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of the
LASL Health Division
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ABSTRACT

This report summarizes research and development activities of LASL's Biomedical and Environmental Research Program for the calendar year 1976. The principal features of the program have been carried out by groups in the Health Division, supported by the Administration for Environment and Safety, Division of Biomedical and Environmental Research of the U. S. Energy Research and Development Administration. Previous annual reports in this series prepared under the auspices of the U. S. Atomic Energy Commission and the U. S. Energy Research and Development Administration are LA-4923-PR (1971), LA-5227-PR (1972), LA-5633-PR (1973), LA-5883-PR (1974), and LA-6313-PR (1975).

Technical information related to the current status of projects in the major program areas of each contributing group is presented in sufficient detail to permit the informed reader to assess scope and significance and to obtain specific information from the publications for 1976 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 LASL Health Division staff will be pleased to provide further information. Inquiries should be directed to the individuals identified throughout the report at the address shown, or they may be reached by telephone by calling 505-667-4281 or 505-667-4218 (FTS 8-843-4281 or 8-843-4218).

OUTLINE OF PROGRAM

The objective of the Los Alamos Scientific Laboratory's (LASL) health and safety research is to serve the nation in areas of developing energy technology where unique capability exists and where disciplines complement basic weapons responsibilities of the Laboratory. In past years, effort was focused largely on problems associated with effects of external and internal radiation. Although the current work addresses a much broader base of environmental health and safety issues in energy technology, the disciplines and methods developed in previous years provide a strong, quantitative foundation for work in nonnuclear areas. The same effects and approaches prevail, and dose-response relationships provide the basis for assessing the hazards associated with exposure to nonnuclear agents. Thus, the quantitative aspects of current inquiry serve to support the basic requirements of technology development by placing hazards in perspective and distinguishing between real problems of toxicity and those perceived to be problems without demonstrable basis.

The strategy for approaching contemporary problems is based on long-standing and well-developed capability in the areas of industrial hygiene and occupational health. This Laboratory takes the position that major consequences of developing technology will be seen first in the work place. An appraisal of industrial practices, sampling, and analysis of process streams and fugitive emissions provides the basis for subsequent evaluation of

environmental and health impacts and identification of areas requiring specific environmental control technology. Recognition of the vulnerability of the work force and its protection by appropriate controls, in turn, provides a significant degree of protection for the public-at-large. Once the dimensions of a problem have been established, recommendations can be made on exposure limits, alternative processes can be evaluated on the basis of acceptability of environmental health and safety risks, and technology development can proceed with optimum economic efficiency.

The Environmental Health and Safety Program of the LASL is now being conducted by five Laboratory Divisions and spans all major funding categories of the ERDA Administration for Environment and Safety (AES). This report summarizes those aspects of the program being pursued by the technical staff of the Health Division. Research in the Chemistry-Nuclear Chemistry, Chemistry-Materials Science, Energy, and Medium Energy Physics Divisions is reported elsewhere. Health Division groups involved are Mammalian Biology (Group H-4), Industrial Hygiene (Group H-5), Environmental Studies (Group H-8), Cellular and Molecular Biology (Group H-9), Biophysics and Instrumentation (Group H-10), and Organic and Biochemical Synthesis (Group H-11). The table of organization shown below identifies the LASL program managers to whom detailed inquiries regarding specific projects can be directed.

TABLE OF ORGANIZATION OF THE LASL HEALTH DIVISION (DECEMBER 1976)

A. Mammalian Biology

Concern for potential health hazards of trans-uranium elements and lack of confidence that current health protection standards are sufficiently conservative are major obstacles to accelerated nuclear power development. Although the industry has in excess of 30 yr of experience, no compelling evidence for carcinogenicity has appeared in exposed populations, and attempts to estimate the carcinogenic risk of plutonium particles in the lung have been initiated in experimental animals in conjunction with a tissue analysis program and expanded epidemiological studies of plutonium workers.

