compared the DNA-binding rates for this DNA specific fluorochrome in ethanol-fixed and unfixed $V_{279-171b}$ cells (Fig. 1). For ethanol-fixed cells (Fig. 1B) the mithramycin staining reached 90% of the maximum fluorescence equilibrium value in 1.2 min and equilibrium by about 4.9 min. In contrast, the kinetics of mithramycin staining was significantly slower in unfixed cells. In the unfixed cells, the staining time to 90% of equilibrium was estimated to be three times longer than for fixed cells. We interpret the slower staining rate for the unfixed cells to be due to the time required for diffusion of mithramycin through the plasma membrane of the intact cells. The results obtained in the leaching experiment (Fig. 2) indicated that, after resuspending mithramycin stained fixed murine spleen cells in PBS, the fluorescence intensity

Fig. 1. Comparison of the mithramycin nuclear DNA staining rates for Chinese hamster lung cells (line V_{279-171b}) unfixed cells (A) and ethanol-fixed cells (B), trypsinized from multicellular spheroids. The two-parameter maps show the time-dependent fluorescence acquired over a 7.3 min period after addition of the DNA fluorochrome to the cells (on-ice). The dots represent locations having five or more cells accumulated, and the isocell contours are shown for elevations of 100 and 200 cells. Note the slower mithramycin DNA staining equilibrium time for the intact, unfixed cells (A) versus fixed cells (B). Note data in both panels were acquired using the same gain settings. Chinese hamster lung cells (line V_{279-171b}) were grown as monolayer cultures using basal medium (GIBCO, no. 420-1300, Grand Island, NY) plus 15% fetal calf serum (Flow Laboratories, McLean, VA). The cells were dissociated with trypsin (0.25% DIFCO, 0152-17, Detroit, MI) and transferred as single cells into spinner flasks at 1.5×10^5 cells per 100 ml basal medium, pH 7.2 - 7.4, with 5% fetal calf serum.^{12,13} Spheroid cultures were maintained routinely for three weeks. Aliquots of suspended spheroids were pipetted at day 21. Single cell suspensions were prepared by trypsinization (0.25%), followed by trypsin inhibition with serum-containing basal medium and two PBS washes on ice using 10 min centrifugations at 150 X g. Cells were kept on ice for up to 1 hr prior to mithramycin staining. The dissociated spheroid cells were subsequently analyzed as either unfixed cells stained with mithramycin in saline just before analysis or as ethanol-fixed cells (2-3 hr fixation, followed by a PBS wash before staining with mithramycin) using mithramycin concentrations similar to that previously described.^{6,14}

Fig. 2. Time dependent fluorescence data showing the behavior of mithramycin leaching from stained mouse spleen cells. Two fluorescence distributions are shown in panel A. The dashed curve is the initial DNA distribution for the cells in a solution of mithramycin. The solid curve shows the cumulative fluorescence distribution for a time period of 7.3 min after the cells were resuspended in PBS. Note the smeared character of this distribution. Panel B shows the corresponding time parameter distribution. The channel numbers on the abscissa in this panel correspond to time intervals instead of fluorescence intensity as in panel A. The count rate is approximately 300 cells/sec. Panel C shows a two-parameter isometric display for the data in Panels A and B. Full scale on the vertical axis is approximately 500 cells. The fluctuation in the number of cells in the modal channel of the G1 peaks in these traces is due to the low number of cells in each peak and is reflected by the different coefficients of variation for these peaks. The $t=0$ trace is the data accumulated during the 0.7 minute start-up time. The two-parameter contour map for the time period of 0.7 to 8.0 minutes after the cells were resuspended in PBS plus another run of 7.5 min for the same sample beginning 6 min after the end of the first run is shown. The dots show locations having five or more cells accumulated, and the contours are shown for elevations of 100, 300 and 600 cells. The dot-dashed lines connecting the two-parameter contours to the fluorescence values at time equal zero are extrapolations drawn to guide the eye. For these experiments, spleens were removed from 129/SV mice, and single cell suspensions were obtained by mechanical dispersion. After one wash in phosphate buffered saline (PBS), pH 7.2, cells were fixed as single cell suspensions in 70% ethanol. Fixation was for a minimum of 24 hours after which the pelleted cells were resuspended in the mithramycin (Pfizer, New York, NY) stain solution (100 $\mu\text{g/ml}$) in 0.15M NaCl with 15 mM MgCl_2 at approximately 500,000 cells/ml. For the dye leaching experiments mithramycin-stained cells were pelleted and resuspended in PBS.

decreased rapidly to one-half the initial intensity in 0.7 min and then slowly to an equilibrium value of 22% of the initial intensity in about 20 min. This equilibrium value is in agreement with previous data obtained for Chinese hamster cells (line CHO) using conventional DNA distribution analysis.⁷ The residual dye plus the biphasic leaching kinetics of mithramycin suggest that there may exist in the mithramycin-Mg⁺⁺-DNA complex two different noncovalent dye binding states as described for mithramycin⁸ and other non-intercalating dyes that bind to DNA.^{9,10}

A recent study has demonstrated the differential uptake of Hoechst 33342 at low concentrations, by unfixed human T and B lymphocytes.¹¹ Following this lead we have initiated studies of the binding of Hoechst 33342 with mouse bone marrow cells. Preliminary results indicate that there are at least three subpopulations of cells showing markedly different rates of dye binding. Sorting experiments are in progress to identify the cells in each subpopulation.

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Software for Flow Cytometry and Sorting

[G. C. Salzman, S. F. Wilkins (E-8), and J. A. Whitfill (E-8)]

A computer-based data acquisition, display, and processing system for flow cytometers has been completed. The LACEL programs and electronic hardware provide the capability to acquire list mode or histogram data for up to eight parameters and control bidirectional sorting based on up to four parameters for each direction. The programs described in this paper also enable the display of single parameter and bivariate histograms and the graphical manipulation of the list mode data. Programs have also been developed for cluster analysis and hidden line isometric displays.

Chromosome Modeling for Centromeric Index

Determination

[G. C. Salzman and L. S. Cram (LS-4)]

A computer model of a chromosome stain distribution has been developed and used to estimate the effect of laser beam width on the

determination of the centromeric index in flow. We have shown that the present beam width of $1.8 \mu\text{m}$ ($1/e^2$) is inadequate to enable centromeric index determination but that a beam width of $0.6 \mu\text{m}$ ($1/e^2$) is sufficient.

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INTRODUCTION

The Genetics Group (LS-3) investigates the nature and regulation of gene expression in eukaryotes, and develops and applies systems for detecting and measuring pollutant-induced alterations in genes and their expression. Such pollutant-induced alterations include:

(1) Those which affect genes whose products (proteins, enzymes) interact directly with the pollutant itself -- binding with it and/or catalyzing its reaction to other chemical forms. The sequelae of this interaction of pollutants and related genes are detoxification and/or activation of the pollutant.

(2) Those which involve interaction of the pollutants with unrelated genes, i.e., those genes whose products do not interact with the pollutant itself. The sequelae in this instance include mutations, terata, morbidity and cancer.

Systems providing defined alterations of the first type provide tools not only for defining protective and activation mechanisms, but also for studying gene expression and its regulation. It is within this context that our group has studied a particular system, the Cd^{++} -induced expression of gene products which bind and detoxify the metal. This system has a number of advantages for studying induced specific gene expression in cells of higher animals: Cd^{++} -induced synthesis of the primary gene product, thionein, is easily measured in vivo and in vitro, is relatively specific, is an ubiquitous response among animal cells, is relatively rapid, of large magnitude, involves a response at the level of gene transcription and is a direct effect. Thus the system promises to facilitate a description of events involved in modulation of gene activity at the transcriptional level. No such description exists for modulation of any eukaryotic gene. The

search for modulators (activators, repressors) of such activity should be facilitated not only by the directness, specificity, rapidity and magnitude of the response, but also by the existence of radiolabeled tracers (^{109}Cd , ^{115}Cd) of the inducer and its presumed effector complexes. Most importantly, cell variants with altered regulation of response are readily derived by growing cells in toxic levels of Cd^{++} to produce resistant sub-populations. We have derived and characterized a number of these. An essential component of such studies involves the production in large amounts of specific DNA sequences complementary to the gene being induced and to its pre-mRNA and mRNA products. To this end, we have acquired expertise in nucleic acid hybridization and recombinant DNA techniques. These have been applied to produce (1) clones of cadmium induction-specific as well as thionein-specific genes, (2) a definition of the specificity and magnitude of the response to cadmium exposure, and (3) the first demonstration of gene amplification as a response to an energy-related toxic agent. An application of these tools to define the Cd^{++} response of cultured cells is described in the first report that follows.

A preponderance of the genetic toxicology work in LS-3 involves the use of in vitro assays to define the mutagenicity of oil shale products and by-products. The systems employed use bacteria (the Ames test), animal cells and human cells as targets for the mutagens. The problems involved in extrapolating the results obtained in such tests to a definition of pollutant's mutagenicity toward man derive primarily from the fact that the ultimate mutagen is often a derivative of the pollutant. The mutagenic derivative is produced as a by-product of cellular enzymic processes which function to solubilize and detoxify many non-physiological organic compounds. Often, the enzymes involved are induced by the very organic molecules whose detoxification they catalyze. This production of mutagenic compounds from non-mutagens by enzymic processes is referred to as "metabolic activation." Bacteria and most cultured animal cells mimic the metabolic activation that occurs in humans poorly or

not at all. Thus enzyme-containing extracts of organs which are most active in this type of response, such as rat liver, are mixed with the target cells to effect activation -- hopefully, in a fashion roughly similar to that which occurs in the human cell. It is apparent that research into this activation process is vital to development and application of valid in vitro mutagenicity assays. Accordingly, much of our work involves analysis, development and use of enzymic activation systems. Another important research effort in this area explores the nature and utility of photoactivation of pollutants. Near ultraviolet light is used to produce mutagenic derivatives of organic pollutants. Such activation has the advantage of greater definition and reproducibility than the metabolic activation procedures. LS-3 investigators are in the forefront in this area. They are studying not only the biological results of such activation, but are also defining the derivatives and DNA adducts formed vis-a-vis those produced metabolically.

Our group believes also that the use of co-cultured human cells as sources of metabolic activation would reduce significantly the uncertainties involved in extrapolating test results to a probability of effects in humans. We have had success in using newborn human foreskin keratinocytes in this context. Ultimately, we seek to use human cells in which both activation and mutation occurs. This necessitates the development of cell cultures which both maintain their metabolic activation properties in vitro and which can be sub-cultured with high efficiency. The development and application of such human cell systems is progressing satisfactorily and will provide a valuable addition to our battery of activation systems.

Thus, LS-3 has in use or in development the components of an exceptional battery of in vitro tests for determining the toxic and carcinogenic potential of energy-related pollutants. Their application to a definition of the cytotoxicity and mutagenicity of shale oils and oil shale byproducts is illustrated in the accompanying reports.

Molecular Mechanisms of Cd Detoxification in Cd-Resistant Cultured Cells: Role of Metallothionein and Other Inducible Factors

[C. E. Hildebrand, M. D. Enger, J. K. Griffith, R. A. Tobey (LS-1), and R. A. Walters]

Studies in our laboratory have utilized cellular and molecular approaches to delineate the role(s) of metallothioneins (MT) and the regulation of their induced synthesis in cellular metabolism of cadmium (Cd^{2+}). Initial studies were focused on the development of stable Cd^{2+} -resistant sublines of the Chinese hamster cell (line CHO).¹ Cd^{2+} -resistant (Cd^r) sublines were derived by continuous exposure of the parental CHO cell to selective pressure with stepwise increases in CdCl_2 levels. Three clonal sublines, designated $\text{Cd}^r2\text{C}10$, $\text{Cd}^r2\text{OF}4$, and $\text{Cd}^r3\text{OF}9$, were isolated and were shown to be resistant to continuous exposures of 2, 26, and 40 μM CdCl_2 , respectively.¹⁻³ The parental CHO line is sensitive to 0.2 μM Cd^{2+} . Survival of CHO cells and the Cd^r sublines during exposure to various concentrations of CdCl_2 is shown in Fig. 1. These sublines retained their Cd^{2+} resistant phenotypes during continuous culture for more than 135 generations in the absence of Cd^{2+} . Measurements of Cd^{2+} uptake by the Cd^r sublines showed that the increased Cd^{2+} -resistance levels were not attributable to decreased Cd^{2+} -transport in the more resistant sublines.^{1,3}

Pharmacokinetic analyses of intracellular Cd^{2+} distribution together with measurement of MT synthesis rates revealed several characteristics which distinguished the Cd^r sublines from one another and from the Cd^{2+} -sensitive CHO cell. (1) All of the Cd^r sublines possessed the capacity to induce MT synthesis and little Cd^{2+} binding to MT-sized proteins could be detected in CHO cells.^{1,3} (2) The more Cd^{2+} -resistant Cd^r sublines ($\text{Cd}^r2\text{OF}4$ and $\text{Cd}^r3\text{OF}9$) induced MT synthesis faster and to a greater maximal rate (50-fold greater than basal rate in 4 h) than the less resistant $\text{Cd}^r2\text{C}10$ cell (25-fold greater than basal rate in 7 h). As a result of the dynamics of the MT induction response, $\text{Cd}^r3\text{OF}9$ and $\text{Cd}^r2\text{OF}4$ cells were capable of sequestering intracellular Cd^{2+} more rapidly than the $\text{Cd}^r2\text{C}10$ cell.^{1,3} (3) Intracellular Cd^{2+} distribution measurements indicated that non-MT

Fig. 1. Survival of CHO cells and Cd^r sublines during exposure to various concentrations of CdCl_2 . In all experiments cells grown in suspension culture were treated with CdCl_2 beginning at 0 h. At the indicated times aliquots were removed from the culture and cells were plated into Petri dishes for determination of cloning ability.

Cd^{2+} -binding ligands (as yet uncharacterized) played a significant role in conferring Cd^{2+} -resistance to the $\text{Cd}^r3\text{OF}9$ subline, but not to the $\text{Cd}^r2\text{OF}4$ or $\text{Cd}^r2\text{C}10$ cells.^{1,3}

Molecular analyses of cellular responses to Cd^{2+} exposure employed measurements of MT synthesis rates, electrophoretic resolution of cellular proteins under denaturing conditions, cell-free translation of poly(A)⁺ RNAs, and nucleic acid hybridization measurements using a radioisotopically labeled complementary DNA (cDNA₃) containing DNA sequences complementary to RNA sequences expressed in response to Cd^{2+} exposure.⁴⁻¹⁰ The cDNA probe was prepared using as the template electrophoretically fractionated, 400 nucleotide (MT) poly(A)⁺ RNA from $\text{Cd}^r2\text{OF}4$ cells maximally induced with Cd^{2+} , and the cDNA complementary to RNA sequences specific for Cd^{2+} -induced cells (induction-specific sequences) was isolated by a series of molecular hybridization fractionations to remove the sequences common to uninduced and induced cells.⁶⁻⁹

MT synthesis rate measurements coupled with cell-free translation determination of thionein mRNA (mRNA_{th}) activity during Cd^{2+} exposure (induction) and following withdrawal of Cd^{2+} (deinduction) in the Cd^r sublines suggested that MT synthesis is

Fig. 2. Nucleic acid hybridization kinetics of ^3H -cDNA tracer containing induction specific sequences with cytoplasmic poly(A) $^+$ mRNA from uninduced (i.e., no Cd^{2+} exposure) CHO and Cd^{2+} 20F4 cells and Cd^{2+} 20F4 cells maximally induced with Cd^{2+} . Hybridization of a globin ^3H -cDNA tracer with its template mRNA (in excess) is included for reference. The value of ERot ("equivalent" RNA concentration $\times$ time of incubation corrected for differences in salt concentration from one reaction to another) at which 50% of the ^3H -cDNA is in hybrid form (i.e., 50% resistant to S_1 nuclease which hydrolyzes single-strand nucleic acid specifically) is indicative of the relative concentrations of the induction specific mRNA sequences present in the various mRNA preparations. Details of these analyses are provided elsewhere.

regulated at the level of transcription of mRNA.^{4,5} Nucleic acid hybridization measurements further demonstrated that regulation of MT induction occurs at the transcriptional level and that the cellular level of induction-specific sequences increased ~2000-fold over uninduced levels during Cd^{2+} -exposure of all Cd^{r} sublines.⁶⁻⁹ Representative results from these nucleic acid hybridization measurements are shown in Fig. 2. Analysis of the hybridization kinetics indicates that 3-4 induction specific sequences (of the 400 NT size class) in addition to the sequences encoding MT are expressed in the Cd^{r} cells response to Cd^{2+} exposure.^{7,9} This prediction was strengthened by resolution of three non-MT, low molecular weight proteins synthesized in a cell-free translation system directed by the purified mRNA from which the cDNA probe was constructed.⁹ In the CHO cell only a portion of the induction specific sequences are expressed, and those expressed are found at only ~100-fold increased level compared to uninduced cells.

The cDNA probe complementary to the Cd^{2+} -induction specific RNA sequences was also

utilized to determine the relative number of the genes coding for those sequences in the genomes of the Cd^{r} sublines and the CHO cell.^{10,11} These measurements indicated that the genes encoding the induction specific RNA sequences were amplified approximately 10 fold and 4 fold in Cd^{r} 20F4 and Cd^{r} 30F9 sublines respectively, and were unamplified in Cd^{r} 2C10 and CHO cells.^{10,11} Thus, amplification of Cd^{2+} -induced genes is not a prerequisite for the development of cellular Cd^{2+} -resistance (e.g., as in the transition of CHO to Cd^{r} 2C10), although specific gene amplification may be important in development of resistance to higher levels of Cd^{2+} .

In addition to MT and other Cd^{2+} -induced, low molecular weight proteins discussed above, high resolution electrophoretic analysis of cytoplasmic proteins in the Cd^{r} sublines with and without Cd^{2+} treatment showed 3-4 high molecular weight (ranging from 25 000 to 100 000 daltons) proteins enhanced by Cd^{2+} exposure. The role and identity of these proteins is under investigation.

In summary, molecular approaches to the study of cellular Cd^{2+} metabolism have provided initial definition of mechanisms involved in Cd^{2+} detoxification. The relative proficiency of cells to induce MT synthesis appears to be a primary factor in the protection of cells against the toxic manifestations of Cd^{2+} . The dynamics of the MT induction response are also a factor in determining the cellular capacity to detoxify Cd^{2+} . In that context, amplification of Cd^{2+} -induced genes may provide a mechanism for enhancing the expression of sequences involved in cellular response to Cd^{2+} exposure. In addition to MT induction, our studies indicate that other proteins are regulated in Cd^{2+} exposure in the Cd^{r} cells. The nature of these responses is currently being studied both in the context of their role(s) in direct detoxification of Cd^{2+} (i.e., by high affinity binding) and of compensatory responses which permit cellular metabolism to accommodate perturbations introduced by the presence of Cd^{2+} . It is likely that the molecular approaches to understanding cellular Cd^{2+} metabolism described here can be applied to the study of Cd^{2+} -modulated cellular responses in human populations. Such studies could provide more accurate assessment of the health risk of

individuals to toxic metal exposure and, further, may lead to an understanding of the molecular defects leading to pathological states involving aberrant essential trace element metabolism (e.g., Zn and Cu).

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Interactions of Photoactivated Polycyclic Aromatic Hydrocarbons with DNA

[D. E. Hoard, R. L. Ratliff, and J. M. Bingham]

It is generally understood that in order to cause cancer potential chemical carcinogens such as the polycyclic aromatic hydrocarbon benzo(a)pyrene [B(a)P] must first be activated, i.e., converted to derivatives that are reactive toward cellular macromolecules.^{1,2} That such activated carcinogens are likewise mutagens³ suggests that the significant macromolecular target within living cells is DNA. Activation of potential chemical carcinogens is generally thought to be accomplished by intracellular metabolic processes, and specifically in man and other mammals through reactions mediated by cytochrome P450-linked mixed-function oxidases.⁴ Published evidence^{5,6} indicates, however, that B(a)P carcinogenicity toward mice is significantly enhanced by concurrently exposing skin-painted animals either to near-ultraviolet radiation (NUV) from an artificial source or to sunlight. This photochemical enhancement of B(a)P carcinogenesis could be the result of direct activation of the hydrocarbon by NUV.

We have demonstrated^{7,8} that exposure, *in vitro*, of DNA to NUV in the presence of ¹⁴C-labeled B(a)P leads to binding of the hydrocarbon to DNA and to the formation of doubly-labeled products (adducts) if the deoxyadenosine (dA), deoxyguanosine (dG), or deoxycytidine (dC) moieties of the DNA are tritium-labeled. Concurrently, destruction of the dG moiety also occurs, as can be measured either by 1:1 labeling of tritium located at the dG eight positions or by a reduction in the amount of unaltered dG recoverable by chromatography following enzymatic degradation of the irradiated DNA. We have now completed an experiment in which one of three otherwise identical solutions of the model DNA poly d-[A,C,(8-³H)G,T] were subjected to 10⁶ J/m²

NUV in the presence of 1/7 molar equivalent of (1) (7,10-¹⁴C) B(a)P, (2) unlabeled benzo(e)pyrene B(e)P, or (3) unlabeled perylene. These three C₂₀H₁₂ isomers vary widely in carcinogenicity, ranging from very strong B(a)P to virtually non-existent (perylene). Photochemical destruction of the dG moiety, measured either as a decrease in tritium specific activity or in the ratio of recovered dG to recovered dT (Table I), was found to be between two and three times as extensive with B(a)P as with either of the remaining hydrocarbons. Chromatograms of nuclease digests of the three treated DNA samples (Fig. 1) demonstrated that doubly labeled hydrophobic products were formed in the case of the ¹⁴C-labeled B(a)P, in agreement with earlier results. When B(e)P was present during irradiation products labeled with tritium were detected in the analogous region of the chromatogram but the recovered activity corresponded to only about one-tenth the amount of dG found when the hydrocarbon was B(a)P. When perylene was the hydrocarbon tritium recovered in the same region of the chromatogram did not exceed background levels.

Fig. 1. Sephadex LH-20 chromatograms of nuclease digests of poly d- [A,C,(8-³H)G,T] after NUV irradiation in the presence of: A (7,10-¹⁴C) B(a)P, B unlabeled B(e)P, C unlabeled perylene. Only the aqueous-methanol gradient portion of the chromatograms is shown. Tritium radioactivity (—); carbon-14, (---); and A₂₆₀ of 4-(p-nitrobenzyl)pyridine marker (.....).