Plutonium-bearing ceramic microspheres have been administered by intravenous or endotracheal routes, and ^{210}Po sols have been insufflated for the purpose of comparing localized "hot" particles and diffuse irradiation on lifetime tumor incidence. Evidence obtained indicates substantially greater risk from diffuse exposure than from intensely radioactive particles irradiating discrete segments of lung parenchyma with comparable total doses. Preliminary descriptions of an expanded toxicology program dealing with hazardous agents of the developing oil shale industry appear for the first time in this report. Approximately half of the group effort now is devoted to nonnuclear toxicity problems.

B. Industrial Hygiene

Inhalation is the most likely and potentially hazardous route for exposure to environmental pollutants, and developmental studies of filter efficiency, particle production characterization, and resuspension are being conducted to define proper containment conditions in the work place. To date, these studies have emphasized containment and characterization of radionuclides but provide the basis for investigating a broader spectrum of industrial pollutants. To determine the extent of plutonium exposure in man, several coordinated studies also are being conducted to measure lung burden distribution in occupationally exposed populations, the amount of plutonium in tissues from the general population, the health of LASL plutonium workers, and assessment of the health impact of plutonium in the environment.

C. Environmental Studies

The Western states and Alaska provide major energy resources and the consequent potential for major environmental impacts related to increased energy production. Ecological aspects of a variety of energy-related activities are being investigated including the trans-Alaska pipeline, Four Corners coal utilization, uranium tailings rehabilitation, the dry geothermal energy demonstration, and radioecological surveys in northern Alaska, Los Alamos and environs, and Trinity Site. Meteorological transport models applicable to the complex terrain of the Western states are being developed using the Los Alamos vicinity as a preliminary test locale.

D. Cellular and Molecular Biology

Model DNA polymers have been employed to measure physico-chemical effects of X irradiation and the influence of known base sequences on the transcription by RNA polymerases. These experiments allow quantitative estimates of the fidelity of transcription in the presence of physical and chemical agents. Cells in culture provide the basic system for studying radiation effects on DNA synthesis, organization of DNA in the nucleus, effects of pollutants on genetic information transfer and gene expression, nucleic acid structure, proliferation capacity, histone phosphorylation, and chromatin structure and function. The biochemical properties of the cell surface have been characterized. The use of flow systems to provide rapid karyotype analysis has been established for relatively simple karyotypes, and a series of cell-cycle-dependent, temperature-sensitive mutant mammalian cell lines have been derived and appear useful for cycle progression and mutagenesis studies.

E. Biophysics and Instrumentation

Rapid single-cell analysis techniques are now routine and have materially expanded the complexity of feasible kinetic experiments. New instrument improvements include multiparameter sensing, an improved light collection system, and the development of a flow-system multiangle light-scattering photometer capable of sensing viable (unfixed and unstained) cells. Parallel developments have been

made in fluorescent dye evaluation and adaptation of dispersion methods to include investigation of ascites and solid tumor cell kinetics. Diagnostic applications include isolation and differential characterization of leukocytes and evaluation of kinetic responses of tumor cells following conventional radiotherapy and exposure to negative pions.

F. Organic and Biochemical Synthesis

Syntheses of biologically and environmentally important tracer compounds utilizing stable isotopes rather than radioisotopes permit investigations in diagnostic medicine and large-scale environmental studies impossible to conduct using radioactive counterparts. The LASL has been designated a National Stable Isotopes Resource, and an inventory of labeled compounds is available. Upon appropriate application to the Stable Isotopes Resource, compounds of interest in specific studies can be provided or synthesized.

In addition to the research activities of the Health Division groups, an active seminar program has been maintained. The LASL Fourth Life Sciences Symposium on "Mechanisms of Molecular and Cellular Toxicology," held September 28-30, 1976, was jointly sponsored by LASL and US EPA/DBER. The proceedings, which will appear in the June 1977 issue of the Journal of Toxicology and Environmental Health, provide an up-to-date summary of the metabolism, interactions, most useful experimental systems, and

repair of DNA damage occasioned by exposure of mammalian cells to environmental carcinogens and mutagens. The practical problems associated with extrapolation of experimental results from molecular, cellular, and intact animal systems to man and the implications of a developing interdisciplinary science are thoughtfully considered. The three final papers of the symposium constitute a valuable commentary on contemporary research philosophy in the life sciences (see Appendix B for a list of attendees and program).

As the specific AES goals have emerged from conferences, planning exercises, and requirements for answers to specific technology-related questions, an extensive third round of reprogramming has occurred in projects specifically able to respond. Thus, the next annual report will reflect different priorities and different descriptions of effort. Because many of the systems reported here adapt readily to toxicology associated with the work place, sufficient continuity in titles will be retained to permit the reader to follow the transition from the traditional focus to the newer projects. To translate planning into productive bench science is the immediate goal of the LASL Health Division. To use the results of that effort for the longer term assessment of health, environmental, social, and economic impacts of accelerated energy production is our target.

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INTRODUCTION

Research in the Mammalian Biology Group (H-4) was totally nuclear-oriented from its inception until FY 1976. Midway in the last fiscal year, a move was made to favor more closely some of the new ERDA goals and to reprogram certain existing capabilities into nonnuclear-oriented research projects. This phase shift was accomplished largely through a reduction in external radiation studies. With this program being phased out, a new project studying health effects of the oil shale industry spanning the spectrum of raw material to finished products and by-products was initiated. "Hot particle" research was selected as being the only true health effects effort that would be maintained in the nuclear field by Group H-4. The radionuclide metabolism program will maintain its capabilities and expertise, but attention will be focused on following retention characteristics of chemical elements

associated with fossil fuel energy development. Many of these elements have already been studied. Thus, within a few months, the group has begun a phase out reprogramming effort that, by FY 1979, should reflect an equal budget and personnel split between nuclear and nonnuclear research. The only non-ERDA program under the management of this group is that concerned with pion radiobiology, funded by the National Cancer Institute through the University of New Mexico. Because of the close association with our ERDA programs, reports on this work are being included this year only.

The newly developing research resulting from reprogramming is not only exciting and relevant to practical problems but will be very profitable scientifically. Immune model systems have been perfected that enable assessment of T- and B-lymphocyte number and function. This will help delineate the effects of carcinogenic agents on the immune

system -- information which is currently unavailable. Our models utilize the Syrian hamster and Wistar/Lewis rat and are adaptable to measuring immune perturbations resulting from the administration of or exposure to toxic substances. They will be used for testing a spectrum of substances ranging from compounds known to be carcinogenic to agents that represent potential hazards in the human situation (e.g., workers in the oil shale industry). Immune function is only one of several parameters that will be used in assessing the potential toxicity associated with oil shale mining and processing. The major route of study on oil shale will be inhalation by small laboratory animals. Hemograms, clinical chemistries and histochemistries, and lung cell karyotyping are a few of the ancillary measurements that will be made. The doses (respirable aerosol concentration x time of exposure) are being varied so as to result in a range of responses from acute death to long term chronic effects due to repeated daily exposure to the aerosols. [The inhalation studies could not have been carried out efficiently without the cooperation and intergroup liaison with the Industrial Hygiene Group (H-5). They have assumed most of the responsibility for aerosol preparation, generation, and characterization.]

As stated, the nuclear-oriented research in mammalian biology will soon be limited specifically to the "hot particle" program. It is anticipated and planned in budget submissions that the external radiation program will be essentially nonexistent by FY 1979. The last replication in the total dose, dose rate, and age at exposure study was begun in the spring of 1976. There should be few animals remaining in that study by the beginning of FY 1979. At that time, all animals from the first replication of exposures should be dead and a suitable means of analyzing the accumulation of multiparameter data perfected. Although some writing and data analysis are expected to continue into FY 1979 on this study, the effort should represent only a

fractional portion of a full-time employee and will not be budgeted through a specific budget request.

The "hot particle" issue will no doubt be of national interest for many years and should be maintained as a viable nuclear program. The observation of tumorigenicity by additional modalities of diffuse exposure has confirmed the suitability of the Syrian hamster as a model for the study of lung carcinogenesis. We have also observed that, when supplemented by intratracheal instillations of ferric oxide, the embedded hot particles give rise to a tumor incidence approaching 50%, compared to only a few percent when the ferric oxide was not given. This striking enhancement by an adjunct is of both practical and fundamental significance. Is an additional stimulus required for tumor induction by radioactive particles lodged in the lung? Culturing and subsequent karyotyping of lung cells from exposed animals have begun as an attempt to measure radiation damage which is not overtly expressed in vivo. Such studies will continue at a much increased pace and should yield useful results during the ensuing year.