TABLE I
10⁶ J/m² NEAR-UV IRRADIATION OF POLY d-[A,C,(8-³H)G,T]
IN THE PRESENCE OF ISOMERIC POLYNUCLEAR AROMATIC HYDROCARBONS

Experiment	Hydrocarbon	dG Specific Activity (³ H dpm/μmol x 10 ⁻⁸)	dG / dT Ratio	dG-Hydrocarbon Adducts (μmol/mol nucleotide)
1	(7,10- ¹⁴ C)B(a)P	1.21	0.39	516
2	B(e)P	3.25	0.75	50
3	Perylene	3.27	0.82	13*

*Includes all tritium activity eluted 350 - 640 cc in chromatogram.

These results suggest that a positive correlation may exist between the potential carcinogenicities of polycyclic aromatic substances and their abilities to affect the dG moiety of DNA under the influence of NUV. With respect to the photosensitization of the dG moiety our results are in conflict with those of Kodama and Nagata,⁹ who were unable to demonstrate any correlation between the carcinogenicities of a wide variety of polycyclic aromatic hydrocarbons and the degree to which dG was destroyed by NUV in their presence. The possibility that the differences in photochemical dG destruction observed in our experiment reflect differences in the ability of the mercury-vapor emission spectrum to excite molecules of the different hydrocarbon isomers has not yet been ruled out, but does not explain the discrepancy in the results; a mercury-vapor lamp was also the source of NUV employed in the experiments of Kodama and Nagata. It would be interesting to extend experiments of this type to include non-hydrocarbon polycyclic aromatic substances, since a variety of such materials are present in novel fossil fuels, such as products derived from oil shale.

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In Vitro Tests for Determining the Carcinogenic Potential of Spent Shale Particulates

[M. D. Enger, R. A. Tobey (LS-1), C. E. Hildebrand, J. Tesmer, and R. Kissane (LS-4)]

Inorganic compounds comprise the bulk of spent shale mass. Thus, in order to test adequately the carcinogenic potential of this product of shale processing, in vitro tests should be developed and applied that measure the activity of inorganic as well as organic carcinogens. Fairly standard procedures exist to measure the organic activity of particulates, i.e., organics are extracted from particles in organic solvents such as CH_2Cl_2 and mutagenic activity is determined using bacterial or animal cell systems. No standard procedure exists for in vitro analysis of particulate inorganic activity. We believe that such procedures may be developed using known inorganic carcinogens as models. That is, by noting the carcinogenic forms and modes of action of known inorganic carcinogens, we should be able to develop in vitro systems that appropriately measure their activity. These systems should be applicable to unknowns as well within a context which, although limited at first, should increase in validity and scope with experience and application as have tests designed primarily for measuring organic carcinogens.

Although numerous inorganics are designated as carcinogens or mutagens in the literature, only As, Cr, and Ni have been shown unequivocally to be carcinogenic to humans.¹ As a starting point then, we consider what is known of their carcinogenic forms and modes of action. Chromium is mutagenic in essentially all test systems.² As the chromate ion, it oxidizes cell walls and membranes, gains entrance as Cr^{+3} , and in this form causes mutations.²

Cr-type activity is thus assayable by applying standard mutagenesis tests to aqueous extracts (leachates) of spent shale. Arsenic also apparently is active as soluble anionic forms. Arsenic activity thus probably also would exist in aqueous extracts; however, the modes of action of this carcinogen and its carcinogenic forms require further definition. They are the subject of a complementary project (L.R. Gurley). The carcinogenic forms and modes of action of Ni are fundamentally different from those of Cr: (1) no forms of Ni are mutagenic, (2) the most carcinogenic forms are the least soluble, and (3) crystal structure is an essential determinant of activity.^{1,3} One of the most potent carcinogens known is nickel subsulfide, Ni_3S_2 . A single intramuscular, intra-testicular or intra-ocular injection results in tumors over 90% of the time.⁴ Inhalation results in nasal and lung cancers. Similar compounds, such as amorphous NiS , are inactive. The question is, how does one determine Ni_3S_2 -type activity in an in vitro system? Several observations are suggestive. First, Ni_3S_2 is extremely effective in transforming SHE cells from a non-tumorigenic to a tumorigenic state.⁵ Non-carcinogenic NiS is not. Secondly, there is a pronounced uptake of Ni_3S_2 by cultured cells.⁶ Even particles similar in size to the cell itself are ingested. Amorphous NiS is not taken up. It has been stated in the literature—perhaps prematurely—that carcinogenic inorganic particles in general are ingested by cells, while non-carcinogenic particles are not.⁷ A complicating factor in this situation, we believe, resides in the fact that, although relatively insoluble in aqueous solvents, Ni_3S_2 rather readily dissolves in physiological fluids such as muscle cell extracts or

serum.^{8,9} The "bio-ligands" formed may actually represent the active species. This possibility we propose to test by determining the effects of bio-ligands formed with Ni_3S_2 and NiS on SHE cell tumorigenicity.

In summation, these observations suggest that a new protocol should be applied to effect a more nearly complete in vitro analysis of the carcinogenic potential of inorganic particulates. First, they suggest that particle extracts should be obtained using serum and other biological fluids as well as using aqueous and organic solvents. These extracts should be tested for their ability to transform cells as well as for their mutagenicity. Further, the toxicity, cell uptake, and transformation activity of the particles themselves should be tested.

Extensive studies have been performed on the solubilization of Ni_3S_2 in culture medium. The cytotoxicity of solubilized Ni_3S_2 and the cytotoxicity and uptake of particulate Ni_3S_2 have been defined. In accord with reported observations, CHO cells take up particulate Ni_3S_2 . Within 14 h, exposure to 50- or 100- $\mu\text{g/ml}$ 2- μ Ni_3S_2 particles results in an average content of one particle per cell. When a similar experiment was performed using Paraho spent shale, it was found that exposure to 100 $\mu\text{g/ml}$ < 5- μ or 5-10- μ particles resulted in an average cellular burden of 1.6 and 1.4 particles/cell, respectively, within 8 h (Fig. 1A). Over 90% of the cells contained particles (Fig. 1B). In light of the suggestion that carcinogenic inorganic particles are taken up by cells,⁷ it appears imperative that the ability of intact or culture-medium-solubilized spent shale to transform non-tumorigenic cells to tumorigenicity be assessed.

Fig. 1A. Mean spent shale particles per cell as a function of time exposed to $<5\text{-}\mu$ ($\circ$) and $5\text{-}10\text{-}\mu$ ($\blacksquare$) particles.

Fig. 1B. Percent of cells containing particles as f(+).

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Mutagenesis In Vitro: Ames/Salmonella Assay of Shale Oils

[J. Nickols and G. F. Strniste]

We have tested for genotoxicity DMSO (dimethyl sulfoxide) extracts of various site-specific shale oils, natural crudes and upgraded products using the *Salmonella*/mammalian-microsome assay developed by Ames et al.¹ Using the standard plate incorporation method, initial screening experiments were performed with and without rat hepatic S9 fraction on bacterial strains TA98 and TA100. Figure 1 shows the mutagenic response induced by a Paraho composite crude shale oil (CDOL 601) and its hydrotreated daughter oil (CDOL 602). The CDOL 601 extract induced a mutagenic response that initially increased linearly with dose in bacterial strain TA98 when activated with S9 fraction (from 0-13.5 μg test material per plate) of the DMSO extracted test material. The non-linear dose response (13.5-270 mg test material per plate) and the substantial reduction of activity at 2700 μg test material per plate are, at least in part, a result of the toxic nature of the sample. No measurable mutagenic activity was found in this crude oil extract, either with or without S9 activation, using TA100. The hydrotreated crude oil extract (CDOL 602) produced no significant mutagenic activity in either

Fig. 1. Mutagenicity of Paraho above ground retort composite crude oil (CDOL 601) and its hydrotreated daughter oil (CDOL 602) as detected in the Ames/Salmonella bioassay using either TA98 or TA100 test strains with rat hepatic S9 fraction. The number of histidine revertants induced are plotted versus the amount of DMSO-extracted material added per plate. See Table I for amounts of oil which partitioned into the DMSO fraction.

bacterial strain tested with or without liver homogenate activation although it was still cytotoxic like its parent composite crude oil.

Due to the minimal response of bacterial test strain TA100 towards these complex mixtures, subsequent screening of samples for mutagenic potential was performed in strain TA98 although cytotoxic measurements were made in both TA98 and TA100. In Table I we list in order of increasing mutagenic potency a representative sampling of natural and synthetic crudes and upgraded products that we have tested to date. In the first column we report the maximum number of his⁺ revertants per plate observed for samples of DMSO extracts tested between 0-100 μ l per plate. All mutagenic samples showed an initial linear dose response, which maximized at varying sample amounts depending, at least in part, on the cytotoxicity of the sample. The order of toxic response is listed in column four, the most mutagenic oils (CDOL 601 and SRC II) were also the most cytotoxic. In the second column we list the number of his⁺ revertants per plate compared to our natural historical background for TA98 (41.0 $\pm$ 8). We can statistically show that

TABLE I

GENOTOXICITY OF VARIOUS NATURAL AND SHALE CRUDE OILS

SAMPLE	his ⁺ rev/plate (max. number)	his ⁺ rev/plate h.b.	% ext. (into DMSO)	cytotoxic (rank)
SAE 90 (100 ml retort oil)	84212	1.3	11.4	11
TOSCO (100 ml retort 100 ml)	87212	1.4	34.5	5
PRHO 602(h.i.) (Departmental Crude Oil)	87217	1.4	16.2	3
OXY rm 3 (100 ml retort 100 ml)	7124	1.7	8.6	7
KCO (Shale crude oil)	7023	1.8	5.0	4
LCO (Shale crude oil)	77213	1.9	12.4	9
TAR(roofing) (Shale crude oil)	79210	1.9	8.1	10
OXY rm 5 (100 ml retort 100 ml)	84213	2.0	17.3	4
LETC (100 ml retort 100 ml)	96220	2.3	27.1	8
PRHO 601 (Shale crude oil)	782241	19.0	44.9	2
SRC II (Coal derived distillate blend)	31121320	76.0	100	1

h.b.: historical background (TA98 + S9) = 41.0 $\pm$ 8

responses greater than 1.6 x natural background are significantly mutagenic. The majority of samples tested to date (including DMSO extracts of natural crude oils) exhibit a marginal but significant mutagenic activity (the exceptions being an SAE 90 refined petroleum product, a TOSCO pilot plant run crude oil and the hydrotreated Paraho composite crude oil). However, two samples show exceptional genotoxic activity, the Paraho composite crude (CDOL 601) and SRC II (a coal derived distillate blend).

We are continuing to test for genotoxic potential other natural and synthetic crudes and upgraded products. Currently we are assessing for mutagenic activity in several samples from the Rio Blanco modified *in situ* (retort 0) and the Geokinetics true *in situ* (retorts 17 and 24) facilities.

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Exogenous Metabolic Activation of Shale Oils in Mammalian Cell Cultures

[R. T. Okinaka and G. F. Strniste]

DMSO extracts of crude oil samples which have been shown to be mutagenic in the Ames test (above) are also being analyzed for cytotoxic and mutagenic properties in a Chinese hamster cell line (CHO) supplemented with S9 activation enzymes. As with other complex mixtures, shale oil samples also exhibit cytotoxic properties in the absence of

Fig. 1. Cytotoxic and mutagenic effects induced by Paraho shale oil extracts (CDOL 601) in CHO cells with and without S9 activation. Open symbols represent cytotoxicity and are a measure of decrease in plating efficiency after treatment. Closed symbols represent mutation frequencies measured as colonies formed in the presence of the drug ouabain. Exposures were for 2 h at 37°C either with or without S9 activation mixtures. Circles = no S9 present, squares = 0.1 mg/ml S9 mix; triangles = 0.3 mg/ml S9 mix (protein).

metabolic activation (Fig. 1, open circles). Although quite cytotoxic the extracts by themselves are not capable of inducing mutations (Fig. 1, closed circles) at the Na^+/K^+ ATPase locus (ouabain resistance). S9 activation, however, can lead to an enhancement in the degree of mutagenesis of this crude oil sample (Fig. 1, closed symbols). These results, however, are complicated by the observation that the addition of S9 results in a concomitant detoxification (cloning ability, open symbols, Fig. 1). The detoxifying effects of S9 are clearly depicted in the three survival-dose curves generated by no S9, 0.1 mg/ml S9, and 0.3 mg/ml S9 proteins.

The accurate assessment of the mutagenic potential of these extracts will require further modifications of S9 activation protocol and methods to separate contributions from toxic, non-mutagenic components; e.g., fractionation schemes.

Mutagenesis In Vitro: Ames/Salmonella Assay Shale Oil Process Waters

[J. Nickols and G. F. Strniste]

Using the Salmonella/mammalian microsome mutagenicity assay developed by Ames *et al.*¹ we have

tested oil shale process waters for mutagenic activity. The three we report on were obtained from 1) an aboveground retort (PRHO), 2) a vertical modified *in situ* retort (OCCD), and 3) a true *in situ* retort (GEOK).

Each water has been examined for cytotoxicity and mutagenicity over a range of 0.1–300 µl of sample per plate using bacterial strains TA98 and TA100, with and without metabolic activation. The activation system used was a S9 liver homogenate prepared from Aroclor-induced rats (1240 µg protein per plate). The standard plate incorporation assay was used to test the three waters, and the GEOK water was also tested (0.1–400 µl sample per plate) using a modification of the standard plate incorporation test that included a preincubation time.²

Using the standard Ames procedure, repeated experiments show that the PRHO sample is clearly mutagenic in the Ames test for both TA98 and TA100 (Fig. 1). The OCCD sample and the GEOK sample were negative in this assay for either bacterial strain tested. However, when pre-induction of the sample with bacteria is incorporated into the assay, the GEOK process water induces a mutagenic response in either TA98 or TA100 test strains (see Table I).

The cytotoxic effects of the PRHO water, particularly observed in TA100, make it difficult to directly compare the mutagenic response of the two bacterial strains. We are currently refining the standard Ames procedure by incorporating a cytotoxicity test to be used simultaneously with the mutagenicity test. This will allow us to report an absolute number of mutagens induced by correcting for percent survival of bacteria treated with complex mixtures which are often cytotoxic.

We have also observed that the mutagenic response in the Ames test varies with the batch or lot number of S9 preparation used. Results from these and other studies should assist us in addressing some of the problems associated with the metabolic activation of complex mixtures.

The two active process waters have recently been fractionated into acid, base and neutral fractions. Upon determining the "genotoxic" fraction(s), HPLC chromatographic separation will enable us to determine what classes of organic

Fig. 1. Mutagenicity of three shale oil retort process waters as detected in the Ames/Salmonella bioassay using both TA98 and TA100 test strains with rat hepatic S9 fraction. The various waters are: PRHO 005, Paraho above ground retort (6 SEPT 78); GEOK 104, Geokinetics true *in situ* retort 17 (15 AUG 79); and OCCD 106, Occidental modified *in situ* room 6 (7 MAR 79). The number of his⁺ revertants induced per plate is plotted versus the amount of process water added per plate.

TABLE I

HISTIDINE REVERTANTS INDUCED IN SALMONELLA TA98 and TA100
BY A GEOKINETIC PROCESS WATER USING THE MODIFIED AMES TEST^{a, b}

<u>μl sample/plate</u>	TA98 ^c		TA100 ^d	
	<u>Revertants per plate</u>		<u>Revertants per plate</u>	
	<u>With S9</u>	<u>Without S9</u>	<u>With S9</u>	<u>Without S9</u>
0.3	36 ± 9	42 ± 5	111 ± 23	150 ± 45
1.0	39 ± 4	31 ± 5	139 ± 10	142 ± 19
3.0	40 ± 12	30 ± 12	124 ± 38	133 ± 41
10.0	41 ± 17	22 ± 9	136 ± 24	147 ± 40
100.0	86 ± 13	26 ± 10	202 ± 29	130 ± 48
200.0	127 ± 31	26 ± 7	277 ± 46	185 ± 23
300.0	175 ± 37	22 ± 6	316 ± 30	176 ± 27
400.0	209 ± 20	20 ± 7	384 ± 21	147 ± 27

^a The above data represents the results of 3 separate experiments in which each data point is the average number of colonies formed (six plates).

^b 1240 μg S-9 homogenate protein per plate.

^c Background revertants (controls) were 38 ± 8 (+S9); 30 ± 11 (-S9) B[a]P control (2.5 μg/ml) 187 ± 56.

^d Background revertants (controls) were 142 ± 35 (+S9); 164 ± 25 (-S9) B[a]P (2.5 μg/ml) 379 ± 68.

compounds are responsible for the genotoxicity of these waters.

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Exogenous Metabolic Activation of Process Waters in Mammalian Cell Cultures

[R. T. Okinaka and G. F. Strniste]

These studies were initiated to measure the mutagenic potency of shale oil process waters in a cultured mammalian cell system (CHO) using exogenous rat liver homogenates (S9) as a source of activating enzymes. This particular cell line and an S9 activation system has been successfully utilized in assessing several known procarcinogens for cytotoxic, mutagenic, and sister chromatid exchange inducing properties.¹

Figure 1 illustrates the cytotoxic and mutagenic effects of a particular retort process water (PRHO, 6 SEPT 78) on Chinese hamster cells. Concentrations of the process water which produce acute cytotoxic responses (2 h exposures) are not sufficient to induce a significant number of mutations in the HGPRT locus of CHO cells (circles). The addition of S9 (squares), at levels which can maximally induce mutations with B(a)P (2 $\mu\text{g}/\text{ml}$), also does not increase mutations to 6-thioguanine resistance. Instead, the addition of the S9 complex causes a detoxification of the cytotoxic responses produced by the process water. This experiment suggests that the conditions for activation of these complex mixtures may require modifications and that toxic, nonmutagenic components in shale oil mixtures may interfere with the normal responses initiated by metabolic activation.

Toxic components in complex mixtures can be removed by simply assessing the mixtures at dilutions which are not toxic to the cells. The

Fig. 1. Cytotoxic and mutagenic responses induced by PRHO water in CHO cells with and without metabolic activation. Open symbols represent plating efficiency after treatment. Closed symbols represent mutation frequencies measured after 8-10 day expression periods followed by selection in media containing 10 μM 6-thioguanine for 7-8 additional days. Exposures were for 2 h at 37°C either with or without S9 activation mixtures.

results depicted in Fig. 2 illustrate the mutagenic responses produced by subtoxic doses of PRHO water in combination with metabolic activation enzymes. When 0.1 mg/ml S9 proteins are used in the activation mixtures, a linear dose response is observed with up to 0.5 parts per hundred concentration of the process water. Although not overwhelming, the level of mutagenesis is still significantly above background. In direct contrast, the use of higher S9 protein concentrations (0.3 mg/ml) results in no increase in mutagenicity and even a slight decrease when compared to background values. These results are not surprising since other studies with polycyclic aromatic hydrocarbons indicate that S9 preparations used in excess can exhibit detoxifying and deactivating capacities as well as activating potential.² Under conditions employed here the maximum induction of mutations with Paraho process water occurs at S9 levels which are three to fivefold below those used to maximally activate a known PAH [B(a)P at 2 $\mu\text{g}/\text{ml}$].

The assessment of the mutagenic potency of shale oil process waters in combination with S9 activation and cultured mammalian cells is much more complicated than studies involving model compounds. Conditions for assessing the mutagenic potential in these waters are being refined and also broadened to include three genetic loci (HGPRT, APRT, and Na^+/K^+ ATPase). Preliminary studies indicate, that the Na^+/K^+ ATPase locus is more sensitive to activated Paraho process water than is the HGPRT locus.

Fig. 2. Mutation induction by subtoxic doses of PRHO water in Chinese hamster cells with S9 activation. Symbols used are: ●-●, mutation frequencies with no activation enzymes present; □-□, mutation frequencies induced by 0.1 mg/ml S9 proteins plus appropriate cofactors; ◇-◇, with 0.3 mg/ml S9 proteins.

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Determination of Direct-Acting Mutagens in Shale Oil Retort Process Water

[D. J. Chen, R. T. Okinaka, and G. F. Strniste]

The mutagenicity of shale oil retort process waters from an above ground retort (PRHO) and a true *in situ* retort (GEOK) has been determined in both the CHO and human cell mutagenesis assay systems (HGPRT locus) established in our laboratory.¹⁻³ In order to determine the direct-acting mutagens in retort process water, assays are performed without rat liver microsomal or light activation. The results are shown in Fig. 1. The data indicate substantial toxic and mutagenic activities for the PRHO water (6 SEPT 78) when tested against human embryonic skin fibroblasts. At 15% surviving fraction of cells there is about a 9-fold increase in 6-TG^R mutants induced (compared to background rates) when the cells are exposed to a 0.5% solution of the process water for 48-h incubation in the dark. The cytotoxic and mutagenic effects observed in CHO cells are less severe under the same experimental conditions. There is no direct-acting mutagenic effect witnessed in either cell exposed to

Fig. 1. Toxic and direct-acting mutagenic effects of surface retort process water (PRHO) in CHO cells and human embryonic skin fibroblasts. 6-TG^R mutation enhancement index = $\frac{\text{observed mutation frequency}}{\text{background mutation frequency}}$. Closed symbols are cytotoxic results; open symbols are mutation results.

as much as 5% GEOK (15 AUG 79) water although a slight cytotoxic effect is observed in the human fibroblasts (data not shown).

Our results indicate that shale oil retort process water contains an undetermined amount of direct-acting mutagens which varies according to the process of retorting. Preliminary data indicate that the PRHO water induces high frequencies of chromosomal aberrations and tetraploid conversions in treated human skin fibroblasts and sister chromatid exchanges in treated CHO cells. However, we have been unable to demonstrate the occurrence of direct-acting mutagens in retort process waters when tested in the standard Ames/*Salmonella* assay (see above). It is possible that these process waters contain certain classes of promutagens which can be metabolized into active intermediates by cultured human skin fibroblasts, even though these early passage fibroblasts do not retain their ability to metabolize polycyclic aromatic hydrocarbons such as benzo(a)pyrene. This fact may relate to the difference in sensitivity between the human and CHO cells, the latter having no detectable metabolic activation capacities. Alternatively, these data could be explained by the fact that human

fibroblasts are more sensitive than CHO cells when treated with complex mixtures.