The pion radiobiology program (preclinical) has progressed this past year, and our group has contributed noticeably to its success. As stated, although this is non-ERDA funded, two reports of its activities are being included in this year's annual report. Our portion of the overall effort in this important phase of high-energy particle radiology has been quite limited and will become more so with the loss from our group of the principal investigator who was associated with the pion program. Some very illuminating results on testes and rectum irradiation by both x rays and the pion beam have been obtained this past year and will be reported later.

The mammalian biology programs of the future appear vital and productive in general, and re-programming is well under way. The balance between operational funding, space, and personnel is perhaps better than in the past few years, but these three parameters are rarely in a steady-state condition.

✓ IMMUNOLOGY STUDIES

A Model System to Determine Toxic Effects on the Immune System

[D. M. Smith, P. A. Baron, G. A. Drake, P. M. LaBauve, J. E. London, and J. S. Wilson]

The immune system plays a pivotal role in the complex fabric of defense mechanisms that sustain life. Immunosuppression and/or incompetence have been established to be major factors in the pathogenesis of a multiplicity of human and animal disease processes. Cancer is one of the diseases characterized by defective immunofunction. Elucidation of events occurring in the immune system prior to and during tumorigenesis is especially important in view of recent alarming increases in deaths caused by cancer. Genetic, dietary, environmental, and occupational exposure factors undoubtedly are important in this increase, possibly by sublimating the protective functions of the immune system.

Clinical investigations and in vitro experimentation have produced valuable insights into the relationship of immune function and carcinogenesis. However, there is a dearth of information regarding the sequence of events in the afferent and efferent compartments of the immune system during tumor initiation and development in mammals with their integrated, interdependent organ systems that maintain homeostasis and ultimately life itself.

Considerable effort has been put forth in our Laboratory to select model systems for studying the effects of environmental contaminants of different energy sources and production by-products on the immune system.^{1,2} Two criteria were central in this endeavor: (1) the model(s) should parallel the human situation as closely as possible and (2) the study should provide a wide spectrum of information regarding as many facets of immune function as possible. To these ends, we have assembled model systems, using the rat and hamster, that provide a detailed insight into perturbations of both T- and B-lymphocyte numbers, functions, and interactions.

Measurement of ³H-uridine uptake provides an assessment of T-cell numbers in the rat. Significant progress is also being made in developing a meaningful, reproducible T-cell rosette formation test in the hamster.

T-cell function is being defined by delayed hypersensitivity skin testing with intradermally injected phytohemagglutinin (PHA) and erythrocyte cytotoxicity measurement. Abilities of T cells to undergo mitogenesis are examined in vitro with the mitogens PHA and concanavalin A (Con A); B-cell mitogens employed are lipopolysaccharide and pokeweed. B-lymphocyte numbers are established by erythrocyte-antibody-complement (EAC) formation, and B-cell function is ascertained by hemolytic plaque assays.

Methodology has been established so that the appropriate tests can be performed with lymphocytes from spleen, thymus, and peripheral blood. These models are especially appropriate and well worth the extra effort entailed with their use. All the aforementioned tests can be performed on a single animal and will facilitate evaluation and assessment of potential health hazards to humans by chemical agents, including those resulting from all phases of synfuel production and utilization.

We plan to continue our investigations relating potential immunosuppression to known chemical carcinogens and instillation of a variety of oil shale products into rats and hamsters, with subsequent monitoring of routine hematology and immunological functions. Application to the "hot particle" animals will be expanded into a much larger effort during the next year. It is felt that some of the puzzling results from this (hot particle) lung tumorigenesis program may find explanation through applying these models.

REFERENCES

1. D. M. Smith and R. L. Gross, "Selection of a Model System to Determine the Effects of Energy Production By-Products on the Immune System," Los Alamos Scientific Laboratory report LA-6238-PR (February 1976).
2. D. M. Smith, "Selection of a Model System to Determine the Effects of Energy Production By-Products on the Immune System," Los Alamos Scientific Laboratory report LA-6603-PR (December 1976).

Survival in Lethally Irradiated Mice Receiving Allogenic Fetal Liver and Thymus

[E. O. Goodrich, M.D. (Mobilization designee to the Medical Research, Development, and Acquisition Command, Office of the U. S. Surgeon General) and J. S. Wilson]

Our preliminary work has confirmed that survival beyond that observed in control animals receiving 950 rad occurs when suspensions of 18-day fetal hematopoietic tissue from allogenic sources are administered intravenously. Survival approaches 100% in syngenic restoration but in allogenic restoration varies with different H-2 combinations.

Using the RFM (H-2f) white mouse primarily as a host, the agouti C3H/HeJ (H-2i) and the black C57B1/6 (H-2b) as both donors and hosts, giving 10 000 000 to 20 000 000 fetal hematopoietic cells intravenously, survival ranged from 20 to 70%. The best combination for restoration was C3H/HeJ fetal liver cells grafted into RFM; the worst was C3H/HeJ into C57B1/6. Successfully grafted animals survived longer than the irradiated, untreated controls but frequently died from 20 to 60 days post-treatment. These studies lead us to suspect that, in the strains being used, chimerism is established but may be temporary and that the delayed deaths may be due to either graft vs host (GVH) or host vs graft (HVG) reactions. This point has not been established and appears to be the next logical area of investigation.

Cytotoxicity and mixed lymphocyte culture techniques currently being developed in this group should identify the source of the rescuing cells in

the survivors and define factors contributing to the hematopoietic restoration. Since fetal stem cells have already been used clinically in the treatment of severely immunodepressed children with evidence of chimerism, we believe this effort may point to broader use of fetal hematopoietic restoration techniques in the treatment of immunodeficient disorders and hematopoietic or radiation damage in humans.

TRACE ELEMENT METABOLISM

Retention of ^{109}Cd in Mice, Rats, Monkeys, and Dogs
[R. G. Thomas, J. S. Wilson, J. E. London, and G. A. Drake]

Several years ago, metabolic studies of cadmium in rodents were carried out using various routes of administration. None of the results have been published in the open literature and, as part of a series of multiroute and multispecies studies with soluble compounds of a variety of elements, data on cadmium in larger laboratory animals were needed for completeness. In the current studies, $^{109}\text{CdCl}_2$ in isotonic saline was administered to 4 female beagle dogs and 4 male rhesus monkeys (*Macaca mulatta*). Three of each species received single intravenous (IV) injections, and the additional subject was fed the isotope absorbed into a

TABLE I

EFFECTIVE MULTISPECIES RETENTION PARAMETERS FOR ^{109}Cd ($T_{1/2}$ IN DAYS)

	Number ^a	a_1^b	λ_1	$T_{1/2}$	a_2	λ_2	$T_{1/2}$	a_3	λ_3	$T_{1/2}$
<u>Intragastric (IG)</u>										
Mouse	8	98.8	1.73	0.40	0.7	0.027	25.7	0.5	0.0025	277
Monkey	1	Data being analyzed								
Dog	1	Data being analyzed								
<u>Intraperitoneal (IP)</u>										
Mouse	6	5.1	0.75	0.92	13.7	0.020	34.7	81.2	0.0023	301
Rat	6	100	0.0023	301						
<u>Intravenous (IV)</u>										
Mouse	11	7.4	1.40	0.50	60.5	0.017	40.8	32.1	0.0029	239
Rat	6	100	0.0033	210						
Monkey	3	27.2	6.93	0.1	72.8	0.0020	347			
Dog	3	100	0.0015	462						

^aNumber of animals in each group used for whole-body analysis.

^bPercent of total administered dose associated with the exponential component shown ($R_t = a_1 e^{-\lambda_1 t} + \dots$).

dog biscuit (canine) or sugar cube (Macaca). The intragastric (IG) dosage was administered approximately every 28 days for 1 yr. All animals were whole-body counted (88 KeV gamma rays from ^{109}mAg daughter) in a consistent geometry at 50 cm from a 12.7 x 22.9-cm diameter, thin-window, sodium iodide crystal. At sacrifice, tissues were obtained for ^{109}Cd analysis. Stable cadmium was administered to one of the IV dogs to observe any influence this might have on the tracer-level distribution and retention pattern.