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Photoactivation of Shale Oil Byproducts

[G. F. Strniste, E. Martinez, D. J. Chen, R. T. Okinaka]

Activation of certain classes of promutagens/procarcinogens can be achieved by exposure to near ultraviolet light (NUV). A health risk of uncertain magnitude exists due to the potential activation by sunlight or artificial NUV sources of ubiquitous environmental pollutants into reactive intermediates which could act directly on target organs such as the skin and lungs. We have been studying the process of photoactivation of polycyclic aromatic hydrocarbons, both at the molecular and cellular levels,^{1,2,3} and have extended these studies toward assessing the problem of photoactivation of shale oil by-products.^{4,5,6,7}

Initial studies using purified DNA as a target indicated that combinations of retort process water and NUV resulted in extensive DNA damage.⁶ The magnitude of damage induced in the DNA was proportionate to the mutagenic potential determined by the standard Ames/Salmonella test for several different retort process waters tested. Because of the simplicity and rapidity of the *in vitro* DNA assay, we are currently adapting it for analyses of multi-fractionated samples of various shale oil byproducts in order to qualitatively define localized "hot spots" (i.e., fractions containing cytotoxic and/or mutagenic components).

We have recently reported results concerning the cytotoxic and mutagenic effects (HGPRT locus) observed in CHO cells when exposed to shale oil retort process water and NUV.⁵ In Fig. 1 we show a plot of 6-TG^R mutations induced versus surviving fraction of cells (as determined by their cloning ability). Although the mutation induction curves are complex, it is evident that combinations of the above ground retort water plus NUV induce 6-TG^R mutants at a frequency about 50% that observed for the potent mutagen/carcinogen, far ultraviolet light (254 nm). Retort process waters produced by two other methods (modified *in situ* and true *in situ*) are much less mutagenic when compared to the above ground retort process water.

We have also examined mutations induced at four other genetic loci (in addition to the HGPRT locus)

Fig. 1. 6-TG^R mutants induced per surviving fraction of CHO cells after exposure to shale oil retort process water and NUV or to FUV only. Duplicate points at the same surviving fraction represent results from two independent experiments. Mutation frequencies have been corrected for plating efficiencies observed at the time of selection. The background mutation frequency in control cells was 1 per 10⁵ cells. Symbols used are: FUV only, (♦); Paraho above ground retort water, 6 SEPT 78, (●); Geokinetics true *in situ* retort process water, retort 17, 15 AUG 79, (■); and Occidental modified *in situ* retort process water, room 6, 7 MAR 79, (▲).

in CHO cells after exposure to retort process waters and NUV (Chen and Strniste, manuscript in preparation). We find resistance to ouabain, a dominant marker which affects the membrane Na^+/K^+ ATPase, is most sensitive for detecting mutagenic potential existing in complex mixtures (retort process waters). For example, at 50% cell survival, the ouabain resistant mutants increased about 60-fold over normal background mutation rates observed at this locus (compared to 10- to 50-fold increases in mutation frequencies observed at the other four loci tested). Another feature in utilizing resistance to ouabain is the minimal time necessary for the maximal expression of mutants (about two days). Thus we have a mammalian cell mutagenesis assay which is compatible with complex mixtures that can be executed in a total time of between 8-10 days.

Fig. 2. 6-TG^R mutants induced per surviving fraction of CHO cells after exposure to DMSO extracts of various shale oils and NUV. Mutation frequencies have been corrected for plating efficiencies observed at time of selection. Background mutation frequency in control, non-treated cells was 1.5 per 10⁵ cells. Symbols and the dilution of DMSO extracts used are: Paraho composite crude oil (1:2500), (●); Occidental modified in situ retort, room 3, 1 JAN 76 (1:3000), (■); and hydrotreated Paraho composite crude oil (1:2500), (▲). Bars represent % one standard deviation.

We have also examined the photo-induced mutagenicity in CHO cells after exposure to DMSO extracts of representative samples of two shale oils, a daughter hydrotreated oil and a refined 90 wt SAE petroleum product oil. The data are presented in Fig. 2. From cell survival studies, it was found that these extracts are very cytotoxic and substantial dilutions were necessary (1:1000 or greater) in order to obtain a treatment condition which by itself was non-toxic to the cells. However, treatment of CHO cells with dilutions of the extracts with subsequent exposure to NUV was found to be cytotoxic. Furthermore, substantial photo-induced mutagenic activity is seen with the Paraho composite crude oil—yet not with its hydrotreated daughter oil. The Occidental MIS (room 3) oil is intermediate in photoactivated mutagenic potency. The SAE 90 wt. petroleum product (1:1000 dilution) did not exhibit any photo-induced toxic or mutagenic activity. (Data are not shown.)

We have also examined the cytotoxic properties of light-activated shale oil retort process water (Paraho, 6 SEPT 78) using cultured human skin fibroblasts as targets (Fig. 3). In normal "repair proficient" (NF) cells, combinations of this water (1:700 dilution) and NUV exhibit a toxic response, similar to that observed in CHO cells. However, in xeroderma pigmentosum (XP) cells, which are "repair deficient" in excision capacities, an increase in cytotoxicity is observed compared to the response seen for the normal cells. This increase in sensitivity for XP is similar to that observed when comparing the responses of these two cell types after exposure to the potent skin carcinogen, far ultraviolet light (see insert curve, Fig. 3). Current studies examining the induction of HGPRT⁻ mutants in these cells induced by photoactivated shale oil process water are in progress.

Fig. 3. Survival of cloning ability of cultured human skin fibroblasts (NF) or fibroblasts obtained from a patient with the genetic disease xeroderma pigmentosum (XP), complementation group A, after 1 h incubation with 1:700 dilution of Paraho above ground retort process water (6 SEPT 78) and subsequent exposure to NUV. The insert shows that survival of the cloning ability of these two cell types after exposure to FUV (principally 254-nm wavelength). Plating efficiencies for NF and XP cells were 50-75% and 25-40%, respectively. All data were normalized to the cloning ability of non-treated cells. Different symbols represent results from independent experiments.

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INTRODUCTION

Bioassay methods for assessing hazards from exposure include whole animal studies (e.g., inhalation studies currently being done by the Toxicology Group) and the use of cell culture models (such as *in vitro* transformation assays). The Experimental Pathology Group pursues studies which provide linkage between these two approaches. Both whole animal studies as well as the cellular screening assays have significant disadvantages if used alone. For instance, it is now generally recognized that the carcinogenic risk associated with complex mixtures, like shale oil, can best be estimated by the combined use of *in vivo* and *in vitro* experimental data. At the same time, programs involving a combined approach offer attractive opportunities for greater understanding of the underlying processes. For these reasons, each of the projects in the Experimental Pathology Group utilizes a combination of whole animal procedures coupled with *in vitro* models.

The studies are in three areas: malignant progression, chromosome damage assessment, and pulmonary damage assessment. These areas overlap

both within the group as well as with important programs in the Toxicology and Genetics Groups. For instance, within the Experimental Pathology Group, fundamental studies of macrophages are pivotal to understanding fibrogenesis in lung as well as the host defense against tumorigenic cells. Similarly, our biochemical studies of chronic lung disease utilize, in some cases, the same animals studied by the Toxicology Group in their spent shale inhalation program. In all studies, the group continues to place emphasis on the application of advanced instrumentation, particularly flow cytometry. These methods, which are undergoing further improvements, have proven critical for our competitive position in chromosome studies, tumor biology and the use of lavage specimens for pulmonary damage assessment. In addition, we are putting other advanced methodologies into service, for projects within the group as well as other Los Alamos needs. These include monoclonal antibody production, and the use of ELISA assays for a variety of enzyme measurements.

Antigen-Specific Stimulation of Amino Acid Transport in Bovine Lymphocytes

[E. H. Tate and L. S. Cram]

Some of the earliest events that occur after treatment of lymphocytes with growth stimulators (mitogens) are changes in the cell membrane. The initial event probably involves binding of the mitogen to the lymphocyte surface. This induces other membrane changes, in particular increases in the uptake of certain ions and nutrients, such as amino acids, glucose, potassium, and calcium.¹ Other cellular changes, such as the onset of DNA synthesis and cell proliferation, occur somewhat later.

Because membrane changes occur so soon after mitogenic stimulation, it has been suggested that at least some of these changes may be prerequisite to growth initiation.^{2,3} Several studies have shown that amino acid uptake (transport) is increased almost immediately in lymphocytes which have been treated with certain nonspecific mitogens that induce cell division in a large proportion (up to 50%) of a normally resting lymphocyte culture.^{4,5,6,7} On the other hand, such transport increases have not previously been demonstrated in lymphocytes treated with specific antigens, which stimulate a much smaller lymphocyte subpopulation, thus, generating a much smaller response than the nonspecific mitogens.

The report presented here demonstrates that lymphocytes from TB-infected or TB-sensitized cattle respond to stimulation by a purified protein derivative (PPD-B) from the causative agent of bovine TB, *Mycobacterium bovis*, by an increase in the transport rates of the non-metabolizable amino acid analogs, α -aminoisobutyric acid (AIB) and α -methylamino-isobutyric acid (MeAIB). The transport increases do not occur when lymphocytes isolated from TB-skin-test-negative animals are cultured with PPD-B.

*Nonmetabolizable analogs of amino acids were used to insure measurement of PPD-B-induced changes in transport only and not changes in protein synthesis.

Fig. 1. Time course of AIB uptake. Lymphocytes were isolated from the peripheral blood of a PPD-B-sensitized animal and incubated in the presence or absence of PPD-B for 24 h. The cells were then harvested and assayed for ³H-AIB transport in amino acid-free medium containing 100 M Na⁺. Each data point is the average of duplicate determinations in a representative experiment. (O) No PPD-B. (●) +PPD-B. Inset. A similar experiment utilizing lymphocytes from an animal which gives a negative PPD-B skin test. The units and scales on the ordinate and the abscissa are the same as those shown in the main figure.

Figure 1 shows that AIB transport is linear with time for 10-20 min at a concentration of 500 μ M AIB in both PPD-B-treated and nontreated peripheral lymphocytes. PPD-B-stimulation for 24 h enhances both the initial AIB transport rate (k) and the total steady-state accumulation of AIB by about twofold. The average enhancement ratio ($k_{\text{treated}}/k_{\text{non-treated}}$) after a 20-24 h treatment of isolated lymphocytes from PPD-B-skin-test-positive animals is 1.9 ± 0.1 (SEM) ($n=17$). This average includes determinations on peripheral lymphocytes from one TB-infected animal and 9 PPD-B-sensitized animals.

Initial rates of AIB transport as a function of AIB concentration were measured in resting lymphocytes and in lymphocytes stimulated with PPD-B for 20 h. A Lineweaver-Burk plot of these data is shown in Fig. 2. The straight lines extrapolate to a common point on the abscissa, demonstrating that the apparent K_m for AIB (1.2 mM) is not altered by PPD-B-treatment. In contrast, PPD-B treatment increases V_{max} 1.6-fold, from 25 to 40 pmol AIB/10⁶ cells/min.

Fig. 2. Lineweaver-Burk analysis of AIB transport in PPD-B-treated and nontreated lymphocytes. Cells isolated from a PPD-B-sensitized animal were incubated for 24 h in the presence or absence of PPD-B and assayed as described in Fig. 1. The initial transport velocity at each substrate concentration was obtained from the slope of the linear uptake curve determined from duplicate 7.5 and 15 min assay points. The data are from a representative experiment, and the error bars are ± 1 standard deviation. The ordinate is the reciprocal of the initial velocity of AIB transport expressed as $\mu\text{mols}/10^6$ cells/min. The data were fit by least squares analysis. (O) No PPD-B. (●) +PPD-B.

Fig. 3. Initial AIB transport rates as a function of incubation time with PPD-B. At the indicated incubation times with or without PPD-B, lymphocyte samples from TB-positive animal #8067 were assayed for AIB transport as described in Fig. 1. Initial transport rates were determined as in Fig. 2. Data are from two separate experiments and were fit by least squares analysis. (O) No PPD-B. (●) +PPD-B.

Figure 3 shows that enhancement of AIB transport in the peripheral lymphocytes of a TB-infected animal increases as a function of PPD-B treatment time but does not change when cells are cultured in the absence of PPD-B. The enhancement process appears to comprise two components. There is a rapid, approximately two-fold increase in transport during the first 18–20 h that corresponds to the increase in V_{max} observed in Fig. 2. The slower component of increase measured after 20 h probably represents proliferation of the PPD-B responsive subpopulation. A generation time of 32 h for such a subpopulation would account for the increase observed.

There have been three major systems for the transport of amino acids described in mammalian cells.^{8,9} Each of these systems, designated as A, L, and ASC, displays specificities for certain

amino acids although some amino acids, such as AIB are transported by more than one system. We performed experiments to determine which system(s) are enhanced by PPD-B by using system specific inhibitors to discriminate among the systems. Table I shows that enhancement of both the A- and L-system components of AIB transport occurred in response to PPD-B, although the A-system was enhanced to a greater degree than was the L-system. After 4 days of PPD-B treatment, A-system transport increased 24-fold while L-system transport was enhanced by 10-fold. System ASC was not enhanced at all by PPD-B treatment.

Further experiments are being carried out in our laboratory to determine whether measurement of amino acid transport increases in lymphocytes in response to antigen treatment is a feasible method for detection of TB in cattle.

TABLE I
COMPARISON OF THE TOTAL AIB TRANSPORT ENHANCEMENT RATIO^a TO THE SYSTEM SPECIFIC
ENHANCEMENT RATIOS AFTER VARIOUS TIMES OF PPD-B TREATMENT

Time of Incubation in PPD-B (days)	Total Enhancement Ratio	System Contribution to Total AIB Transport (%)			System Enhancement Ratios		
		A	ASC	L	A	ASC	L
0	1.0	23	20	57	1.0	1.0	1.0
1	2.2	28	21	51	2.7	2.3	2.0
2	3.9	43	5	52	7.2	1.0	3.6
3	6.6	37	5	58	10.4	1.7	6.7
4	11.1	50	0	50	24.0	0	9.8

^a Enhancement Ratios = $\frac{\text{AIB transport rate, PPD-B treated.}}{\text{AIB transport rate, nontreated}}$

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Production of Monoclonal Antibody Against Metallothionein

[G. C. Saunders, C. E. Hildebrand, and A. Martinez]

The first major undertaking of the recently established Life Sciences hybridoma facility has been to produce monoclonal antibody to metallothionein (MT). Metallothioneins are low molecular weight, sulfhydryl-rich, metal-binding proteins which are involved both in essential trace metal metabolism in mammalian cells and in detoxification of toxic heavy metals.¹ Mechanisms which regulate metal-induced expression of genes encoding metallothioneins have been a prime focus in DOE programs in this laboratory.² A spinoff of this project may be the development of a rapid mutagenesis assay in which the marker will be a shift from MT induction positive, MT⁺, to MT induction negative, MT⁻. This assay depends on the availability of MT specific antibody so that identification of MT containing cells is possible. MT antibody raised in animals is not a well-defined reagent in that it is multispecific in regard to MT type, has a variable affinity constant from animal to animal, and is difficult to obtain in high titer. A family of monoclonal antibodies specific to each (or both) MT types, with known affinities could be used as a standard reagent available to many laboratories in defining MT positive cells. We report success in obtaining monoclonal antibody to MT.

Balb C mice were immunized IP with a mixture of monomeric and glutaraldehyde cross-linked MT's

(in complete Freund's adjuvant) obtained from MT inducible CHO cells. One month later a second injection of MT antigen was given and four days later the spleen was removed. A single cell suspension was obtained and mixed with SP 2/0 mouse myeloma cells at a ratio of 10 spleen cells to 1 myeloma cell. The cells were fused using polyethylene glycol according to the method of C. Reading (personal communication). Two weeks later supernatant culture fluid obtained from hybrid positive cultures was screened using an in-house developed indirect EIA procedure for the presence of MT antibody. Screening antigen was a mixture of two major isometallothionein (isoMT) species obtained by ion exchange column chromatography of partially purified MT obtained from cultured Chinese hamster cells proficient in MT synthesis.³ Each isoMT represents a distinct MT⁴ (and unpublished results). Four culture wells out of 100 tested were initially screen positive. Pure populations of cells producing MT antibody will be obtained from each screen positive culture through a series of limiting dilution cloning steps. We are near the final cloning stage for one of these hybrids, which appears to have specificity for only one of the isoMTs (See Fig. 1). The remaining hybrids appear to have specificity against both isoMT antigens. Work will continue until purified monoclonal antibody is obtained unique to 1) each antigenic isoMT species and 2) common determinants present on each isoMT species.

Fig. 1. Test fluids were reacted against MT antigen 2 or 4. Hybridoma fluid specific to sheep red blood cells tested in duplicate did not bind to either antigen (marked -). Fluid from cloned MT screen positive cells shows binding to antigen 2 only (marked +) while heterologous MT rabbit antiserum bound to both antigens (marked R).

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Sensitive Novel Method for Detecting Elastolytic Activity Using Peroxidase Labeled Elastin [G. C. Saunders and Z. V. Svitra]

In our studies related to mechanisms of pulmonary fibrogenesis a sensitive assay for determining both intracellular and extracellular elastase and collagenase activity is required. Available elastolytic techniques including those using dye labeled¹ or isotopically labeled elastin² have not proved suitable; the former is not sensitive enough and the latter involves all the logistical problems of using radioisotopes. However, the principle of measuring the release, due to elastolytic activity of an agent bound to elastin is appealing. Earlier work done at Los Alamos³ suggested the possibility of binding the enzyme horseradish peroxidase to elastin particles via linkage between aldehyde groups put on the peroxidase and epsilon amino acids present on the elastin. Initial experiments employing this principle have been quite successful, and should also be applicable to other lytic systems, e.g., collagenase action on collagen.

Aldehyde groups were put onto peroxidase by the method of Nakane.⁴ Five mg of peroxidase-aldehyde were reacted overnight at 4°C with 200 mg of bovine neck elastin, 100-400 mesh. Unbound peroxidase-aldehyde was removed by numerous washings in normal saline. Washing was considered

complete when supernatant fluid was negative for peroxidase activity in the presence of a mixture of 0.005% H_2O_2 in 0.4 mM ABTS (2,2'-azine-di-(3-ethyl-benzothiazoline-6-sulfonate). The conjugate was then incubated for 24 hr in the presence of trypsin and chymotrypsin to remove moieties capable of reactivity with non-specific proteases. Very little enzyme activity was lost in this procedure. After removing the proteolytic enzymes with several saline washes, the elastin-HRP conjugate was stored in saline at 4°C until use. The conjugate retained stability for at least 1 month. Before each use the conjugate was washed twice in saline.

Standard elastolytic activity curves using 1 ng to 100 ng elastase were generated as follows. All reactions took place in 0.01M Tris, pH 8.6 in the presence of Ca^{++} . To tubes containing 1 ml of diluted elastase, 10 μ l of suspended elastase-HRP conjugate (100 mg conjugate/ml) were added. After varying times of incubation at 37°C the peroxidase activity of the supernatants was determined by placing 0.1 ml aliquots into 1 ml of ABTS- H_2O_2 mixture. This reaction was stopped³ after 10 to 30 min and the absorbance of the reaction product read at 414 nm. Absorbance values were plotted against elastase concentrations to obtain a standard curve. Over at least a 2 log range of concentration linear results were obtained.

The sensitivity range of the assay could be manipulated by varying any or a combination of the following: amount of elastin-HRP added, time of incubation with conjugate, or time of incubation with ABTS- H_2O_2 . We found that ng/ml of elastolytic activity could be detected in as little as two hours of incubation at 37°C (Fig. 1). Sensitivity of 50 picogram elastase/ml has been achieved by incubating this amount of elastase with conjugate for 12 hours and allowing the ABTS-HRP reaction to proceed for 30 minutes. To our knowledge no other published technique surpasses this level of sensitivity.

We are adapting this procedure to a microplate assay such that rapid screening and quantitation of lung lavage fluids for elastolytic activity can take place. Also we are developing a similar assay to detect collagenase activity.

ELASTASE/ELASTIN INCUBATED 2 HOURS AT 37°C

Fig. 1. Standard curve of reaction product absorbance at 414 nm versus elastase concentration following a 2 hr incubation of conjugate with elastase, and a 10 minute reaction of the supernatant fluid with ABTS- H_2O_2 .

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Effects of Process Waters from the Oil Shale Industry on In Vitro Induction of Sister Chromatid Exchange

[E. W. Campbell, F. A. Ray, and L. L. Deaven]

We have established a screening laboratory to study materials from the oil shale industry for in vitro induction of sister chromatid exchange (SCE). Day-to-day background levels over a period of 8 months varied from 5.84 ± 0.46 to 9.00 ± 0.69 SCE/cell with an average of 7.67 ± 0.18 . Process waters in this report are derived from three sources (Geokinetic, Occidental, and Paraho), and are described in Table I.

TABLE I
MATERIALS STUDIED

<u>Sample</u>	<u>Los Alamos Repository Date</u>	<u>Serial No.</u>	<u>Sample Description</u>	<u>Total Hydrocarbon</u>
Geok watr	15 Aug 79	104	product water	2.15
OCCd watr	7 Mar 79	106	room 6 oxy product water	3.17
PRHO watr	6 Sept 78	005	product water	5.7

SCE induction was determined by treating cultured Chinese hamster cells (line CHO) with three or more concentrations of each sample tested, in the presence of 5-bromodeoxyuridine. Each assay required an evaluation of cytotoxicity using the mitotic index as an indicator for dose selection. Metaphase chromosome preparations were made after allowing the cell monolayers to grow for two replication cycles, and then blocking them with Colcemid. The resulting 2nd division chromosomes were stained differentially,¹ and SCE rates were determined by counting the exchanges in 25 cells from each test.

Experimental Results

A Los Alamos field study group collected water from the Paraho Demonstration Project at Anvil Points, Colorado. The water was separated from surface - retorted shale oil in a holding tank. Then the test material was filtered through Whatman #42 filter paper and a 100- μ m Millipore filter to remove particulates. For the SCE tests, it was also filtered through a 0.45- μ m sterilizing filter. This water, at a concentration of 0.2% (v/v) in the culture medium induced SCE levels to approximately three times the background frequency. Cell cycle perturbation by this concentration of water resulted in a 50% decrease in the mitotic index (M.I.). A water concentration of 0.5% results in a M.I. of 1.2%. At this concentration, none of the metaphase cells were differentiated, indicating that they did not traverse through 2 cell cycles by the 29-hour collection time.

The Geokinetic process product water did not elevate SCE rates significantly over background rates using concentrations as high as 5% (v/v) in the culture medium. At a concentration of 7.5% (v/v), the chromosomes were not differentiated, indicating that the cells did not progress through two cell cycles.