Some of the raw data on the large animals are yet to be analyzed completely. However, there is enough information available to give a good inter-species comparison. Table I shows the kinetic parameters of retention that are available. After the last feeding to the monkey and dog, the whole-body retention was followed for about 6 wk. When these are analyzed, they will be included in the table.

Of greatest application to the recommendation of standards (MPCs) are the long-term retention constants in Table I. Although there appears to be variation between route for a given species, particularly the mouse, it should be remembered that the parameters have large statistical uncertainties. For instance, there is perhaps no real difference between the long-term mouse half-lives of 277, 301, and 239 days following IC, IP, and IV administration, respectively. It does appear certain, particularly in the case of the dog, that there is a trend toward longer half-lives of retention with increasing body weight. In fact, the IV dog shows an effective half-life which is essentially equal to the physical half-life of ^{109}Cd .

Tissue data from IP injection in the mouse are shown in Fig. 1 as percent initial body burden/g. These clearly indicate a gradual increase in kidney concentration (Fig. 1A), while other tissues (see Figs 1A and 1B) decrease with time out to 410 days post-injection. The data are expressed on a per gram basis on the assumption that this is proportional to the resulting injury. However, this assumption is apt to be much more valid with radio-nuclides than with toxic chemicals (stable isotopes). These data will be submitted for publication very shortly after analysis of a few more data.

Fig. 1. Tissue distribution of ^{109}Cd following intraperitoneal administration to mice. Data are expressed as percent of initial body burden/g and are corrected for physical decay.

Cesium-137 Whole-Body Content in a Normal New Mexico Population

[R. G. Thomas, E. C. Anderson, and C. R. Richmond (now at the Oak Ridge National Laboratory)]

Interest in ^{137}Cs as a global contaminant from atmospheric testing of nuclear weapons has continued since its presence in man was first identified by whole-body counting techniques.¹ Body burdens of ^{137}Cs in selected populations have been monitored for many years by several laboratories, and the behavior and fate of ^{137}Cs have been studied in a variety of organisms and ecosystems. Because of the long time span over which these measurements have been made, their continuation provides unique data of considerable ecological importance. A

comprehensive understanding of ^{137}Cs movement through the biosphere and its temporal variations in man is of particular importance as it represents the largest body of knowledge available on the subject of environmental contamination by a radioactive material and its ultimate deposition in man.

Approximately 40 normal Los Alamos Scientific Laboratory employees were whole-body counted periodically (weekly or monthly) during the years of 1956 through 1976. Two primary energy channels are used to analyze the body content of ^{137}Cs and ^{40}K . The counters (HUMCO I and HUMCO II) are 4π liquid scintillation types with a geometrical efficiency of nearly 100%.^{2,3} The subject remains prone in a cylindrical cavity 18 in. in diameter and 72 in. in length inside the scintillation fluid-containing vessel. Counting time is 200 sec, and two consecutive counts are recorded. All data are computer-analyzed and normally reported as pCi $^{137}\text{Cs/g}$ K. Each subject is weighed prior to counting so that the total mass of K/kg body weight can be compared.

During the summer of 1976, a total of 100 subjects were counted to gain an accurate insight into the present counter accuracy and ^{137}Cs distribution at the lower levels encountered under present limited atmospheric testing conditions. After remaining constant from 1969 through 1973, the levels of ^{137}Cs have fallen considerably during the past 3 yr, and there was concern that the counter (perhaps due to deterioration of fluid or phototubes) was giving inaccuracies. By counting this number of subjects over a short period of time, it was felt that many seeming inequities might be assessed.

The data from counting the 100 individuals this year are presented on a probability plot in Fig. 1 as pCi $^{137}\text{Cs/g}$ K. These are shown in this manner to indicate the normal distribution obtained when a large number of individuals from a heterogeneous population by weight, size, and sex are counted. Similar distributions were obtained for the body potassium and total ^{137}Cs contents. The mean values for these were 139 g and 1.3 nCi, respectively.

The $^{137}\text{Cs/K}$ ratios for the years 1956 to the present are shown in Fig. 2. Also indicated are time spans during which the "dirtiest" atmospheric

Fig. 1. Concentration of whole-body ^{137}Cs as pCi/g K. Data are plotted on linear cumulative probability paper to indicate their normal distribution.

Fig. 2. Concentration of whole-body ^{137}Cs as pCi/g K. Individual points were generally the mean of fewer than 100 counts during any one year.

weapons testing occurred. Data in Fig. 2 demonstrate the time-lag of ^{137}Cs buildup following the late 1950 and early 1960 atmospheric tests. The time to reach a peak level after each large series appears to be about 2 yr. The decline is at approximately the same rate as the increase, but there may not be an obvious correlation between the two. A leveling off occurred between 1969 and 1973, at which time another decrease occurred. Although most testing during this period was in the Southern Hemisphere, there was apparently enough fallout in the Northern Hemisphere to maintain this level. The decline from 1973 to the present stable levels probably represents the response to a reduced rate of atmospheric injection. The pattern and absolute

values presented in Fig. 2 agree very closely with those presented recently by Lewis *et al.*,⁴ which also show the decline that began in 1973.

The worth of such data as presented is not always obvious. However, obtaining the information such as was done at LASL in the summer of 1976 requires little time, effort, or money. The project will continue until such time that levels are too low to interpret or that the collection of further data appears meaningless. Correlations with environmental factors have been attempted in the past and will continue as part of the future effort.

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OIL SHALE TOXICITY

[L. M. Holland, M. I. Tillery (H-5), D. M. Smith, J. R. Prine, and R. G. Thomas]

Oil shale is an important potential source of energy which will eventually be exploited on a large scale as other sources of fossil fuel are depleted and when the associated technical, environmental, and economic problems are overcome. As the industry develops, there will be a parallel concern over the possible health hazards of oil shale and

its products. Most of the existing analytical data are addressed to the crude fuel content (caloric value) of the shale, and there are few reports about the kinds and amounts of organic materials present.^{1,2} Furthermore, the make-up of the retorted shale and the crude product varies with the type of retorting process employed. However, some type of mining and crushing operation is common to all methods, and industrial exposure to raw and retorted shale particulates will result.

Inhalation Experiments

Inhalation exposures of Syrian hamsters to both raw and retorted shale were begun in April 1976 and are summarized in Table I. The aerosol concentrations ranged from 13 to 150 mg/m³ respirable mass, and the exposure times varied from a few hours to several weeks. Aerosols were generated from a Wright dust feed after the material was reduced to a fine dust by ball-milling. All material used in the inhalation studies was obtained from the Laramie Energy Research Center (LERC). The raw shale was from Anvil Points, Colorado, and the retorted material was Anvil Points shale from the LERC 150-ton retort.

As a pilot experiment, half of the animals in Group 7-1-1 (Table I) were used for serial sacrifice in an effort to study early lung response and hematological values. Total white blood cell counts, differential counts, red blood cell counts, packed cell volumes, and hemoglobin values did not differ significantly from control animals during or after exposure.

Intratracheal Experiments

In an effort to deliver more precise doses and to enhance the response, several groups of

TABLE I

INHALATION EXPOSURE GROUPS (IN PROGRESS)

Experiment Number	Material	Concentration ^a (mg/m ³)	Exposure	Animals ^b	
				Experimental	Control
7-1-1	Raw shale - LERC ^c	13	4 h/day; 4 days/wk; 4 wk	25	12
7-1-2	Raw shale - LERC	33	4 h/day; 4 days/wk; 4 wk	54	20
7-1-3	Retorted shale - LERC	24	4 h/day; 4 days/wk; 6 wk	80	20
7-1-4	Retorted shale - LERC	150	14 h in 24	12	0

^aRespirable mass.

^bSyrian hamsters.

^cLaramie Energy Research Center.