The Occidental process water 106 induced SCE rates at almost twice background levels at a concentration of 7.5% (v/v). A concentration of 10% (v/v) prevented the cells from progressing through two cell cycles by the 29-hour collection time, and therefore, no differentiation occurred.

Figure 1 compares the results of all 3 water samples using various plots to determine if the differences in SCE rates were merely due to differences in dilution. The results were normalized according to percent hydrocarbons in the sample, volume of water and mitotic index. The graphs show that the higher elevation in SCE rates of the Paraho sample cannot be solely attributed to a higher concentration of identical components.

Under our storage conditions, waters have given similar results over a period exceeding 3 months.

These results, although limited, raise a number of procedural questions. For one thing, the SCE rate observed with any given sample is partially dependent upon cytotoxic or cell cycle perturbing components that may or may not induce SCE's. For instance, our results with the Occidental water, mentioned above, must be interpreted within the context that a third water (from the Geokinetics process) was untestable, due to cell cycle delay when mixed with medium to the

Fig. 1 Paraho water 005, 6 Sept 78, 5.7% hydrocarbon (●) Geokinetic water 104, 15 Aug 79, 2.15% hydrocarbon (▲) Occidental water 106 106, 7 Mar 79, 3.17% hydrocarbon (◆) mean SCE/ cell results normalized according to percent hydrocarbon, volume of water and mitotic index.

concentration of 7.5%. Hence, whether the Geokinetics water was as active as the Occidental specimen could not be determined.

Of more general interest, however, is the question of how the type of data generated by this screening effort can be made applicable to oil shale technology development. An obvious objective will be to determine the activity of a range of samples within the context of realistic exposure scenarios.

All tests described herein are measurements of directed acting components. *In vitro* metabolic activation studies using S-9 microsomal fractions, that are currently being used in our Ames testing laboratory, have been added to the SCE analysis

program. Initial studies were conducted using cyclophosphamide, an agent known to increase SCE frequencies after metabolic activation. A 30-minute exposure of the culture to 0.25 mg/ml cyclophosphamide plus an S-9 mixture² induced an SCE frequency of 24.72 per cell, and a 2-hour treatment increased the frequency to over 75 SCEs per cell. Preliminary studies indicate that 2-hour exposures of the three process waters in the presence of S-9 do not increase the frequencies above those observed after a 2-hour treatment with the waters only. At the present time our results indicate that most of the SCE inducers in shale oil materials are direct acting; however, further studies with S-9 are necessary before any conclusions can be made.

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Studies on Growth and Survival of Hoechst-stained Cell Populations Following Analysis and Cell Sorting

[H. A. Crissman and R. J. Kissane]

Arndt-Jovin and Jovin¹ first reported the use of Hoechst 33342 (10 g/ml for 1 hr) as a vital DNA-binding fluorescent stain and demonstrated its applicability for viable cell staining and cell sorting. Using CHO cells as a model cell system, we initiated a series of studies designed to optimize the Hoechst (HO) staining procedure for cell types and instrumentation to be used in our laboratory. These studies included determination of (1) minimal dye concentrations required to provide quality FCM-DNA for cell cycle analysis and likewise maintain high levels of cell viability, (2) possible dye-induced mutagenicity, (3) kinetics of dye uptake and dye release by viable cells, and (4) photochemical toxicity and

Fig. 1. Uptake of Hoechst 33342 (2 $\mu\text{g/ml}$) by CHO cells over a 90 minute time period. Relative fluorescence intensity (ordinate) is proportional to the G_0/G_1 modal channel values at the various time periods.

physical damage to stained cells during UV laser exposure (365 nm) and electronic cell sorting.

Our studies indicate that CHO cell populations maintained in culture and treated with 2 $\mu\text{g/ml}$ HO 33342 for 1.5 hr provide FCM-DNA profiles with CV's averaging about 9.5% (range 7.5-11.5%). Stained cell populations retain viability near 100% of levels obtained for unstained control cells. Furthermore, no mutagenic effects were noted under these conditions. Cell growth is retarded slightly during the dye exposure period, but when stained cells are placed in drug free medium, growth rates parallel control cell rates after about 4 hours and continue to do so for at least 5 days thereafter when the growth study is discontinued.

Based on the fluorescence intensity of cells immediately following addition of the dye, there is a rapid exponential uptake of the dye by CHO cells (Fig. 1). At 7 minutes cells reach 15%, at 15 min 35%, at 30 min 60% and at 45 minutes 90% of their final maximum fluorescence intensity. From 45 to 90 min improvement in the S and G_2+M structure of the DNA cell cycle distribution is noted. The fluorescence distribution remains

quite stable for at least 3 hr thereafter, which is an added advantage for cell sorting over long time periods where fluorescence intensity shifts could adversely affect the purity of sorted cells.

Hoechst 33342 is likewise quickly released from stained CHO cells. Cells analyzed at various time periods after removal from the stain solution showed a rapid decrease in fluorescent intensity. Following 10 min in dye-free medium the intensity decreased 30%, at 1 hr the decrease was 80% and at 4 hr the intensity had decreased 95% compared to cells maintained and analyzed in the dye solution. These results indicate that the dye is not tightly bound to the chromatin of cells, a factor which probably accounts for the high viability levels and the lack of mutagenicity found in these studies.

Survival of stained cell populations analyzed and sorted at 100 or 200 milliwatts laser power was about 85% of unstained and non-sorted cells; however over the range of 300, 400, and 500 mW survival values fell rapidly and exponentially to 2% survival at 500 mW (Fig. 2). Sorted, unstained cells maintain 85% survival at 500 mW laser power. These data provide an estimate of

Fig. 2. Effects of laser power (milliwatts, mW) and electronic cell sorting on survival of CHO cells following 90 minutes staining with Hoechst 33342 (2 $\mu\text{g/ml}$). Closed triangle ($\blacktriangle$) represents survival of unstained cells exposed to 500-mW laser power and sorting.

Fig. 3. DNA distribution profiles showing the effects of BudR quenching of Hoechst 33342 fluorescence in stained colon 26 cells. Colon 26 cells were stained with Hoechst 33342 (2 μ g/ml for 90 minutes in cell culture without treatment (A) or following treatment with BudR (30 μ M for 26 hours). CV=coefficient of variation (B). Cells in A and B were analyzed at 200-mW laser power and were tumorigenic in host Balb/C mice following electronic cell sorting.

the photochemical toxicity of the dye to cells when exposed to the high intensity of the narrowly focused laser beam.

Cells cultured in non-toxic levels of bromodeoxyuridine (BrdU) will incorporate BrdU into DNA in place of thymidine. As first shown by Latt² such incorporation results in quenching of fluorescence when the cells are subsequently stained with HO 33258. We are using this approach in combination with viable staining to distinguish traversing from non-traversing cells. Only traversing cells will incorporate BrdU and following staining will exhibit quenching of the HO dye. Conversely, HO would not be quenched in subpopulations of non-traversing cells. We have

succeeded in adapting this method to the analysis of colon 26 cells in culture.

Colon 26 cells cultured for 28 hr in the presence of BrdU, and subsequently stained with HO 33342 exhibited a 54% decrease in fluorescence intensity compared to cells maintained in BrdU-free medium (Fig. 3). BrdU treated cells were tumorigenic in host Balb/C mice. These studies support the feasibility of detecting and separating viable traversing and non-traversing subpopulations of tumors.

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A Comparison of Biochemical Methods of Analyzing Collagen Content of Mammalian Lungs for the Assessment of Pulmonary Damage

[M. S. Halleck]

We are developing assay systems to determine the biochemical response to possible lung injury caused by inhaled pollutants generated by the oil shale industry. One of the principal damage indicators in lungs exposed to environmental pollutants may be the development of pulmonary fibrosis, a debilitating, often fatal, chronic disease recognized as a derangement of collagen metabolism, occurring as an end result of lung injury.¹ An estimate of amounts of lung collagen can be made through measurements of the amino acid hydroxyproline, which occurs almost exclusively in collagen protein, although small amounts of this amino acid are found in elastin and the C1q component of complement.² Hydroxyproline may be measured by a number of different colorimetric methods, as well as by amino acid analyzer or by gas chromatography. We have made comparative studies of hydroxyproline content of rat lungs using several of these methods.

In order to study the biochemical progression of pulmonary fibrosis in experimental animals, total lung hydroxyproline, as well as other amino

acids, was measured in rats using a known model for pulmonary fibrosis, silicosis. The silica model was chosen, since oil shales have a relatively high free silica content (8-15%)¹ and the fibrotic response to dusts containing free silica is well known.³ Fisher 344 rats were exposed intratracheally to 25 mg silica (Min-U-Sil, $\geq 5\mu\text{M}$) in 0.5 ml physiological saline. For purposes of comparison, six weeks following silica exposure left lungs from 3 treated and 3 control animals were freeze pulverized, hydrolyzed and analyzed for their content of hydroxyproline and other amino acids (by methods listed in Table I). Right lungs were sectioned for histological examination.

The results of the hydroxyproline analysis are shown in Table II. Although the total amount of hydroxyproline measured in control and treated rat lungs was somewhat variable, depending upon the method of analysis, all 4 methods demonstrated a similar increase in hydroxyproline content of silica-treated lungs relative to controls. Microscopic analysis of histological sections from these lungs, stained with special collagen stains (Masson's trichrome), also demonstrated the presence of excess collagen in granulomas, indicating fibrosis.

Simple measurements of total lung hydroxyproline do not provide complete information concerning the pulmonary response to instilled silica, however. A 170% increase in lung weight (Table III) relative to an approximately 50% increase in collagen content (Table II) suggests

TABLE II
COMPARISON OF METHODS FOR MEASURING HYDROXYPROLINE

	HYDROXYPROLINE CONTENT OF LEFT LUNG (μg)			
	METHOD A	METHOD B	METHOD C	METHOD D
CONTROL	621	752	394	614
TREATED (6 weeks after silica injection)	927	1160	593	850
Increase Relative to Controls	49%	54%	51%	38%

that other biochemical processes form a major part of the response. Silicotic fibrosis is known to be an inflammatory process, characterized by the production of large granulomas.⁴ Although eventually leading to the deposition of collagen and fibrosis, the silicotic process is dominated early in the first weeks by inflammation. Evaluation of other amino acids might then prove useful in following the progression of this reaction, characterized by the production of proteinaceous fluids and cells. One of our early attempts to measure these changes was to analyze the proline content of lungs (proline being an amino acid common to many proteins). Using the colorimetric analysis shown as method D, we were able to measure proline to hydroxyproline ratios, shown in Table IV. Proline content of the silica-treated lungs was greatly elevated, compared to controls, in the early stages of the silicotic disease. Amino acid analysis, as well as gas chromatography, was also used to quantitate proline to hydroxyproline ratios. All three methods gave very similar results (Table IV). The

TABLE I
METHODS FOR HYDROXYPROLINE MEASUREMENT

METHOD A	COLORIMETRIC METHOD OF PROCKOP AND UDENFRIEND (1966) ANAL. BIOCHEM. 1: 228.
METHOD B	COLORIMETRIC METHOD OF GRAHNT (1965) J. CLIN. PATH. 12: 685 USING AUTOMATIZER.
METHOD C	GAS CHROMATOGRAPHY OF N-HEPTAFLUOROBUTYRIC AMINO ACID N-PROPYL ESTERS ON A FUSED SILICA CAPILLARY COLUMN.
METHOD D	COLORIMETRIC METHOD OF BLUMENKRANTZ AND ABSCOE-HANSEN (1975) ANAL. BIOCHEM. 63: 331.
METHOD E	ANALYSIS OF AMINO ACIDS ON A BECKMAN 21 AMINO ACID ANALYZER USING THE PHYSIOLOGICAL FLUIDS PROGRAM WITH LITHIUM BUFFERS.

TABLE III
RESPONSE OF LUNG WET WEIGHT TO
SILICA INSTILLATION

	Left Lung Wet Weight (mg)
CONTROL	406
TREATED (6 weeks after silica injection)	1096
Increase Relative to Controls	170%

TABLE IV
COMPARISON OF METHODS FOR MEASURING
PROLINE/HYDROXYPROLINE RATIOS

	<u>PROLINE/HYDROXYPROLINE</u>		
	<u>METHOD C</u>	<u>METHOD D</u>	<u>METHOD E</u>
CONTROL	2.5	3.0	3.5
TREATED (6 weeks after silica injection)	3.4	4.3	4.8
Increase Relative to Controls	36%	44%	37%

3 methods for measuring proline/hydroxyproline also demonstrated a similar increase in these ratios in silica-treated lungs relative to controls (Table IV); thus demonstrating that the increased weight of the silicotic lungs was probably also due to fluids and other proteins besides collagen.

We have found gas chromatographic analysis of hydrolyzed lungs to be an extremely useful way to characterize their amino acid content. A comparison of gas chromatograms from a control rat lung and a rat lung 6 weeks following instillation of silica is shown in Fig. 1. Of the 14 amino acids we have been able to identify in these lung hydrolyzates, it can be seen that most of them are significantly elevated in the silica-treated lung, again, probably due to the inflammatory response. These results suggest that gas chromatographic analysis will prove to be a very useful method, allowing us to establish an amino acid "fingerprint" at various stages during the progression of pulmonary damage and fibrosis.

Development of these methods for the assay of pulmonary fibrosis is an ongoing process. We are currently working on the development of a micro-titer colorimetric assay for hydroxyproline, which should prove to be an extremely sensitive as well as rapid method, for quantitating pulmonary collagen content. This, in conjunction with gas chromatography, will allow us to carefully assess the progression of lung damage in not only the silica model, but also the possible damage caused by inhalation exposure to oil shale dusts and their derivatives.

Fig. 1. Gas chromatograms of identical amounts of acid hydrolyzates from control and silica-treated Fisher 344 rat lungs, 6 weeks following silica instillation. Samples prepared according to Method C.

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Multi-Laser Excited Fluorescence Measurements on Single Mammalian Cells

[J. A. Steinkamp]

Experiments often require more than one fluorescent dye to simultaneously quantitate various biochemical and functional properties of cells, such as DNA, RNA, protein, cell surface moieties, viability, enzymes, and phagocytosis of fluorescent latex particles. Since fluorescent stains have different wavelength excitation spectra, a single laser will most likely excite only one fluorochrome. However, two or more lasers operating at different wavelengths, with spatially separated beams, can be used to sequentially excite different fluorochromes bound to cells.¹⁻⁵

A three-laser excited instrument for multiparameter measurement of cells stained with fluorescent dyes having separated fluorescence excitation spectra has been developed. This instrument also contains additional electronic cell volume and optical light scatter sensors and it was a developmental outgrowth of a previously described single-laser excited cell separator.^{4,6} To permit versatility in fluorescence measurements, two argon and one krypton laser beams are independently focused onto the cell stream within the flow chamber using separate pairs of beam-shaping optics (Fig. 1). A new three-color fluorescence sensor, which uses a single collecting lens, contains a unique dichroic filter-mirror arrangement for positioning the optical sensing region of one photodetector onto, above, or below the other for single-laser or sequential multiple-laser excited fluorescence measurements. A maximum of six signals from electrical and optical sensors are processed by hardwired multiparameter signal processing electronics. Selected signals are displayed as frequency distribution histograms using a multi-channel pulse-height analyzer or LSI-11 computer. This versatile system can be used for single or multiple-laser induced fluorescence and other optical measurements of microscopic particles and cells at wavelengths ranging from the uv to ir.

As illustrated in Fig. 1, cells stained with fluorescent dyes suspended in normal saline are

Fig. 1. Cutaway view of the multiparameter cell separator flow chamber illustrating three-laser excitation with spatial laser beam positioning.

analyzed sequentially (1000 cells/sec) as they pass through the flow chamber. On entering the chamber they first pass through an electronic cell volume sensing orifice and then intersect two argon and a krypton laser beam of appropriate wavelength causing bound fluorochromes to fluoresce and cells to scatter light. The three beams, which have Gaussian power density profiles (TEM_{00} mode), are focused into elliptical slits at the cell stream-laser beam intersection by separate sets of crossed cylindrical lenses. Micromanipulator lens mounts allow each beam to be raised or lowered such that cells can be sequentially illuminated. This arrangement permits each beam to be independently focused onto the cell stream. Fluorescence emitted by stained cells intersecting the spatially separated laser beams is measured with sensors capable of detecting light in one, two, or three wavelength regions as selected by various filter combinations.

To demonstrate the measurement of cells stained with fluorescent dyes having separated excitation spectra, pulmonary alveolar macrophages which had phagocytized green fluorescent latex spheres were stained for DNA content (Hoechst 33342) and total protein (Rhodamine 640). Sprague-Dawley rats were euthanized, the trachea surgically exposed, the thoracic cavity opened,

and 15 ml of 1.75- μ m-diam green fluorescent spheres (5×10^7 per ml) instilled into the lungs hydrostatically. Thirty minutes later the lungs were lavaged with saline to remove alveolar macrophages. Lavage cells were then washed, fixed in 70% ethanol, stained with Hoechst 33342 and Rhodamine 640 for DNA content and total protein, respectively, and analyzed for fluorescence by sequentially exciting in the uv (near 350-nm, 457-nm, and 568-nm wavelength). Figure 2 shows the excitation spectra of Hoechst 33342, green spheres, and Rhodamine 640. Since the three spectra are well separated, each fluorochrome must be separately excited. Figure 3 shows the DNA content, phagocytosis, and protein frequency distribution histograms. The DNA content distribution (Fig. 3A) of blue fluorescent Hoechst-stained lavage cells consists of two major peaks. The first peak represents cells having 2C DNA content and the second peak, binucleates and doublets, having twice the DNA content. A very small percentage of cells synthesizing DNA

Fig. 2. Fluorescence excitation and emission spectra of Hoechst 33342 (—) bound to calf thymus DNA; 1.75- μ m-diam green fluorescent spheres (---); and Rhodamine 640 (---) bound to bovine serum albumin.

Fig. 3. DNA content, phagocytosis, and total protein frequency distribution histograms recorded from rat pulmonary alveolar macrophages.

(S phase) are located in the region between the two peaks. Macrophages that have phagocytized green fluorescent spheres are illustrated in Fig. 3B. Peaks 1-4 represent alveolar macrophages having phagocytized 1 to 4 spheres, respectively, as identified by cell sorting.⁷ In this particular experiment, 90% of the macrophages had phagocytized one or more particles. The total protein distribution of red fluorescent Rhodamine 640-stained cells is shown in Fig. 3C. This distribution is very broad and typical of cells having a wide range of size.

This new technology has great potential for experimental cell research, with the range of applications expanding as the methodology is utilized in the laboratory. Primary emphasis will be placed on the development of assays for

determining damage resulting from exposure to toxicants related to the production and utilization of synthetic fuels.

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Cytogenetic Effects of Shale-Derived Oils and Related By-Products in Mice

[J. Meyne]

Shale-derived oils contain many known mutagenic and carcinogenic compounds. Because of the expected development of an oil shale industry it is important to assess the occupational risk to workers and the hazards of toxic by-products of this technology that may be introduced into the environment. *In vivo* cytogenetic analysis is one of the recommended methods for evaluation of potential environmental hazards. This method involves direct examination of metaphase chromosomes for detection of cytologically detectable chromosomal damage expressed as structural changes (aberrations). *In vivo* metaphase analysis not only allows objective evaluation of insults to the genetic material, but also takes into account the

activation and detoxification mechanisms of mammalian metabolism. Although any actively proliferating tissue can be used for *in vivo* metaphase analysis, bone marrow is the tissue of choice in mammalian systems because of the relatively high mitotic index, the low and consistent frequency of spontaneous aberrations, and the relevance to the type of damage implicated in human exposure to mutagens.

Table I shows the results of metaphase analysis on bone marrow cells from female Swiss (CD-1) mice which had been treated with three intraperitoneal injections of crude shale oils diluted in olive oil. The injections were given at 24-h intervals and the mice were killed and metaphase preparations made 4 h after the last injection. All 3 doses of Paraho crude shale oil induced increased frequencies of both breaks and gaps observed in metaphase chromosomes. The frequencies of breaks induced by the 0.5 ml and 1.0 ml/kg doses were about three times the background levels, while the 2.0 ml/kg dose showed about a five-fold increase above control frequencies. The hydrotreated Paraho crude shale oil demonstrated the same trend of aberration induction, but at lower frequencies, e.g., about a two-fold increase at the low and middle doses and about a three-fold increase at the high dose. The Occidental crude shale oil induced the highest frequency of breaks at the 0.5 ml/kg dose, and elevated but lower frequencies of breaks at the 1.0 ml and 2.0 ml/kg doses.

Table II lists the frequencies of aberrations induced by treating Swiss (CD-1) female mice with three oil shale retort waters, using the same schedule as for the crude shale oils. The Paraho and Occidental retort waters gave similar responses, with all 3 doses being in the range of two-fold increases above control frequencies for the number of breaks observed. The Geokinetic retort water induced a two-fold increase at 0.5 ml/kg, but the 1.0 ml/kg dose was not increased above control frequency and the 2.0 ml/kg dose intermediate.

The same treatment schedule and doses were also used to determine the frequency of induction of sister chromatid exchanges (SCE's) *in vivo*. The Paraho crude oil demonstrated a slightly

TABLE I
CYTOGENETIC ANALYSIS OF BONE MARROW OF FEMALE SWISS (CD-1) MICE AFTER
INTRAPERITONEAL INJECTION OF CRUDE SHALE OILS^a

Treat- ment	Total Cells Scored ^b	Number of Aberrations ^c				Breaks per Cell	Gaps per Cell	Mitotic Index
		b	B	g	G			
untreated	500	4	0	11	2	0.008 ±0.008	0.026 ±0.008	4.54 ±0.59
olive oil	1000	8	0	25	2	0.008 ±0.006	0.027 ±0.008	4.83 ±0.59
0.5 ml/kg PCSO	500	9	2	21	1	0.022 ±0.011	0.044 ±0.011	4.46 ±0.82
1.0 ml/kg PCSO	500	10	3	20	5	0.026 ±0.011	0.050 ±0.007	3.62 ±0.88
2.0 ml/kg PCSO	500	19	3	24	6	0.044 ±0.017	0.060 ±0.016	3.60 ±0.60
0.5 ml/kg PCSO-HT	500	8	0	16	0	0.016 ±0.009	0.032 ±0.008	4.88 ±0.45
1.0 ml/kg PCSO-HT	500	6	1	12	0	0.014 ±0.009	0.024 ±0.006	4.94 ±0.43
2.0 ml/kg PCSO-HT	500	11	3	21	4	0.028 ±0.016	0.050 ±0.026	4.90 ±0.88
0.5 ml/kg OCSO	500	16	2	22	3	0.036 ±0.020	0.050 ±0.016	3.72 ±0.28
1.0 ml/kg OCSO	500	8	2	12	1	0.020 ±0.012	0.026 ±0.013	4.60 ±0.41
2.0 ml/kg OCSO	500	8	1	12	0	0.018 ±0.005	0.024 ±0.006	3.72 ±0.84

^aThree injections of crude shale oil diluted in olive oil were given at 24-h intervals. Mice were sacrificed 4 h after the last injection. PCSO, Paraho crude shale oil; PCSO-HT, hydrotreated PCSO; OCSO, Occidental crude shale oil.

^bA total of 100 cells from each of 5 mice. Solvent control is mean of two groups of 5 mice each.

^cb-chromatid breaks; B-isochromatid breaks; g-chromatid gaps; G-isochromatid gaps.

increased frequency of SCE's. The hydrotreated Paraho oil, Occidental crude oil, and all 3 waters showed no increase above control frequency. It appears that the oil shale products tested are not effective inducers of SCE's.

TABLE II
CYTOGENETIC ANALYSIS OF BONE MARROW OF FEMALE SWISS (CD-1) MICE AFTER
INTRAPERITONEAL INJECTION OF OIL SHALE RETORT WATER^a

Treatment	Total Cells Scored	Number of Aberrations ^c				Breaks per Cell	Gaps per Cell	Mitotic Index
		b	B	g	G			
untreated	500	4	0	11	0	0.008 ±0.008	0.022 ±0.016	4.26 ±0.40
saline	1000	7	0	25	1	0.007 ±0.007	0.026 ±0.017	4.00 ±0.49
0.5 ml/kg PHRO	500	6	0	18	1	0.012 ±0.008	0.038 ±0.016	3.64 ±0.34
1.0 ml/kg PHRO	500	7	0	14	1	0.014 ±0.017	0.030 ±0.012	3.70 ±0.67
2.0 ml/kg PHRO	500	5	1	14	3	0.012 ±0.011	0.034 ±0.011	2.90 ±0.43
0.5 ml/kg OCCD	500	7	0	13	1	0.014 ±0.006	0.028 ±0.008	3.42 ±0.41
1.0 ml/kg OCCD	500	5	0	13	0	0.010 ±0.010	0.026 ±0.009	3.38 ±0.38
2.0 ml/kg OCCD	500	7	1	15	0	0.016 ±0.009	0.030 ±0.010	3.52 ±0.48
0.5 ml/kg GEOK	500	8	0	16	0	0.016 ±0.009	0.032 ±0.008	3.18 ±0.36
1.0 ml/kg GEOK	500	3	0	13	0	0.006 ±0.009	0.026 ±0.006	4.02 ±0.48
2.0 ml/kg GEOK	500	5	0	12	0	0.010 ±0.007	0.024 ±0.015	3.88 ±0.36

^aThree injections of retort water diluted in saline were given at 24-h intervals. Mice were sacrificed 4 h after the last injection. PHRO-Paraho retort water; OCCD-Occidental retort water; GEOK-Geokinetic retort water.

^bA total of 100 cells from each of 5 mice. Solvent control is mean of two groups of 5 mice each.

^cb-chromatid breaks; B-isochromatid breaks; g-chromatid gaps; G-isochromatid gaps.

Chromosome Instability and Tumorigenesis

[L. S. Cram and M. Bartholdi]

Do deviations from the normal diploid chromosome constitution alter a cell's biologic behavior in ways compatible with the neoplastic state? The answer to that question is influenced by the findings of Wells *et al.*¹ that low passage post crisis nude mouse cells become tumorigenic at a time when none of the classical *in vitro* transformation properties are evident but karyotype changes are observable. To investigate the role of karyotypic changes occurring in parallel with the onset of tumorigenicity, we have started a series of cell lines from whole Chinese hamster embryos (WCHE) and started development of a chromosome high resolution imaging sorter (CHRIS). The Chinese hamster cells, grown in culture on both monolayer and sponges, acquire, spontaneously, tumorigenic properties. These cells can be studied for aspects of their growth which escape regulation without the addition of chemicals or virus. At successive passages tumorigenicity assays are done, *in vitro* transformation properties studied, and the karyotypes analyzed. For quantitative karyotypic analysis that is sensitive to low-frequency events, an instrument that performs high resolution DNA content measurements on individual chromosomes has been built.

Nineteen separate Chinese hamster embryo cell lines have been established. Cells are passaged on both solid substrate as monolayers and in the fenestrated volume of sponges. The cells are injected, or sponges inbedded, in athymic mice to assay tumorigenicity. The growth curve for one WCHE line grown on monolayer is illustrated in Fig. 1. After a brief increase in the number of cell doublings per day, the cells enter crisis, a period of significantly reduced growth. Rodent cells, in contrast with most other mammalian cells, notably human, emerge from crisis spontaneously with an increased number of doublings per day. At passage 14, coinciding with emergence, the tumorigenicity assay was first positive. With successive passage the cells are believed to become more highly tumorigenic. The spontaneous and progressive nature of the system

Fig. 1 Number of population doublings per day as a function of passage number. WCHE 10 M showed a distinct crisis followed by a rapid increase in growth rate with a final plateau of about 1.3 doublings per day. There are approximately 4.25 doublings per passage after crisis.

makes it ideally suited for determining early karyotype changes associated with tumorigenicity.

In Fig. 2 the distribution of chromosome number per cell and passage number is illustrated. A shift in number occurs at passage 11, coinciding with crisis.

The application of the technology of flow cytometry to chromosome analysis is shown in Fig. 3. Metaphase chromosomes are isolated from blocked cells and stained with propidium iodide. The chromosomes flow oriented through a laser beam and the fluorescence intensity measured. The chromosomes are resolved by peaks based on differences in DNA content. Note that in the WCHE 10 M p6 (passage 6) the fifth and sixth peaks from the left tentatively assigned to the homolog pair of chromosome 8 has merged into a single peak by passage 26 (WCHE 10 M p 26).

By measuring isolated chromosomes of cells using flow cytometric techniques we hope to obtain greater sensitivity for detecting chromosome alterations than is feasible with classical manual karyotypes. This is reasonable because of the quantitative data obtainable using flow cytometric analysis and its inherent capability to detect low frequency events.

Fig. 2. Distribution of chromosome number per cell at sequential passages. The onset of aneuploidy (instability in the number of chromosomes per cell) appears to coincide with cell crisis.

Further work in better defining the earliest passage number with the onset of tumorigenicity and correlating flow karyotypes with banding studies will help establish the sequence of chromosome changes in relation to tumorigenicity.

Fig. 3. Flow karyotypes of propidium iodide stained chromosomes isolated from WCHE cells at passage 6 (precrisis) and passage 26 (post-crisis). Fluorescence intensity is proportional to DNA content per chromosome. A difference is evident by the fifth and sixth peaks from the left merging into a single peak by passage 26. Data were taken on a specially designed flow cytometer with a triple nozzle producing a 2- μ m flow stream which intersects a laser beam focused to 3 μ m by a microscope objective. The fluorescence is collected by a 40X, 0.75 NA water immersion objective coupled with glycerol to the flow nozzle. Capabilities to be added include sorting and chromosome scanning for centromeric index determination.

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Tumorigenicity Assays: Studies of the Fate of Implanted Cells

[P. M. Kraemer, G. L. Travis, and G. C. Saunders]

Cell culture models of cancer have suggested that in vitro malignant change, like the in vivo counterpart, involves a series of steps.¹ We have previously shown that the earliest point in this series that can be considered neoplastic depends upon the methodology used for the tumorigenicity assays.² In that study, the spontaneous malignant change of mouse fetal cells, which occurs upon prolonged passage, can be detected at earlier passage levels if the cells are implanted into nude mice in the form of "sponge cultures," that is, cells growing on small cylinders of collagen surgical sponge (Gelfoam®).

However, our studies, as well as others,³ suggest that tests yielding either negative results or tumors with latent periods of many months are difficult to interpret. In particular, the question arises as to what happens to the implanted cells in those instances. Our sponge culture methodology allows this question to be approached. Specifically, we can remove and study the entire host-implant arena at various times after implantation and then characterize the participants in vitro.

Preliminary experiments utilized sponge cultures of Balb/c3T3, labeled with ^{14}C -thymidine in vitro, then implanted subcutaneously into nude mice. Sponges removed at various times were assayed for radioactivity. In three experiments comparing a tumorigenic subline (3T3A) with a nontumorigenic subline (3T3N) the loss of radioactivity was more rapid in the latter. However, both types of implanted sponge cultures lost >90% of their radioactivity within 7 days. At the same time, parallel sponge cultures carried in vitro showed no loss of radioactivity over a ten day period. These preliminary experiments suggested that early loss of most of the test cells could account for insensitivity of the assay as well as raising the possibility of false negatives in this type of test.

More recently, we have continued these studies with Chinese hamster cells, malignant or normal. In this case, we have utilized additional parameters, namely, following the

Fig. 1. Nontumorigenic Chinese hamster cells (WCHE/10sp p5) were grown in replicate on Gelfoam sponges, labeled with ^{14}C -thymidine, and implanted into nude mice at time 0. At various times, sponges were retrieved and either assayed for radioactivity (diamonds) or dissolved and assayed for cell number and species of origin. Chinese hamster cells are designated with triangles, host cells with circles.

number of implanted cells and host cells in sponges retrieved at various times over a 72-h period, then dissolved in collagenase and trypsin. An indirect fluorescence method, using mouse anti-Chinese hamster antibody, was used to distinguish host cells of the nude mice from the implanted Chinese hamster cells.

Results of two such experiments are illustrated in Figs. 1 and 2. Figure 1 illustrates the results when pre-crisis cells derived from whole Chinese hamster fetuses, passaged on sponges five times, and the fifth passage sponges, each containing 4.5×10^5 labeled cells, were implanted and retrieved. Both radioactivity and cell number fell sharply within the first 24 hours. The infusion of host cells was progressive over the 72 hours; microscopic examination of these cells indicated that polymorphonuclear leukocytes were the predominate host cell to enter the sponges in the first 24 hours. The data in Fig. 1 also suggest that a stable subpopulation of the WCHE/10 sp cells persists but does not grow, following the initial phenomenology.

Fig. 2. Similar experiment as Fig. 1, except that highly tumorigenic Chinese hamster cells (line CHO) were used.

With one exception, the results for the highly tumorigenic cells (CHO) illustrated in Fig. 2, are similar. Again, most of the implanted CHO cells are lost and lost quite promptly. This phase is followed by an emergent subpopulation that proceeds to grow and subsequently forms a rapidly growing progressive tumor.

Our results suggest that unexpected host defense mechanisms may decimate the cell populations being tested for tumorigenicity. Such mechanisms may compromise the sensitivity and accuracy of such tests. In addition, it would be of considerable interest to identify the nature of this defense, that is, whether it is cellular, immunological, or whatever. Experiments are being pursued to broaden our understanding of early tumor formation or its failure.

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Effects of Acute Inhalation Exposure to Silica

[A. P. Stevenson, D. M. Koelle, J. S. Wilson (LS-1), G. C. Saunders, C. C. Stewart, and P. M. Karemer]

The development of oil shale as an energy source poses potential health hazards to mine workers and others associated with the industry. Accordingly, the health effects and safe exposure levels of shale dusts and retort products must be determined. The most obvious target organ for manifestation of these effects is the lung. The present report describes results obtained after an acute exposure of Fisher 344 rats to a highly respirable silica aerosol. The study was performed to explore the usefulness of silica (a constituent of both raw and spent shale) as a model system to study the effects of raw and spent shale inhalation exposure.

The animals (age seven weeks at the time of exposure) were given nose-only exposures for 6 h in the Los Alamos silica exposure chamber. The concentration of silica in the chamber was 14.8 mg/m^3 ; the mean mass aerodynamic diameter was $2 \text{ }\mu\text{m}$, with a geometric standard deviation of $1.6 \text{ }\mu\text{m}$. The silica was in the form of pure α -1 quartz, and eighty percent (by weight) of the dust was in the respirable size range (given by the respirable dust curve of the American Conference of Governmental Industrial Hygienists). Exposed rats were sacrificed and studied at 3, 6, 14, 21, 28, and 36 days after exposure. Results were compared to control (sham-exposed) rats sacrificed on day 3 and on day 38.

Pulmonary lavage was performed by a modification of the tracheobronchial lavage method¹ and lavage contents were analyzed for total cell number, cell viability, differential cell type, and DNA content per cell (measured by flow cytometry). Lung sections were prepared in order to detect possible fibrotic responses as well as for comparison to lavage findings. The labeling index of the lavaged cells was determined by autoradiography following the injection of tritiated thymidine.

Results indicate significant changes in number and differential composition of lavageable cells two to three weeks after exposure and a substantial return to control values by five weeks. Cell number was significantly increased at

21 and 28 days after silica exposure, rising to a peak of $7.52 \pm 1.07 \times 10^6$ cells per lavage on day 28 and returning toward control values on day 35 (Fig. 1). Large changes were observed in the differential cell counts two to four weeks after exposure, with a trend toward return to control values by day 35. Figure 2 shows the changes in the total numbers of macrophages, lymphocytes, epithelial cells, and polymorphonuclear leukocytes (PMN) as a function of time after exposure. There was a very large influx of PMN on days 14 and 21 and a small increase in the number of lymphocytes on day 21. Both the number and percentage (not shown) of epithelial cells increased on day 28. Histological appearance of lung sections followed a similar trend of maximum deviation from control animals by three weeks after exposure. Examination of thin lung sections from day 38 control animals showed evidence of an inflammatory response. Future experiments should include sacrifice of control animals at periodic intervals during the experiment.

There did not seem to be a proliferative response on the part of the resident alveolar macrophages that were lavageable, as measured by autoradiography and flow cytometry. However, preliminary experiments measuring the *in vitro* uptake of tritiated thymidine by lavaged macrophages indicate that these cells are highly stimulated to divide on glass or plastic when nutrient medium

Fig. 1. Total number of cells obtained per lavage as a function of time after inhalation exposure of F344 rats to silica. Each data point for the controls (●) represents the mean number of cells from three animals, and each data point for the exposed animals (○) represents the mean number of cells from four animals. Bars represent the calculated standard deviation.

Fig. 2. Total number of macrophages, lymphocytes, epithelial cells and polymorphonuclear leukocytes as a function of time after inhalation exposure of F344 rats to silica. Each data point for the controls (●) represents the mean number of cells from three animals, and each data point for the exposed animals (○) represents the mean number of cells from four animals. The mean number of cells from each animal was obtained by multiplying the percentages from differential cell counts times the total number of cells obtained from the individual animal. Bars represent the calculated standard deviation.

containing rat embryo fibroblast-conditioned medium is present. (About 55% labeled cells were observed at 36 hours.) This in vitro proliferative capacity is now being tested as a possible parameter to measure the macrophage proliferative capacity following inhalation of oil shale particulates.² The tumoricidal capacity of resident alveolar macrophages from normal rats was also examined. In both qualitative³ and quantitative⁴ assays these cells showed in vitro cytotoxic activity against an established cell line (CHO) known to produce solid tumors in nude mice (unpublished results). This tumoricidal capacity is now being evaluated to determine whether this function is abrogated by exposure to oil shale by-products and/or their constituents, e.g., silica particles.

No evidence of fibrosis was found in the lung sections of exposed animals, and the particulate matter could be totally cleared by 12 weeks. While it is too early to make any firm conclusions as to the long term effects of an acute exposure, we believe that short term exposures to toxicants may be totally reversible but that long term

exposures may lead to irreversible lung damage. Accordingly, the thrust of this program will be to define precisely the duration of exposure at which long term severe lung damage (as manifested by fibrosis) is no longer reversible as well as to elucidate the cellular mechanisms invoked with this process.

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INTRODUCTION

In Group LS-5, there are two areas of programmatic research — the stable isotopes of carbon, oxygen, and nitrogen (ICONS) program and the Los Alamos National Laboratory oil shale program. The major activity in Group LS-5 is the ICONS program, which has as its objective facilitation of the use of naturally occurring stable isotopes in the physical and life sciences. Stable isotopes can be used as nonradioactive tracers to monitor biochemical, agricultural, environmental, metabolic, and chemical processes. Prerequisite to these applications of stable isotopes are the availability of separated isotopes and the availability of compounds labeled with stable isotopes. At Los Alamos, the separation of carbon, oxygen, and nitrogen isotopes is conducted by the Inorganic Chemistry Group (CNC-4) of Los Alamos' Chemistry and Nuclear Chemistry Division. The synthesis of compounds labeled with stable isotopes is conducted in Group LS-5. Both chemical syntheses and biochemical syntheses are used to prepare labeled

compounds. We are also investigating stereoselective processes for preparing the naturally occurring L-isomer of amino acids. At present, the use of compounds labeled with stable isotopes in the physical and life sciences is encouraged through the National Stable Isotopes Resource (SIR), which was established at Los Alamos by the National Institutes of Health. Outside investigators may apply to the SIR for labeled compounds, analytical services, training, or cooperative research with applications being reviewed and evaluated by an advisory committee.

The second programmatic activity in Group LS-5 is the analytical chemistry of shale oil program. This program has two objectives. The first is to provide analytical data on the samples being studied by other elements of the Los Alamos program to assess the health effects of shale oil production. The second objective is to develop new analytical methods when they are required to address questions from health effects studies. Because of the chemical complexity of shale oil and related

samples, analyses employ chromatographic procedures, particularly the combined techniques of gas chromatography-mass spectrometry.

Another area of study by LS-5 personnel (C. T. Gregg and J. C. Hutson) has been the prenatal

toxicology of oil shale product water. These results are presented in the LS-1 (Toxicology) section of this report for programmatic descriptions and to reflect administrative changes made after the report period.

Organic Synthesis with Stable Isotopes

[R. M. Blazer, G. H. Daub, V. N. Kerr, D. L. Williams, and T. W. Whaley]

In describing organic syntheses with stable isotopes, it is useful to classify them as either developmental or nondevelopmental. Nondevelopmental syntheses include routine preparations that provide isotopic starting materials for other work and preparations that employ known or easily modified procedures. In the past year we conducted nondevelopmental syntheses of methanol- ^{13}C , acetic- ^{13}C acid, bromoacetic- ^{13}C acid, glycine- ^{13}C , acetylene- $^{13}\text{C}_2$, lithium acetylde- $^{13}\text{C}_2$ ethylenediamine complex, ethylene- $^{13}\text{C}_2$, polyethylene- ^{13}C , glucose- ^{13}C , and ribose- ^{13}C .

Syntheses in which reactions must be investigated for applicability to isotopic labeling can be classified as developmental syntheses. A five-step synthesis of triolein- $^{13}\text{C}_3$ was accomplished in the past year. The synthesis started with 1-bromo-8-heptadecyne and proceeded through 9-octadecylenitrile- ^{13}C , 9-octadecynoic- ^{13}C acid (stearolic- ^{13}C acid), methyl stearolate- ^{13}C , *cis*-9-octadecenoic- ^{13}C acid (oleic- ^{13}C acid), and triolein- $^{13}\text{C}_3$. The method for semihydrogenation of the triple bond in stearolic acid to the *cis*- double bond in oleic acid was improved from our previous work by employing a hydroboration procedure using disiamylborane. The condensation of glycerol and oleic- ^{13}C acid to afford the labeled triglyceride was accomplished with dicyclohexylcarbodiimide and 4-dimethylaminopyridine.

The last annual report described a nine-step synthesis of D,L-histidine- $^{15}\text{N}_2$ from glycine- ^{15}N and potassium thiocyanate- ^{15}N . This same synthesis was employed to prepare D,L-histidine- ^{15}N . A modification in which 4-imidazolecarboxaldehyde was condensed with hydantoin to afford an alkylidenehydantoin as a substrate for a chiral hydrogenation reaction was investigated, but was unsuccessful.

Work on a seven-step developmental synthesis of L-ornithine- ^{13}C was also conducted. The synthesis started with glycine- ^{13}C , which was esterified and

condensed with benzophenone to give the Schiff's base 1. The Michael addition of acrylonitrile to 1

followed by acid hydrolysis of the diphenylmethylidene group gave ethyl 2-amino-4-cyanobutyrate- ^{13}C (2). This compound

was acetylated, and the resulting amide was hydrogenated in acetic anhydride to afford D,L-diacetylornithine- ^{13}C ethyl ester (3).

In preliminary experiments, enzymatic hydrolysis of the ester in 3 by chymotrypsin and subsequent acid hydrolysis of the amides gave ornithine (4) that was

greater than 90% L-isomer. In the synthesis with labeled material, ornithine- ^{13}C that was 60% L-isomer and 40% D-isomer was obtained. This result, while unfortunate, can be remedied by conversion of ornithine to the diacetyl derivative 5

and resolution with acylase I. This work is currently in progress.

Biochemical Synthesis with Stable Isotopes

[V. H. Kollman and T. W. Whaley]

We are continuing to employ algal systems as sources of labeled organisms and labeled compounds in our biochemical syntheses. We have continued to use Agmenellum quadruplicatum for the biosynthetic production of D-glucose- $^{13}\text{C}_6$. The growth of this organism under iron- and nitrogen-limiting conditions to promote accumulation of cyanophycin starch has been described in previous reports.

We have also continued to grow Chlorella pyrenoidosa labeled with carbon- 13 , nitrogen- 15 , and deuterium. The use of protein hydrolysates from

this organism to detect receptor synthesis in cell culture by density centrifugation techniques is an interesting use of stable isotopes. Adaptation of the algae to growth on D₂O was accomplished by successive growth in D₂O-H₂O mixtures (i.e., 55, 75, 87, 94, and finally 99 mol % D). Carbon and nitrogen labeling was accomplished with carbon-¹³C dioxide and potassium nitrate-¹⁵N. After adapting to deuterium and obtaining a sufficient culture volume (ca. 18 liters), the organisms were transferred to three shaker boxes and brought to a total culture volume of 54 liters. Continuous production of triply labeled algae in the three shaker boxes afforded 120 g of dry cells in 3 months.

In a collaborative project with group CNC-4 personnel, the production of L-glutamic acid by *Microbacterium ammoniaphilum* has been investigated. A series of experiments using a variety of labeled substrates (i.e., D-glucose, acetic acid, urea, sodium bicarbonate, and ammonium sulfate) have been conducted. By nuclear magnetic resonance spectroscopy analysis of the labeling pattern in L-glutamic acid when various substrates are employed, the operation of both the tricarboxylic acid cycle and the glyoxylate pathway can be demonstrated.

Asymmetric Syntheses of Amino Acids

[R. M. Blazer, G. H. Daub, D. L. Williams, and T. W. Whaley]

In our two previous annual reports we have described a project for investigating asymmetric synthesis of amino acids, i.e., reactions that afford a preponderance of the naturally occurring L-isomer. We are investigating two approaches to this problem — chiral hydrogenation reactions and chiral alkylation reactions.

Hydrogenations of α-acylamino-α,β-unsaturated carboxylic acid derivatives using rhodium catalysts complexed to bisphosphine ligands have been reported to give excellent optical yields. One such liquid is (-)-1,2-bis(o-anisylphenylphosphino)ethane. We have prepared the actual hydrogenation catalyst [a tetrafluoroborate salt of a bisphosphine-cyclooctadiene-rhodium (I) complex] and conducted preliminary hydrogenation experiments. The hydrogenation of the Z-isomer of

α-acetamidocinnamic acid (6) in ethanol gave the L-isomer of N-acetylphenylalanine (7) having an

optical purity of 92% (i.e., 92% L-isomer, 8% racemic), a result in accord with literature data. We had planned to hydrogenate two alkylidene hydantoins (8a and 8b) that would give tryptophan and histidine, respectively). Hydrogenations in

these systems with catalysts such as Raney nickel are known; however, attempts to hydrogenate the alkylidene hydantoins with the bisphosphine-rhodium catalyst were unsuccessful. In the case of α-acetamido-α,β-unsaturated carboxylic acids, the rate of hydrogenation of the Z-isomer with bisphosphine-rhodium catalysts is several orders of magnitude faster than the rate of hydrogenation of the E-isomer. We suspect that the alkylidene hydantoins have the E-configuration about the double bond.

In order to synthesize amino acids by alkylating glycine, it is necessary to mask the carboxylic acid group and the amino group for compatibility with the alkylation conditions. Based on literature precedent, we have chosen an ester and an imine for this purpose. In a formal sense, an iminoester reagent is formed from glycine and a hydroxyketone reagent. The chirality of the alkylation reaction results from a chiral hydroxyketone, which can be recycled. We have described the synthesis of three hydroxyketone reagents, 9, 10, and 11. Conversion

of these reagents to the corresponding glycine iminoester requires two transformations — formation of an ester and formation of an imine. We have studied the esterification of 9, 10, and 11 under a variety of conditions (i.e., acetic anhydride,

12

13

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3. H. G. Floss -- Department of Medicinal Chemistry and Pharmacognosy, Purdue University -- Stereochemistry of Polyketide Biosynthesis -- 15g Lithium Acetylde- $^{13}\text{C}_2$ Ethylenediamine Complex.
4. M. J. Gelfand -- University of Cincinnati College of Medicine -- Diagnosis of Malabsorption by Breath Analysis after Oral Administration of ^{13}C -Triolein -- 20g Triolein-1',1'',1'''- $^{13}\text{C}_3$.
5. E. W. Gertz -- Veterans Administration Medical Center, San Francisco -- Myocardial Carbohydrate Metabolism: Uptake and Oxidation of Glucose and Lactate -- 13g Zinc L-Lactate- $^{13}\text{C}_3$.
6. R. M. Grainger -- Department of Biology, University of Virginia -- The Fate of Oocyte Proteins during Development in *Drosophila* -- 25g [^{13}C , ^{15}N] Chlorella pyrenoidosa.
7. C. R. Hutchinson -- School of Pharmacy, University of Wisconsin -- Stereochemistry of Erythromycin Antibiotic Biosynthesis -- 10g Ethyl-1- ^{13}C Iodide.
8. D. G. I. Kingston -- Department of Chemistry, Virginia Polytechnic Institute & State University -- The Biosynthesis of Virginiamycin M₁ and Antibiotic A-2315 -- 0.5g L-Serine- $^{13}\text{C}_3$.
9. P. D. Klein -- Department of Pediatrics, Texas Childrens Hospital -- 0.1g L-Histidine-2- ^{13}C .
10. M. D. Lane -- Department of Physiological Chemistry, The Johns Hopkins University School of Medicine -- Regulation of Insulin Receptor Level in Animal Cells -- 32g [^2H , ^{13}C , ^{15}N] Chlorella pyrenoidosa.
11. E. Leete -- Department of Chemistry; University of Minnesota -- 10g Lithium Acetylde- $^{13}\text{C}_2$ Ethylenediamine Complex.
12. K. L. Rinehart, Jr. -- Department of Chemistry, University of Illinois -- A Study of the Mode of Incorporation of D-[U- ^{13}C] Glucose- $^{13}\text{C}_6$.

13. R. G. Shulman -- Department of Molecular Biophysics and Biochemistry, Yale University -- ^{13}C NMR Studies of Metabolism *in vivo* -- 3g D-Glucose-1- ^{13}C , 0.5g D-Glucose-6- ^{13}C .
14. J. H. Siegel -- Department of Surgery, Buffalo General Hospital -- The Role of Occult Myocardial Failure on Human Shock: I-Myocardial Preservation in Cardiac Surgery -- 1g D-Glucose- $^{13}\text{C}_6$.
15. M. Walser -- The Johns Hopkins University School of Medicine -- Fate of Ketoacids in Uremic Patients -- 2g L-Valine-1- ^{13}C , 2g L-Leucine-1- ^{13}C .
16. M. Yudkoff -- Division of Biochemical Development and Molecular Disease, Children's Hospital of Philadelphia -- Effects of Folic Acid on Galactose Metabolism by Galactosemia Patients with Uridyltransferase Deficiency -- 6g D-Galactose- $^{13}\text{C}_6$.

Analytical Chemistry of Oil Shale

[W. D. Spall and T. W. Whaley]

During this year, we have concentrated on three areas related to the Los Alamos National Laboratory oil shale program. The first area involves airborne particulate matter generated during oil shale mining and processing, which presents a potential worker hazard. We have studied the NIOSH cyclohexane extraction procedure that is used in the analysis of these particles. The second area of study was two crude oils -- a Paraho crude oil and its hydrotreated daughter oil, which have widely different biological activities. This activity difference apparently results from differences in concentration of specific compounds in the two oils. The third area of study was waters associated with oil shale production. The product water from the Paraho process has proven to be the most biologically active water studied so far at Los Alamos. We have identified the major organic components of this water using gas chromatography - mass spectroscopy.

The process of mining and retorting oil shale generates large amounts of dust. The organic material in the dust presents a potential worker

hazard. One method for the extraction of the organic material from airborne particulate shale material is the NIOSH cyclohexane/sonication procedure. We have studied this commonly used procedure to determine the amount and nature of organic material extracted from Anvil Points raw shale used in animal inhalation studies at this laboratory. The initial results of this study are illustrated in Fig. 1 which shows the amount of organic material extracted as a function of sonication time for two different sizes of particles. The particles were collected with an Andersen impactor. The upper curve is for particles less than 0.2 microns in diameter, and the lower curve for 10 micron diameter particles. Sizes between these limits were also investigated. All of the extraction curves are hyperbolic of the form $1/\text{concentration} = a + b (1/\text{time})$. The magnitude of the parameter "b" was found to vary linearly with the logarithm of the particle diameter, thus allowing appropriate extraction times to be computed for various sized particles. For bulk samples sized at 100 mesh or less, nearly 3.5 hours of extraction is required for 95% extraction of the sample. The NIOSH procedure recommends a 5 minute sonication of airborne particulate matter, which is not sufficient for complete extraction of oil shale dusts.

Since the biological activity of Paraho crude shale oil and the nitrogen content of the oil both decrease upon hydrotreatment, one might expect,

Fig. 1. NIOSH sonication of raw shale.

to a first approximation, that the observed decrease in biological activity results from the removal or alteration of nitrogen containing compounds upon hydrotreatment of the oil. We have begun the identification and quantitation of nitrogen compounds by glass-capillary gas-chromatography, (GC)², using a detector which responds to a portion of nitrogen. The chromatogram (Fig. 2) shows the persistence of some nitrogen compounds (peaks B, D and E) and the removal of others (peaks A and C) after hydrotreating. Identification of individual compounds (nitrogenous as well as nonnitrogenous) was accomplished by (GC)²MS, and the quantitation of these compounds was carried out using the standard addition technique developed earlier by us. Table I shows selected results for the two oils.

Consideration of all of the data for 114 compounds representing 30 chemical classes leads to the following tentative conclusions. Exocyclic nitrogen compounds such as aniline are nearly eliminated by hydrotreating, as are primary amines and nitrites. Endocyclic compounds such as pyridine and quinoline remain essentially unchanged. PAH concentrations are slightly reduced as are alkyl substituted aromatic compounds. Partial hydrogenation of aromatic compounds and hydrogenation of olefins appears to be taking place. Identification of the organic components of oil shale product water from the Paraho process has been carried out using

TABLE I
CONCENTRATIONS OF SELECTED COMPOUNDS IN PARAHO
REFINERY RUM CRUDE AND HYDROTREATED
CRUDE OIL

Compound	Concentration in ppm	
	Crude	Hydrotreated Crude
naphthalene	397	203
anthracene	480	231
phenanthrene	526	221
benzo[a]pyrene	13.6	17.7
benzo[e]pyrene	3.1	5.6
3-methylcholanthrene	1.3	0.8
1-naphthylamine	17.6	0.3
2,6-dimethylaniline	21.2	0.5
1-aminofluorene	14.1	0.2
quinoline	1773	1487
acridine	3.0	3.7
carbazole	14.7	12.8
benzo[f]quinoline	27.8	26.6
phenol	186	93
o-cresol	427	102
o-ethylphenol	581	440
thiophene	422	214
benzo[c]thiophene	631	202
decalin	822	1947
tetralin	622	847
1-methylnaphthalene	1047	811
2-methylnaphthalene	993	176

Fig. 2. Glass-capillary gas-chromatograms of Paraho crude oil and hydrotreated crude. Nitrogen selective detector. Upper trace is crude oil; lower trace is hydrotreated crude.

(GC)²MS techniques. The Paraho water was found to contain high concentrations of carboxylic acids, amides, and nitrites. These three classes of compounds account for nearly 85% of the total organic carbon content of the water, and their high concentrations precluded identification of other materials present. The carboxylic acids and amides were removed chromatographically and a variety of furans, pyragenes, ketones, thiazoles, phenols, thiophenes and alcohols identified by their mass spectral fragmentation patterns. The major difference between the Paraho water, and similar waters collected from Occidental and Geokinetics is the high carboxylic acid, amide and nitrite levels of the Paraho water. The other compounds appear to be quite similar in concentration.

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INTRODUCTION

The Environmental Science Group is a multidisciplinary organization that conducts research relevant to the identification and resolution of environmental issues associated with emerging energy technologies, nuclear waste management, and the Los Alamos National Environmental Research Park (LA/NERP). A primary focus of the group's activities is upon the requirements for information about air, water, and land disturbances resulting from energy developments. Such information applies directly to environmental impact and alternatives assessment, development of control technologies, facilities siting, and other decision areas in the development of new energy sources.

Current research activities include pollutant source characterization, identification of mobile contaminants, transport of pollutants in the environment, assessment of the effects of environmental pollutants, and the identification or development of methods to control environmental contaminants. Specific research programs in support of developing energy technologies include environmental assessment of geothermal systems, conditioning and stabilization of uranium mill tailings, deer

population dynamics near prototype oil shale development areas, disposal and control of coal fly ash/scrubber sludges, and transport of contaminants by air- and water-borne sediments. In the area of nuclear waste management, projects include field experiments on erosion control, bio-barriers, water management and advanced engineering concepts, unsaturated transport modeling, alternatives to shallow-land burial, the attenuation of radionuclides in liquid wastes by soil, and the assessment of the environmental control technologies in the US Department of Energy (DOE) nuclear waste management program. LA/NERP projects include a survey of endangered plant species, raindrop transport of contaminated soil, animal population dynamics, elk nutrition/vegetation dynamics, Ponderosa pine regeneration, and the assessment of nitrogen stress in the environment.

The Environmental Science Group consists of about 45 scientists, consultants, graduate students, and technicians. Contracts are also negotiated with regional universities for supporting research. At this time, contracts are in effect with the University of New Mexico, University of Arizona,

New Mexico State University, and Colorado State University.

A broad base of scientific disciplines is represented within the Group, including biology, botany, chemistry, ecology, archaeology, hydrology, meteorology, soil sciences, geology, and statistics.

Research support in the Environmental Science Group comes from several parts of DOE, Nuclear Regulatory Commission (NRC), and US Environmental Protection Agency (EPA). The purpose of this report is to summarize the work done for the Assistant Secretary for the Environment (ASEV).

The Mechanisms of Hydrologic Transport of Soil Contaminants in Mortandad Canyon

[T. E. Hakonson and G. C. White]

The sorting and differential transport of soil particles by rain and run-off are important mechanisms of soil erosion that influence contaminant distribution and inventory. For example, small-sized particles, generally containing much higher contaminant concentrations, are likely transported at a faster rate over longer distances than coarse-sized particles.

The initial focus of this research will be on the selective sorting and transport of soil particles as they relate to altering the distribution of contaminants in soils and sediments. Several field experiments employing radionuclide-labeled soil particle size fractions are planned to accomplish research objectives.

During the experiments in 1979, tracer-labeled soil particles were made by batch reacting different radionuclides (^{182}Ta , ^{141}Ce , ^{124}Sb , ^{46}Sc) with four soil particle size fractions. In 1980, the tracers used to label the soil particles were ^{182}Ta , ^{160}Tb , ^{181}Hf , and ^{46}Sc . The size fractions, which consisted of silt-clay ($<53\mu\text{m}$), fine sand ($53\text{--}106\mu\text{m}$), medium sand ($106\text{--}495\mu\text{m}$), and coarse sand ($>495\mu\text{m}$), were produced by mechanical sieving of several hundred kilograms of study area sediment.

Specific objectives for the initial experiments on sediment transport in Mortandad Canyon were:

- to determine the effect of particle sorting on the transport of different-sized sediment particles,

- to determine relationships between particle transport and run-off variables,
- to determine long-term effects of particle sorting on sediment distribution, and
- to develop relationships that can be used for prediction where possible, for use in surveillance, control, and mitigation of soil contaminants subject to hydrologic soil erosion processes.

The sediment transport studies conducted during the summers of 1979 and 1980 each used about 500 kg of tracer-labeled sediment particles. Labeled sediment was added to a 5 m reach of the Mortandad Canyon stream channel before the late summer rainstorm season about 20 m and 800 m upstream from two high resolution recording water gauging stations.

Careful measurements were made on a variety of watershed characteristics (e.g., slope, area, channel width), run-off volumes through time, suspended sediment loads and tracer concentrations in run-off, and tracer concentrations in channel sediments as a function of distance downstream. Tracer concentrations in samples were measured with an automated GeLi gamma ray counting system.

During 1979, three runoff events occurred in Mortandad Canyon after placement of the labeled soil in the stream channel. Two of the events (storms 1 and 2) were small and resulted in peak flows at the labeling location of 0.01 and 0.008 m^3/s . The third event (storm 3) was substantially larger, although still a relatively small flow, and resulted in a peak flow of 0.24 m^3/s . Flows in excess of 2.8 m^3/s have been recorded in Mortandad Canyon.

Our data on runoff and particle transport distance as a function of cumulative number of runoff events (Fig. 1) clearly demonstrate that particle sorting by runoff occurs and that smaller particles are transported further downstream than larger particles during a given runoff event. For example, labeled silt-clay ($<53\mu\text{m}$) particles were transported at least ten times further downstream (Fig. 1) than were the medium to coarse sands ($106\text{--}495\mu\text{m}$ and $>495\mu\text{m}$) during a given runoff event. The highly mobile nature of the silt-clay particles is further indicated by their presence in the stream channel

Fig. 1.

Runoff and sediment particle transport distance after three rainstorm runoff events in Mortandad Canyon during 1979.

at locations where surface water runoff from a given event ceased.

Depletion of the labeled soil from the label location was most rapid for silt-clay particles (Table I) consistent with the high mobility of this size fraction. Following the first runoff event, maximum concentrations of ^{182}Ta (<53 μm particles) in the label location were only 13% of the initial concentrations of ^{141}Ce , ^{124}Sb , and ^{46}Sc and could still be detected at the label location after the first run-off event. However, after three run-off events, <3% of the initial concentrations of any of the tracers were detected at the label location. These data imply that a point source of contamination in the intermittent stream channel in Mortandad Canyon is rapidly diluted and/or transported downstream. The rate of depletion, initially, is more rapid for the highly mobile silt-clay size fraction; however, this depletion is nearly complete

for all size fractions after as few as three relatively small run-off events.

TABLE I

PER CENT OF THE INITIAL CONCENTRATION OF TRACERS IN SEDIMENT AT THE LABEL LOCATION FOLLOWING THREE RUNOFF EVENTS IN MORTANDAD CANYON IN 1979

	Runoff Event Number		
	1	2	3
^{182}Ta (<53 μm)	13	13	2.4
^{141}Ce (53-106 μm)	88	16	0.56
^{124}Sb (106-495 μm)	100	17	0.53
^{46}Sc (>495 μm)	65	28	1.2

The relationship between particle transport distance and peak runoff discharge is given for the four sediment size classes in Fig. 2. Although it is tempting to conclude that a positive relationship exists between the distance a particle moves and peak discharge, the lack of data at intermediate runoff discharges (i.e. 0.06 to 0.20 m^3/s) precludes that interpretation. Obviously, more data are needed in the intermediate runoff discharge range.

Dr. Leonard Lane, from the US Department of Agriculture (USDA) Southwest Watershed Research Center, has been retained on an inter-governmental personnel agreement to use the runoff and sediment transport data collected in Mortandad Canyon in a simple hydrologic model to estimate runoff volumes and peak rates of runoff. Additionally, the channel morphology data (slope, area, channel

width) from Mortandad Canyon along with flow will be used to estimate discharge per unit width at any point in the channel. Channel morphological data, when coupled with existing total sediment bed equations, provide a means of estimating sediment transport for particles of any given size.

In summary, particle sorting by rainstorm runoff does occur and is characterized by large downstream movement of silt-clay particles with relatively smaller movement of coarser particle sizes. Furthermore, the maximum transport distance of silt-clay particles coincides with the maximum distance downstream that surface water runoff occurs. Transport of labeled soil particles from a point source is most rapid for silt-clay particles; however, after as few as three relatively small runoff events, <3% of any of

Fig. 2.

Relationship of particle transport distance to peak discharge for three runoff events in Mortandad Canyon during 1979.

the labeled particles remained at the label location. Thus, contaminants, particularly those associated with silt-clay size fractions, that are released to an intermittent stream channel would be rapidly transported downstream during rainstorm runoff events.

The Accumulation of Soil Particles on Vegetation Surfaces Resulting from Rain Splash

[T. E. Hakonson, M. Dreicer, and G. C. White]

Wind and water resuspension of soil are processes that result in surface contamination of vegetation in the environment. Observations based on our field studies of plutonium at Los Alamos and Trinity Site suggest that raindrop splash of soil is a major source of contamination on vegetation surfaces. Very little is known about the mechanisms and importance of this transport process despite the usefulness of this information in environmental surveillance, waste control, and hazard assessment activities.

The purpose of this study is to increase our understanding of the mechanisms by which surface soil particles are transferred to and retained by foliage surfaces. Specific objectives of the study are to measure the accumulation and retention of soil particles by vegetation as a function of

- soil particle size
- rainfall intensity
- height of foliage above the ground
- surface area and canopy cover of plant
- upper versus lower leaf surface
- type of particle collection surface

The study is being conducted by Colorado State University and Los Alamos National Laboratory, and it employs the radionuclide-labeled soil particle technique described for the sediment transport study. In addition, a scanning electron microscope was used to verify the labeled particle technique. Samples of leaf surfaces were visually inspected in a defined viewing field to quantify the size and number of particles present as a function of previously discussed experimental variables.

Surficial accumulation and retention are dynamic, simultaneously occurring processes, which require separate measurement for proper data inter-

pretation. Therefore, two separate experiments were conducted, one involving accumulation of particles by foliage following ground surface application and one involving retention of particles applied directly to the foliage. These experiments were conducted simultaneously in separate but otherwise similar study plots.

An experimental plot was partitioned into four subplots to quantify the resuspension and retention of tracer-labeled soil particles exposed to rain and no-rain treatments. A roof over one-half of the plot created the no-rain, or control, treatment.

Barriers were erected around the plot to preclude wind as an experimental variable; three anemometers were located within the plot to verify the effectiveness of the wind barriers. Natural precipitation events were measured with recording tipping bucket rain gauges.

The labeled soil particles, characterized by size, density, and tracer content, were applied to the soil surface (accumulation treatment) or directly to the foliage surface (retention treatment). The ground surface in the retention plot was covered with burlap to prevent resuspension of particles that had washed off the plants.

Tomato plants, along with an artificial particle collector, were used as the particle collection surfaces. The artificial collectors consisted of glass slides covered with felt on both upper and lower surfaces. The slides were fastened to a 2.5-cm-diam dowel rod at heights ranging from 15 to 80 cm above the ground surface.

Foliage and glass slides were collected after each of four rainstorm events. A total of about 4000 samples was collected during the experiment. Analyses for the respective tracers were performed on GeLi gamma-ray counting systems.

The following discussion emphasizes the particle accumulation portion of the splashup study. The accumulation data will serve as the partial requirement of an M.S. degree for a Colorado State University graduate student. Specific objectives of this portion of the experiment were to characterize soil accumulation on tomato plant surfaces as a function of soil particle size, rainfall intensity, height of foliage above the ground and plant surface area, and canopy cover.

In order to measure the buildup, through time, of particles on plants, tomato plants were sampled after every rainstorm, as well as after exposure to several storms.

Four rainstorms occurred in the study plot following the placement of the tracer labeled soil. The results of the Analysis of Variance (ANOVA) tests for tomato plants exposed to individual storms indicated that no particles greater than $105\mu\text{m}$ were deposited on plant surfaces when compared to controls and that the differences in amounts of these larger particles with height of foliage above the ground and storm number were not significant ($p < 0.05$).

Background concentrations of the two larger ($>105\mu\text{m}$) size fractions on plants averaged about 1 to 2 mg soil/g wet plant.

Conversely, concentrations of $<53\mu\text{m}$ and $53\text{--}105\mu\text{m}$ soil particles on plants exposed to single storms as a function of treatment (rain vs no-rain), height of foliage above the ground, and storm number were all significantly different ($p < 0.05$). Concentrations of both size fractions on plants exposed to rain averaged about 1 mg soil/g wet plant compared to about 0.2 mg soil/g wet plant for the control treatment.

Greater concentrations of soil were found on plants sampled from the 0 to 40 cm (above the ground surface) height strata than from the >40 cm strata. For silt-clay particles ($<53\mu\text{m}$), the lower strata contained about twice the soil mass per gram of plant than did the upper (>40 cm) strata. This finding is consistent with soil flux-height relationships that have been observed during wind resuspension studies.

The amounts of the two smaller soil size fractions on plants also differed significantly between storms; the average intensities of the four storms differed by nearly a factor of 10.

The differences in the amount of soil accumulated by plants as a function of plant surface area and canopy cover were not significant ($p < 0.05$) for any of the particle size fractions. The uniformity of the size of the tomato plants used in this study probably contributed to this latter finding.

Attempts to relate the amount of $<53\mu\text{m}$ particles on plants to rainstorm parameters (i.e.

maximum intensity, average intensity, duration, etc.) were unsuccessful. However, the mean concentration of $<53\mu\text{m}$ soil particles (mg soil/g wet plant) on tomato plants was highly correlated to time ($y = 0.038 + 0.0045x$, $n=4$; $r^2 = 0.94$) where y is the amount of soil and x is time from the beginning of the experiment. The significance of this observation is not immediately apparent and requires further evaluation. On the other hand, the relationships between the amount of 53 to $105\mu\text{m}$ soil particle on plants and rainfall parameters was highly significant. For example, the linear regression of mass of 53 to $105\mu\text{m}$ particles versus maximum (peak) rainfall intensity (mm/min) resulted in the equation: $y = 0.7 + 1.9x$ ($n=4$; $r^2=0.93$), where y is the amount of soil in mg/g wet plant and x is the rainfall intensity in mm/min. Thus, increasing rainfall intensity resulted in increased amounts of 53 to $105\mu\text{m}$ particles on plant surfaces.

Data from plants exposed to one to four rainstorms were more revealing than the data from individual storms because of greater soil loading on the plants, which facilitated detection of the tracers. Results of the ANOVA test revealed that the two smaller size fractions contributed essentially all of the soil that was deposited on tomato plants after exposure to multiple rainstorms. Concentration of the smaller size fractions on plants exposed to one to four storms was significantly ($p < 0.03$) greater than controls. Coarse particles ($>105\mu\text{m}$) were either not resuspended by rainfall or, if resuspended, were not retained by the plant surfaces.

Differences in the amount of soil accumulated by plants as a function of height of foliage above the ground surface were significant ($p < 0.05$) for both the $<53\mu\text{m}$ and 53 to $105\mu\text{m}$ particles. In both cases, samples from the lower height strata contained as much as 10 times more soil than the upper height strata. Differences in soil accumulation between storm sequences were only significant ($p < 0.05$) for silt-clay particles. In no case were significant differences found in the amount of soil detected on plants as a function of plant canopy cover and surface area.

The relationships of silt-clay particle accumulation on plants to rainstorm parameters and time were significant in most cases. For example,

the amount of silt-clay particles accumulated on tomato plants as a function of total rainfall, accumulative mean rainfall intensity, accumulative mean rainfall during the first 30 min of the storm, and time increased with increasing values of the latter parameters (Fig. 1). From this preliminary analysis of the data, it would appear that relationships using some expression of rainfall intensity have the greatest potential for predicting silt-clay soil mass on vegetation.

Curve (c) in Fig. 1 provides a basis for calculating a soil accumulation rate constant for silt-clay particles under the assumption that at the end of the fourth storm the amount of soil on the plants was approaching equilibrium. The linear model that serves as the basis for this calculation is:

where:

S = amount of soil available for resuspension, calculated as 1250 g/m^2 based on 1 mm layer over 1 m^2 with a bulk density of 1.25 g/cc

P = grams of soil/ m^2 plant surface area, calculated as 0.96 g/m^2 , estimating $0.0025 \text{ g soil/g wet plant}$ at equilibrium (Fig. 1c) and 385 g/m^2 biomass

k_1 = accumulation rate constant calculated as $P (\text{equilibrium}) \times k_2/S$

Fig. 1.

Relationship of the amount of soil accumulated by tomato plants as a function of rainstorm parameters and time.

k_2 = loss rate constant from plant surface, calculated as 0.1197/day using 5.8 d half-time (from Fig. 1).

The calculated value for k_1 was 9×10^{-5} /day. This value represents the first empirical estimate of a soil accumulation rate constant for plants under field conditions. A similar calculation for the 53 to 105 μ m size fraction yielded a k_1 value of 2.3×10^{-4} /day. These estimates of the accumulation rate constant have been incorporated into the DOE-funded pathway analysis for the study "Human Radiation Exposures Near the Nevada Test Site."

The results of the study using the scanning electron microscope to characterize size and number of particles accumulated and retained by tomato leaves essentially confirm the results obtained by the labeled particle technique. For example, silt-clay represented nearly 100% of the

particles observed on leaf surfaces, and the lower height strata accumulated more soil than did the upper strata.

The number of particles present per unit area of leaf surface steadily increased with time (Fig. 2) in plants exposed to multiple rainstorms, whereas plant surfaces initially contaminated with soil (retention plot) lost soil through time (Fig. 2). The loss of particles from plant surfaces was likely caused by wash-off from impacting raindrops. The retention and accumulation curves in Fig. 2 appear to reach an equilibrium at about the same level, suggesting that the leaf surface had reached its soil loading capacity (i.e. loss = input). Retention (weathering) half-times roughly calculated from the data (Fig. 2) were about 7 days, based on the retention curve and 13 days based on the accumulation curve. This compares with 5.9 day half-time calculated from the data obtained by the tracer methods.

Fig. 2.

Mean number of particles accumulated and retained by tomato leaves as a function of time from the beginning of the experiment.

The particle splash-up study confirms that small, highly mobile particles are the major component of soil retained by plant surfaces. Calculations readily show that relatively small amounts of silt-clay particles, which contain as much as 10 times higher contaminant concentrations, could account for the relatively high radionuclide plant/soil concentration ratios observed in field studies.

Adsorption of Soluble Contaminants from Uranium Mill Tailings by Marine Shales

[M. L. Marple, E. T. Louderbough,* L. D. Potter,* D. R. Dreesen, and E. J. Cokal]

Leaching of contaminants from uranium mill tailings piles is an environmental concern because of potential contamination of groundwater. The adsorption of these contaminants by strata underlying the tailings piles could reduce the impacts of leaching. Many tailings piles, both inactive and active, are located in the Colorado Plateau region, which has extensive formations of Cretaceous marine shales. The Mancos shale and the equivalent Tropic shale have high levels of sodium-saturated montmorillonite clay and sodium, which increases surface sealing upon wetting. Thus, the shales could be valuable as sealants underlying tailings ponds. The purpose of this study was to evaluate the adsorptive capacities of four shales for a variety of major and trace element constituents in aqueous tailings leachates and to evaluate the potential usefulness of such shales in reducing the leaching transport of environmental pollutants.

Four shales from the Colorado Plateau, which exhibit a wide range of chemical and physical characteristics, were chosen for this study. Mancos shale samples from three sites were selected. The shales from Austin and Loma in western Colorado are dark and platy. The Austin shale has a very acidic pH of 3.5 and a 60:40 ratio of illite to montmorillonite. The Loma shale has a pH of 8.0 and a 75:25 ratio of illite to montmorillonite. The shale found near Thompson in eastern Utah has a highly alkaline pH of 9.9. It has an illite

to montmorillonite ratio of 60:40. The Tropic shale was collected from an area near Kanab, Utah, and has a 10:90 ratio of illite to montmorillonite and a pH of 8.0. Soils derived from the Tropic shale are saline-sodic.

Two soils from the Grants Mineral Belt of New Mexico were used for comparison with the shales in the adsorption trials. The soil from Ambrosia Lake is a clay loam with a pH of 7.9. The Bluewater soil is a clay with a pH of 8.3.

A leaching column of each of the six substrates (shales and soils) was prepared for each of the two kinds of input tailings leachate. Fine shale material (<0.074 mm) and medium soil material (0.074 to 2 mm) were used. Each column was made of a 14-cm length of PVC pipe with a 10-cm diameter, and filled with 320 g of material (shale or soil).

Tailings from two inactive uranium mill sites were processed to produce two input leachates. The carbonate tailings from the Ambrosia Lake site in New Mexico were chosen because of their high total and leachable molybdenum and selenium contents. The acid tailings were from Mexican Hat, Utah. These tailings were slurried with distilled water, shaken daily for 25 days, and vacuum filtered.

1 column was saturated by infiltration of the leachates and were maintained at saturation. Approximately one pore volume (125 ml) of output leachate was collected daily from each column, as well as 125 ml of the input solution. The carbonate leachate study lasted 26 days, except for the Tropic shale column, which was terminated after 22 days. The acid leachate study lasted 24 days.

Both input and output leachates were analyzed daily for pH. The tailings leachates and subsequent column leachates obtained on days 1, 5, 10, 15, 20, and 25 (24 for acid tailings) were analyzed for major elements and ions at the University of New Mexico and for trace elements at the Los Alamos National Laboratory. Analyses of calcium and magnesium (using additions of lanthanum) and of sodium were performed by atomic absorption spectrophotometry. A Technicon autoanalyzer was used for analysis of sulfate and chloride. For the carbonate samples, molybdenum and selenium were analyzed using flame atomic

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absorption analysis (hydride generation). For the acid samples, cobalt, copper, iron, manganese, and nickel were determined using flame atomic absorption analyses.

Analytical results of major and trace constituents showed little variation over time. Thus, means of the six analyses over time were computed for each column and for each constituent. Mean concentrations in the input tailings leachates were tested for equality with those in the effluent leachates collected from the shale and soil columns using an approximate t-test in which the variances of the means are assumed to be unequal.¹ The equality of the mean concentrations of leachates from different shale or soil columns was also tested using the approximate t-test.

Concentrations of both calcium and sulfate in carbonate effluents were significantly greater ($p < 0.1$) in shale leachates than in soil leachates. Leachates from shale columns differed significantly from one another and from soil columns. Leachates from the Tropic shale had significantly greater sodium ($p < 0.001$) for both acid and carbonate leachates and significantly greater chloride ($p < 0.1$) for the carbonate leachate. The pH values were significantly lower ($p < 0.1$) for both acid and carbonate leachates from the Austin columns compared with all other columns.

Concentrations of trace elements and the per cent change in carbonate leachates from shale and soil columns compared to the input tailings solution are shown in Table I. The pH of the leachate was significantly lower ($p < 0.001$) than the input solution only for the Austin shale column because of the acidity of the shale. Molybdenum showed the same result, a significant ($p < 0.02$) change (44% reduction) only in the Austin leachate, which can be attributed to the low solubility of the element under acidic conditions.² Selenium content of all the shale and soil leachates was significantly ($p < 0.02$) lower than that of the input solution. But the reduction in selenium content (because of adsorption) was significantly ($p < 0.05$) less for the Thompson shale than for other materials.

Concentrations of trace elements and their per cent change in acid leachates are shown in Table II. Both soils showed significant ($p < 0.01$) attenuation of all five elements. Three shales--Loma, Thompson, and Tropic--showed significant ($p < 0.05$) attenuation for all elements, but the reductions were significantly ($p < 0.01$) less than those of the soils for all elements except iron. The Austin shale differed ($p < 0.01$) from the other three shales for all elements except cobalt. It showed less attenuation or even an enrichment (of

TABLE I
VALUES OF pH AND CONCENTRATIONS OF Mo AND Se (ppm) IN INPUT AND OUTPUT CARBONATE LEACHATES

	pH	Molybdenum		Selenium	
		ppm	% ^a	ppm	%
Input Solution	7.0 ± 0.5	0.52 ± 0.04	100	0.19 ± 0.06	100
Soil Leachate: Ambrosia Lake	7.7 ± 0.2	0.49 ± 0.14	94	0.03 ± 0.03	14
Bluewater	7.6 ± 0.6	0.46 ± 0.12	88	0.03 ± 0.04	32
Shale Leachate: Austin	5.1 ± 0.8	0.29 ± 0.14	56	0.06 ± 0.05	30
Loma	7.5 ± 0.3	0.44 ± 0.12	85	0.04 ± 0.03	21
Thompson	7.3 ± 0.4	0.52 ± 0.05	100	0.09 ± 0.03	47
Tropic	7.3 ± 0.6	0.52 ± 0.09	100	0.05 ± 0.01	23

^a% = (concentration in effluent leachate/concentration in input solution) X 100.

TABLE II

VALUES OF pH AND CONCENTRATIONS OF Co, Cu, Fe, Mn, AND Ni (ppm) IN INPUT AND OUTPUT ACID LEACHATES

	pH	Cobalt		Copper		Iron		Manganese		Nickel	
		ppm	% ^a	ppm	%	ppm	%	ppm	%	ppm	%
Input Solution	4.5 ± 0.2	2.1 ± 0.02	100	7.9 ± 0.2	100	0.08 ± 0.03	100	12 ± 0.2	100	1.1 ± 0.3	100
Soil Leachate: Ambrosia Lake	7.4 ± 0.4	0.02 ± 0.01	1	0.04 ± 0.02	1	<0.01 ± 0.01	13	0.8 ± 1.0	7	0.05 ± 0.03	5
Blue-water	7.4 ± 0.2	0.1 ± 0.1	4	0.1 ± 0.1	1	<0.01 ± 0.0	13	1.3 ± 1.2	11	0.06 ± 0.08	5
Shale Leachate: Austin	4.3 ± 0.1	1.9 ± 0.5	90	3.8 ± 0.8	48	0.20 ± 0.08	250	11 ± 0.3	92	1.2 ± 0.02	106
Loma	6.7 ± 0.3	1.5 ± 0.1	70	1.6 ± 0.8	20	0.02 ± 0.01	25	9.0 ± 1.0	77	0.8 ± 0.1	72
Thompson	6.0 ± 1.3	1.1 ± 0.3	50	1.2 ± 0.7	15	0.03 ± 0.03	38	7.6 ± 1.0	65	0.6 ± 0.2	53
Tropic	6.8 ± 0.5	1.3 ± 0.3	63	1.8 ± 0.9	23	0.01 ± 0.01	13	8.1 ± 0.9	70	0.7 ± 0.1	63

^a% = (concentration in effluent leachate/concentration in input solution) X 100.

iron and nickel) in leachates because of the acidic pH, which favors the solubility of these elements in soils.²

In conclusion, shales showed varying capacities to adsorb trace element contaminants from tailings solutions. The acidic shale from Austin showed large adsorption of two contaminants common in carbonate tailings solutions, but little adsorption, if any, of contaminants from acid tailings. Two other types of Mancos shale from Loma, Thompson, and Tropic shale showed moderate adsorption of contaminants from acid tailings, but fine-textured local soils had greater adsorptive capacities than the shales. These results indicate that the adsorptive capacities of shales as substrates beneath a tailings pond depend on the chemical characteristics of both the shales and the uranium mill tailings. Although such shales could be useful in reducing seepage from tailings ponds, individual assessments for specific tailings and specific shales must be made to characterize accurately the adsorption of aqueous tailings contaminants by shales from the Colorado Plateau.

Acknowledgments

A major portion of this study was performed under a subcontract to the University of New Mexico from the Los Alamos National Laboratory. It was performed in partial fulfillment of the requirements for the Doctor of Philosophy degree of E. T. Louderbough and in conjunction with research supported by the Rocky Mountain Forest and Range Experiment Station of the U. S. Forest Service (Contract No. 43-82FT-8-1231).

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Plant Uptake Assay of Uranium Mill Tailings

[D. R. Dreesen, M. L. Marple, and E. J. Cokal]

Previous studies at the Los Alamos National Laboratory have shown that native plant species can absorb and translocate appreciable quantities of trace elements and radionuclides from uranium mill tailings.¹⁻⁴ The levels of Mo and Se found in stems and leaves of plants grown in tailings under greenhouse conditions are much greater than the levels considered to be toxic to grazing animals.³ High concentrations of U and ²²⁶Ra have also been found in some native plants growing in tailings under field and greenhouse conditions.^{3,4} The assimilation and translocation of these hazardous constituents could provide a pathway for the transport of contaminants into the environment if plant roots penetrate covers used to stabilize inactive uranium mill tailings piles. Results of previous experiments indicate that this absorption and translocation depend on the species and on the chemical characteristics of the tailing material. Thus, a technique was needed to rapidly assay the contaminant uptake potential of a wide variety of native and agronomic plants grown in a variety of tailings.

Important criteria for a successful assay technique of plant uptake are (1) the ability to perform the assays rapidly (i.e. sufficient biomass production in a time span of 2 to 3 months), (2) the need to minimize the influence of the growth media mixture on uptake, and (3) the desire to provide conditions that promote the germination and growth of native plant species of the arid western United States. The developed method was a synthesis of nutrient absorption techniques used by Stanford and DeMent⁵ and a potting mixture for Western shrubs and forbs used by Ferguson and Monsen.⁶ The Stanford and DeMent technique grows plants in a sand culture periodically watered with nutrient solution. When a sufficient root mass has developed, the bottom of the container is removed and the roots are placed in contact with soil containing the various nutrient treatments. We have revised this technique by substituting a potting mixture containing slow release fertilizers and calcareous (alkaline) amendments for the sand culture. Rapid plant growth is

achieved with periodic irrigation using only deionized water. When an appreciable root mass has developed (Stage I of Fig. 1), the container bottom is removed and the root mass is nested into another container having a layer of tailings or a control substrate of soil or potting mixture (Stage II of Fig. 1). The roots grow into the tailings for a period of at least one month, and then the above-ground plant material is harvested.

A preliminary proof-of-concept experiment was performed with the same grass (*Sporobolus airoides*) and shrub (*Atriplex canescens*) species used in our previous uptake experiments.³ The assayed material was fine tailings (slimes) from an inactive alkaline leach mill at Ambrosia Lake, New Mexico. These tailings have been extensively investigated in earlier uptake and mobility studies.¹⁻⁴ Several diluents of the tailings were investigated to determine whether they would promote root penetration and growth in the tailings layer. These diluents included the peat moss/vermiculite potting mix and fine vermiculite. Two potting mixtures were evaluated: the peat moss/vermiculite mix of Ferguson and Monsen⁶ and the same mix minus peat moss with fine vermiculite replacing the coarse vermiculite. The water soluble organics leached from the peat moss could affect the uptake of contaminants; therefore, peat moss was eliminated from one of the potting mixtures.

One month following germination, the plant roots were placed on the tailings treatments or the control substrates. After growing for another month, the aboveground plant material was harvested, air dried, acid digested, and analyzed for Mo by flame atomic absorption spectrophotometry. Molybdenum was chosen as the indicator element because of its bioavailability in the tailings material, as shown in earlier studies.³

The Mo content of the aboveground plant material is reported in Table I for the two species, the two potting mixes (controls), and the three tailings treatments. Concentration ratios have been included comparing the Mo concentration of the plants grown in tailings versus the plants grown in the controls. In addition, Mo concentrations and concentration ratios from an earlier experiment³ are presented.

Fig. 1.

Configuration of two stages of the assay technique for plant uptake.

The lesser Mo concentrations in vegetation from the uptake assay compared with our earlier greenhouse experiment are readily apparent. The most probable reason is the difference in longevity of growth (one month for assay vegetation and six months for greenhouse vegetation). The concentration of assimilated elements is expected to increase with plant age or over the course of a growing season.⁷ Even though the absolute Mo concentrations differ between the two experiments, the concentration ratios cover a similar range of values. If appropriate control materials are assayed for all experimental designs, concentration ratios can be calculated to provide the best measure of bioavailability. The next set of experiments will use the vermiculite potting mixture, because of the apparent difference with peat

moss, and undiluted tailings to simplify the assay system. Future attempts will be made to harvest clean roots from only the potting mixture layer. By analyzing the roots, we may be able to ascertain if certain elements are absorbed by the roots but not translocated to stems and leaves. These experiments will assess concentration as a function of age (i.e., time grown in assay material) and will evaluate the uptake of contaminants from tailings from three inactive mill sites (Salt Lake City, Utah; Shiprock, New Mexico; and Durango, Colorado).

The plant uptake assay technique holds promise as a generic method to assess contaminant uptake from solid wastes and will provide a rapid means to evaluate uptake potential of tailings contaminants by important plant species of the arid western United States.

TABLE I

MOLYBDENUM CONTENT OF ABOVEGROUND PLANT MATERIAL FROM THE UPTAKE ASSAY AND THE
GREENHOUSE EXPERIMENT^a
(in µg/g dry weight basis)

PLANT UPTAKE ASSAY

Potting Mixture	<i>Sporobolus airoides</i> (Alkali Sacaton)		<i>Atriplex canescens</i> (Four-Wing Saltbush)	
	<u>Vermiculite</u>	<u>Peat Moss/ Vermiculite</u>	<u>Vermiculite</u>	<u>Peat Moss/ Vermiculite</u>
Control	0.21	0.61	0.36	0.36
Tailings	5.8 (28)	9.4 (15)	11.0 (31)	39.5 (110)
Tailings + Peat Moss/Vermiculite		4.9 (8)		29.1 (81)
Tailings + Vermiculite	3.7 (18)	4.8 (8)	12.3 (34)	9.3 (26)

GREENHOUSE EXPERIMENT^b

	<i>Sporobolus airoides</i> (Alkali Sacaton)			<i>Atriplex canescens</i> (Four-Wing Saltbush)		
	<u>Sand</u>	<u>Loam</u>	<u>Clay</u>	<u>Sand</u>	<u>Loam</u>	<u>Clay</u>
Control	6	8	13	10	12	2
Mean		9			8	
Tailings	147 (25)	173 (22)	78 (6)	273 (27)	181 (15)	147 (73)
Mean		133 (15)			200 (25)	

^aConcentration ratio of plant conc. (tailings)/plant conc. (control) in ().

^bRef. 3.

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Thermal Water Supplies to Surface Waters as
Related to Hydrothermal Energy Development
in the Jemez Mountains

[J. M. Williams, L. E. Hersman, and G. J. Langhorst]

The Jemez caldera provides an excellent source for the production of energy by thermally heated water. Two technologies are currently being developed there: hot dry rock or HDR (by DOE/Los Alamos National Laboratory) and hydrothermal (by Union Geothermal, Public Service Company of New Mexico and DOE). HDR will use a closed, recycled water system in a hot but dry underground zone. The hydrothermal process, on the other hand, will pump water from a hot underground aquifer. Most of the water will be pumped back into the ground, but about 27% will be lost to the atmosphere, primarily through cooling tower vapors. Interested groups have raised questions about the impact that such a water loss, at flow rates needed for power genera-

tion, will have on the supply of surface water for agriculture and recreation in the Jemez Mountain area.

The principal, observable, thermal releases to surface waters in the Jemez Mountains are in the Soda Dam-Jemez Springs area (Fig. 1). This is downstream from the Redondo Creek/Sulfur Creek area where hydrothermal energy development will occur. Because the prime source of certain chemical ions in the surface waters of the region is thermal water, the amount of thermal water flow can be evaluated from the concentration of these indicator ions. Purtymun et al.¹ have been monitoring water quality in this area for a number of years. Their data provide an excellent source for determining thermal flow to the Jemez Pueblo (Position 6 in Fig. 1). Water Resources Associates, Inc. used arsenic to determine that $0.0103 \text{ m}^3/\text{s}$ (0.365 cfs) of thermal water is flowing into

Fig. 1.

Jemez River thermal supplies.

the Jemez River from sources above Jemez Pueblo.* W. P. Balleau used 5 ions and obtained a value of $0.023 \text{ m}^3/\text{s}$ (0.81 cfs).** Our analysis using 6 ions is shown in Fig. 1. The thermal water supply from the hydrothermal development area above Soda Dam is minor [$0.00105 \text{ m}^3/\text{s}$ ($0.037 \pm 0.04 \text{ cfs}$ or $26 \pm 29 \text{ acre ft/y}$)]. Thermal inputs to the Jemez River above Jemez Pueblo, including those from Soda Dam and Jemez Springs (major contributors) and Rio Guadalupe, amount to $0.023 \text{ m}^3/\text{s}$ ($0.81 \pm 0.34 \text{ cfs}$). The thermal water supplies in this region appear to be defined reasonably well.

Thermal water flows from most of the other Jemez Mountain areas are poorly defined. Water quality data are less abundant than for the upper Jemez River, and the flow systems are complexed by dams, which can serve as evaporating points and thus as ion concentrators. An attempt has been made by Balleau, however, to apply the ion concentration method described above.** His analysis shows that no thermal flow is being produced from the east quadrant of the Jemez Mountains, but that $0.14 \text{ m}^3/\text{s}$ (0 cfs) is being produced in the half quadrant between this quadrant and the Jemez Springs area discussed above. The lack of appreciable water flow of any kind through the latter area makes this analysis suspect. We believe that a better approach is provided by the following method, which treats the Jemez Mountain drainage system as a set of proportionate sectors.

The drainage fields from the Jemez Mountains can be divided into four sectors based on ground water flow directions (see Fig. 2). In this manner, the hydrothermal development area and the upper portion of the Jemez River (that including the Jemez Pueblo) form one sector and account for 35.8% of the flow area. Using the thermal supply value [$0.024 \text{ m}^3/\text{s}$ (0.85 cfs)] of this sector and assuming that the thermal supply in each sector is proportional to its share of the total area, the thermal water supplied by each sector can be estimated (see Table I).

The total thermal flow from the Jemez Mountains, based on proportionate sectors, is

Fig. 2.

Thermal water supplies to surface waters in the Jemez Mountains.

TABLE I
THERMAL WATER SUPPLIES TO SURFACE WATERS IN
THE JEMEZ MOUNTAINS

Sector	Fraction of Total Area (%)	Thermal Water Supply m^3/s (cfs)
A	35.8	0.024 (0.85)
B	26.7	0.018 (0.63^a)
C	25.3	0.017 (0.60^a)
D	12.2	0.0082 (0.29^a)
A to D		0.067 (2.37)

^aValue based on proportionality of area to Sector A.

*J. R. Erickson, "Hydrology--Jemez Mountains, New Mexico," Report by Water Resources Associates, Inc., for Union Geothermal Division of Union Oil Company (March, 1977).

**W. P. Balleau, "Geothermal Hydrology at Jemez Mountains--Ion Balance and Flow Depletion," report for Department of Interior/Bureau of Indian Affairs (March, 1980).

approximately $0.068 \text{ m}^3/\text{s}$ (2.4 cfs) with about half [$0.031 \text{ m}^3/\text{s}$ (1.1 cfs)] flowing down the Jemez River system (sectors A and D). The total is somewhat lower than the $0.215 \text{ m}^3/\text{s}$ (7.6 cfs) estimated by Purtymun and Johansen in 1974.² The flow down the Jemez River system appears fairly reliable, however, and places an upper limit on the extent to which a hydrothermal development can influence water flow in that system. Thus, complete stoppage of thermal water supplies to the Jemez River would reduce the mean flow rate of $0.77 \text{ m}^3/\text{s}$ (27 cfs--Erickson, 1977) by $0.031 \text{ m}^3/\text{s}$ (1.1 cfs) or about 4%.

A complete stoppage of thermal water supplies is probably unlikely. A significant reduction, however, could cause mineral spas to close and take croplands out of production in this water-limited land. Further work will be performed to evaluate the extent to which various levels of reduction will influence crop irrigation and to address the complex problem of just how much thermal water flows will be reduced by hydrothermal development in the Jemez Mountains.

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Activities of the Los Alamos National Environmental Research Park, 1980

[K. V. Bostick]

The Los Alamos National Environmental Research Park (LA/NERP) was dedicated in 1976 as a field laboratory set aside for ecological research, for study of the environmental impacts of energy development, and as a source of public information on environmental issues. The emphasis of research on the park is to develop criteria that facilitate energy development in ways that are least harmful to the environment.

The philosophy of the LA/NERP is also guided by the Charter For The Life Sciences at Los Alamos National Laboratory. Part of this charter is:

"As a necessary basis for effective long-term performance in meeting the obligations and commitments associated with its primary goal, develop life sciences and environmental research at Los Alamos National Laboratory to a status of national and international recognition as a center of excellence with emphasis on the need for a continuing program of sound supporting research.

Develop close relationships with universities and research institutions, especially with the University of California, on a scientific and academic level."

The LA/NERP encompasses approximately 111^2 km of DOE land at Los Alamos. The steep elevation gradient (1500 m in 25 km) and Canyon/Mesa terrain give the research park a wide spectrum of South-western habitat types in a compact area. Another unique feature of the LA/NERP is that some areas within the park have been protected from activities such as agriculture and lumbering for nearly 40 years. Pre-Columbian Indian ruins are in better condition than similar ruins on adjoining U. S. Forest Service lands because of this history of restricted access.

Studies conducted on the LA/NERP primarily involve Laboratory staff and graduate and undergraduate students from regional universities contracted to conduct specific studies. Some of the work is conducted in cooperation with Federal and state agencies, such as the National Park Service and the New Mexico Department of Game and Fish.

Research projects are selected by the LA/NERP Advisory Committee from proposals solicited within the Laboratory and at regional universities. Members of the Advisory Committee are E. M. Wewerka, LS-6; W. R. Hansen, H-8; R. F. Crombie, ENG-11; and G. J. Nunz, G-DO. Wewerka and Hansen are the LA/NERP Co-coordinators. K. V. Bostick is the Assistant Coordinator.

The La Mesa fire in June, 1977 burned over 60 km^2 on Department of Energy, National Park Service, and US Forest Service land. Many

of the studies conducted by the LA/NERP are related to this fire. These studies and other LA/NERP sponsored studies conducted in 1980 are described below.

Natural Regeneration of Ponderosa Pine as Related to Fire History and Land Use

[L. D. Potter, T. S. Foxx, and F. Barnes]

Natural regeneration of ponderosa pine is dependent on a number of environmental factors such as species competition, canopy cover, soil types, and climatic conditions. In general, ponderosa pine forests of the Southwest are slow to recover after wildfire. Artificial restocking is routinely carried out.

Because of differing philosophies in land use management, lands under jurisdiction of each agency were treated somewhat differently after the La Mesa fire. Lands controlled by the National Park Service were not logged after the fire, and no artificial restocking is anticipated. Lands under jurisdiction of the United States Forest Service were logged and restocking is scheduled for 1981. All lands, with the exception of 0.7 km² on the LA/NERP, were seeded with six native grasses soon after the fire to prevent severe soil loss.

Two of the six seeded grasses became well established. They included sheep fescue (*Festuca ovina*) and slender wheatgrass (*Agropyron trachycaulum*). There is evidence that these grasses will severely suppress natural pine regeneration by competing for water, sunlight, and nutrients. It was therefore decided to look at natural pine regeneration as related to various land management practices on the Pajarito Plateau and to determine the microsite conditions that favor pine establishment. To further understand the grass-pine seedling competition, the water status of sheep fescue, slender wheatgrass, and mountain muhly (*muhlenbergia montana*) was studied during the growing season and compared with soil water at several depths.

The following treatment areas were surveyed for the presence or lack of presence of ponderosa pine seedlings.

- (1) Areas seeded with grasses and not logged post-fire,
- (2) areas with native grasses not logged post-fire,

- (3) areas seeded and logged post-fire,
- (4) areas thinned post-fire and not seeded,
- (5) areas not logged post-fire and not seeded, and
- (6) areas with known fire history, which burned before 1977 and which have had ample time for natural succession.

In addition to determining the numbers of seedlings per km², site specific information was gathered for individual seedlings. Thus far, such information has been tabulated for 1385 seedlings. Presently, field surveys have been completed, and data tabulation and correlation are in progress.

Niche separation of sheep fescue, slender wheatgrass and mountain muhly were studied during the 1980 growing season by periodic measurements of leaf water potential, per cent soil water and root structure.

The relationship between leaf water potential and soil water at different depths is currently being studied for each species. Small ponderosa pine seedlings (1 to 2 years) will be excavated to determine their root structure and comparisons made with grass root structure and water usage.

Biotelemetry Studies on Elk

[G. C. White]

The movements of Rocky Mountain elk (*Cervus elaphus nelsoni*) in the eastern Jemez Mountains in north-central New Mexico were studied from 1978 to 1980. Thirty-six elk were trapped, marked, and released, and thirty of these animals were radio-collared.

The La Mesa fire created a wintering habitat that was used heavily by the radio-collared elk. The 10-year-old clear cuts on Cerro del Medio on Baca Land and Cattle Company property were used for calving and nursing areas. In general, radio-collared elk used areas in an early successional state, and they did not use areas at Los Alamos National Laboratory where there was human activity.

Results of the study through 1980 are published in "Biotelemetry Studies on Elk" (Los Alamos National Laboratory report, LA-8529-NERP, 1981). A motion picture, "Elk Biotelemetry at the Los Alamos Environmental Research Park" (Y-355) was also produced based on this study.

Comparative Winter Nutrition of Elk in the Jemez Mountains, New Mexico

[M. M. Rowland, A. W. Alldredge, J. E. Ellis, and G. C. White]

Studies of winter diet quality and nutritional status of elk were conducted in the Jemez Mountains in 1979-1980. A recently burned montane area, including LA/NERP lands, and an unburned area were selected for winter range comparisons. Composition of elk diets in the two areas, determined by fecal analysis, was markedly different. Elk consumed significantly more grass in the burned area, while consumption of browse was significantly greater in the unburned area. *In vitro* organic matter digestibility of grass forages was greater in the burned area, but other forage quality comparisons revealed no differences between areas. Although dietary protein was similar between locations, digestibility on the burn was significantly greater than in the unburned area. Observed differences, between areas, in diet quality were attributed, in part, to changes in forage availability following the fire in Bandelier National Monument, rather than increases in forage quality. A simulation model of ruminant energy and nitrogen balance was used to predict effects of diet quality on elk nutritional status. Greater energy deficits were predicted for elk in the unburned area, effecting greater losses of fat and lean body in this area. Superior dietary nitrogen was insufficient to compensate for these losses.

Fire plays an important role in maintaining quality habitat for elk in the Jemez Mountains. Other winter ranges for the Jemez Mountain elk include proposed geothermal sites. Knowledge of winter range qualities associated with good winter range for elk may assist in mitigation of potential losses of habitat caused by energy development in the Jemez Mountains.

The Influence of Winter Range Quality on Elk Condition

[B. J. Weber and G. C. White]

Condition of elk utilizing portions of the area burned by the La Mesa fire was established from animals live-trapped at Bandelier National Monument (BURN, N=20) during winter, 1980. Values obtained from elk at the BURN were compared to

those of elk trapped at a similar, but unburned habitat (BACA, N=16) during the same time period. Animal condition was evaluated on the basis of weight, depth of back fat (DBF), and several blood parameters. Pregnancy rates were determined by rectal palpation. Observed age and sex ratios did not differ significantly between areas ($P>0.73$). Differences between locations for weight, DBF, and blood values were analyzed using an analysis of variance. Elk sampled at the BURN weighed significantly more than elk at the BACA ($P<0.01$). DBF was greatest at the BURN, and back fat was detected in only one BACA elk. Blood variables indicative of nutritional status (alkaline phosphates, triglycerides, albumin, mean corpuscular volume) were all significantly higher at the BURN ($P<0.05$). Significantly higher ($P<0.05$) serum levels of CO_2 and total bilirubin found in the BACA elk suggest that these animals were under greater stress. Pregnancy rates were 100% for adult female elk at both locations. No yearling elk were captured at the BACA, and only one of four trapped at the BURN was pregnant.

Pinon-Juniper Habitat Types

[F. J. Barnes]

The aim of the pinon-juniper habitat study is to determine a graded series of habitat types in the pinon-juniper woodland of the LA/NERP and adjacent lands. These studies will provide the basis for an eco-physiological investigation into the distribution of *Pinus edulis* and *Juniperus monosperma*, the dominant tree species of the woodland. The final study will provide information for developing a management tool to determine production potential of the various habitat types of pinon-juniper woodland of the Southwest.

Extensive reconnaissance of the LA/NERP lands has been completed, while reconnaissance of the Bandelier National Monument woodland is still underway. Sample stands of woodland are being selected and study plots (25 by 15 m) are being established. To date, 15 plots have been staked. The density and cover of pinons and junipers are being recorded by basal diameter classes. Because junipers branch extensively from the base, all basal branches are recorded by diameter classes,

and an equivalent basal diameter is calculated using the formula

$$DAB = \sqrt{d_1^2 + d_2^2 + \dots + d_n^2},$$

where DAB is the equivalent diameter at base, and d_n is the diameter of the nth branch. Representative trees on each plot are cored for age determination and their heights recorded. Over 150 specimens of grasses and forbs have been collected and pressed, and identification of these specimens is under way.

Status of the Flora of the Los Alamos National Environmental Research Park

(T. S. Foxx and G. D. Tierney)

Results of the survey of the LA/NERP for endangered, threatened and rare plant species have been published in LA-8050-NERP, Vol. 1 "Status of the Flora of the Los Alamos National Environmental Research Park" (1980).

Under the Endangered Species Act of 1973, it became necessary to locate critical habitats of plant species in danger of extinction on state and Federal lands. A study to provide information regarding locations of possible endangered, threatened, protected, and rare plant species within the LA/NERP was initiated in August, 1977.

Only one species, grama grass cactus (*Cylindropuntia pappophoroides*), presently on proposed Federal endangered and threatened species lists (1975, 1978), was found in the area adjacent to the LA/NERP. The population was not large, and various human activities are contributing to the deterioration of its habitat.

Fourteen plants on the New Mexico State protected list were located. Only one, larkspur violet (*Viola pedatifida*) appears to be of any significance. It is a rare peripheral, which has been collected infrequently in New Mexico. It was found in a small population, and its habitat could be damaged by logging, herbicides, or surface water depletion. All other species present in the area that are on the protected list are enumerated for information purposes. None of those plants were considered rare or in need of protection from Laboratory activities other than to help preserve the natural flora of the area.

During the survey, 322 species representing 64 taxonomic families were collected or noted in Mortandad, Effluent, and Water Canyons. Many of these species have not previously been reported for the area.

Much of the area surveyed was heavily disturbed because of activities before and after the establishment of the Laboratory. Various stages in plant succession were noted.

The upper portion of Water Canyon was subjected to the La Mesa fire. Post-fire plant succession showed increased size in many plant species. Heavy browsing of most shrubs, some trees, and many forbs was observed.

Assessing Nitrogen Stress in Local Ecosystems (V. P. Gutschick)

Ammonia transferred in air at normal parts-per-billion levels may have a large impact on long-term nitrogen balances of ecosystems. The transfer itself has proved difficult to assess because accurate field measurements are both cumbersome and lengthy, hence few in number. Thus, a small and rapid detector was designed, in which selective absorption of ammonia should occur from an air flow onto a quartz oscillator crystal. The change in oscillator frequency by mass-loading will quantify the ammonia level in the air, upon equilibration for several minutes. Fabrication of the electrical and mechanical assemblies is now complete, and evaluation of crystal coatings for sensitivity and ammonia selectivity has begun.

The ecological studies in discerning broad principles behind nitrogen-acquisition activities of plants have progressed considerably. A number of concepts have been developed and are ready for quantitative testing in the laboratory and in the field.

Other Activities

One other LA/NERP-funded study, "Rainfall as a Mechanism for Soil Contamination of Plant Surfaces," is described above. In addition to these studies, the LA/NERP also provided support to James N. Hill, UCLA, conducting archaeological surveys on the Pajarito Plateau and to LeRoy D. Hacker of the US Soil Conservation Service, com-

pleting verification and followup work on a soil-type map of Los Alamos County.

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Overview Assessment of Nuclear Waste Management

[B. W. Burton, B. A. Perkins, and J. G. Steger]

In October 1979, at the request of the Environmental Safety and Engineering (ESE) Division, Los Alamos National Laboratory began an evaluation of the effectiveness of environmental control practices in the DOE waste management program. Our role in this activity is to assist ESE to conduct an independent evaluation of the environmental control technologies applied to nuclear waste management, and to advise ESE where these controls seem to be inadequate, or where further studies are needed to determine adequacy.

After reviewing the environmental control technologies associated with nuclear waste management programs, we have identified the most urgent problems requiring further action or follow-up. These are listed, in order of decreasing importance.

Surface Land Disposal Technology Development.

There is insufficient understanding of radionuclide release and transport mechanisms, which is compounded by the location of many burial sites in areas of complex geology. Regulatory standards and criteria seem to be conflicting and, in any case, may not be adequate to provide safe disposal. In addition, there seems to be no coherent plan for ensuring site integrity after closure. We recommend further research in the areas of hydro-geologic conditions at existing sites and radionuclide release and transport mechanisms. A close evaluation of limiting concentrations in burial grounds (maximum and minimum allowable concentrations) is needed. Also needed are programs to address waste treatment and volume reduction. Land use evaluation schemes to be used in new facility siting should be developed.

Active Uranium Mill Tailings Piles. Remedial action and the NRC licensing requirements addressing old and new tailings piles are promising, but their long-term effectiveness has yet to be proven. Environmental controls for currently active piles are not adequate, but these facilities are already licensed. It is reasonable to assume that successful developments in remedial action programs and NRC regulations regarding stabilization/rehabilitation at the close of operations will be applied to these active piles. However, currently active milling operations, which were not subject to the new NRC requirements at the time they were licensed, should be comprehensively studied to determine the extent to which their current practices are affecting the environment and to determine the cost/feasibility of ameliorative action.

Uranium Mine Dewatering. Pumpage of uranium mine water results in changes in the aquifer, dispersal of contaminants (both radioactive and toxic), and loss of water, itself a valuable resource. Mine water control is not very effective, and these activities are poorly documented. Environmental health and safety regulations and division of authority are not clear. Carefully coordinated field studies to determine the overall potential health and environmental impact of current uranium mining practices with particular attention to implications of gaps in regulatory authority and enforcement capacity of both state and federal authorities should be undertaken.

Site Decommissioning. Some of the decontamination and site decommissioning activities scheduled for the near future are potentially hazardous. They will involve large quantities of radionuclides, will take place in populated areas, and are novel. Relevant documents do not address methods of establishing priorities, development of cleanup criteria, or methods for the disposal of the waste. These issues must be addressed before operations are allowed to begin.

INEL TRU Exhumation/Treatment. This activity is discussed in some documents but not in others, so the current status of the project is not clear. The operations will be innovative, the quantity of

radionuclides involved is large, and the environmental controls are not known. This activity is potentially hazardous and, if it is still scheduled, DOE should ensure that careful plans are made and the environmental controls are adequate before exhumation is allowed to begin.

Uranium Mine Spoils. Mine spoils piles are a potential source of wind- and water-borne contamination (both radioactive and toxic). There are very little data available regarding mine spoils. Although spoils piles are poorly regulated, an effective reclamation program can probably be developed under RCRA. These spoils piles are outside DOE jurisdiction, but ESE should pay close attention to developments in this area.

Medical/Institutional Wastes. Large volumes of very low activity wastes are produced by the medical/institutional community. Enforcement of environmental controls has been ineffective, and much of the waste is disposed to municipal sewer and refuse systems. Because of the low activities involved, the hazards of institutional wastes are biological and chemical rather than radiological. Large volumes of toxic and organic liquids are buried in commercial low-level burial grounds, presenting the potential hazard of chelating and mobilizing radionuclides. Although these waste generators are not under DOE jurisdiction, there is a real need for increased development of waste treatment and volume reduction systems.

The Nuclear Regulatory Commission (NRC) has recently proposed a rulemaking to deregulate some biomedical wastes.* If adopted, the new rule would exempt liquid scintillation media and animal carcasses from NRC regulation and increase the amount of uncontrolled release of tritium and carbon-14. The ultimate effects of this decision are not entirely clear at the present time.

We feel that the following areas need not be of immediate concern.

*Federal Register, Vol. 45, No. 197, pp. 67018-67020 (October 8, 1980).

Conversion/Enrichment/Fabrication. Because of the small amounts of waste produced by these facilities, we feel they should receive a low priority.

These facilities have been in operation for some time, and no environmental releases of any consequence have been reported. We perceive the major problem to be onsite holding ponds, but these will not become a concern until the facilities are decommissioned.

Reactors/Fuel Storage Operations. These operations have been under licensed supervision for many years and we feel there is no immediate problem in these

areas other than the need for more efficient waste volume reduction practices at reactors.

Terminal Isolation. These programs are relatively new, and it is probably too early to assess the potential control technologies at this time. We have noted that post operational monitoring and accident recovery are not adequately covered. Also, there may be conflicts in the guidelines. We are somewhat concerned about the potential misuse of probability estimates of future geological and climatological events.

APPENDIX
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