TABLE II

INTRATRACHEAL EXPOSURE GROUPS (IN PROGRESS)

Experiment Number	Material	Dose (mg)	Frequency/Time	Animals ^a
7-1-7	Retorted shale -- Fischer assay	5	Daily for 4 days	20
7-1-8	Retorted shale -- LERC ^b	5	Once	6
7-1-9	Retorted shale -- Fischer assay	5	Weekly for 7 wk	20
7-1-10	Retorted shale -- LERC	5	Weekly for 7 wk	24
7-1-11	Retorted shale -- Fischer assay	5	Weekly for 10 wk	20 ^c
7-1-12	Raw shale -- LERC	5	Daily for 4 days	25

^aSyrian hamsters.^bLaramie Energy Research Center.^cRats.

animals were exposed to shale material by intratracheal instillation as summarized in Table II. The ball-milled raw and spent shales prepared for aerosol generation were suspended in saline and delivered by direct tracheal intubation. In addition, two groups of animals were treated with Fischer assay spent shale obtained from TOSCO Corporation of Golden, Colorado. With the exception of one group of rats to be used for immunology studies, all animals were Syrian hamsters.

Animals from Groups 7-1-7 and 7-1-8 (Table II) were used to study the uv fluorescence of the

organic material in shale as a means of identifying and locating the material in histological sections. Our first observations suggest that this technique may be useful for studying macrophage response and the mechanisms responsible for foreign body elimination (Fig. 1).

Results and Discussion

Except for those sacrificed for specific purposes, most of the animals in these early groups are still alive and definitive results cannot be reported. Early histopathology observations on the inhalation animals have shown a minimal response to

Fig. 1. Ultraviolet fluorescence of processed (spent) oil shale in a hamster lung section. The material was from the LERC 150-ton retort and was administered by intratracheal instillation.

Fig. 2. Epithelial reaction to intratracheally administered oil shale. The large oil shale particles are indicated by arrows.

oil shale, with a slight increase in macrophage activity being the only change noted. When the material is given in large amounts by direct tracheal intubation, macrophage involvement is more pronounced and one animal had a pulmonary epithelial response resembling bronchiolar adenomatoid proliferation after only 2 doses of spent shale (Fig. 2).

Planned Experiments

With acquisition of new aerosol exposure chambers in early 1977, long-term inhalation exposures will be initiated. Using the threshold limit value (TLV) for coal dust (2 mg/m^3) as a base or an assumed TLV for oil shale of 1.5 mg/m^3 respirable dust,³ exposure groups extending out to life span and up to 100 times the TLV will be started. Intratracheal and inhalation studies using silica dust as a fibrogenic control are already under way, and experiments involving crude shale oil are planned. Experiments using intestinal tract exposure, skin application, and eye irritation will also be initiated.

Fibrogenesis and carcinogenesis are the end points of primary interest and will be studied by the standard methods (e.g., histopathology, histochemistry, hematology, and blood chemistry). A model for testing immune function has been developed at LASL⁶ and will be applied to the oil shale toxicology effort. Our experience with trace element metabolism will be especially useful for the trace mineral problem per se, as well as for studying tissue distribution and translocation. Whole-animal mutagenesis experiments utilizing cytogenetic techniques and dominant lethal assay (DLA) procedures are already under way.

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Histochemical Evaluation of Lung Tissue

[J. L. Dodd and D. M. Smith]

Histochemistry is a useful tool with which enzymes can be observed at their sites of activity in tissue. Enzyme changes occur as integral components of disease pathogenesis, including tumor initiation and growth. Histochemistry can facilitate means by which the transition from normal to abnormal (e.g., carcinogenesis) can be studied. This methodology additionally might be used clinically in corroboration with biopsy procedures to increase the probability of correct diagnosis. We are developing histochemical techniques to detect changes induced by pure carcinogens and by-products of oil shale technology on small animals (rats, hamsters).

Raw shale, shale oil products, and retorted shale all have potentially toxic constituents, including some that are carcinogenic. Therefore, it is important to know the toxicity (exposure limits) of these products and by-products so that the now infantile oil shale industry can incorporate the appropriate technology to minimize environmental and health hazards or, when toxicity is obscure, to relax some now stringent regulations. Early definition of any toxic manifestations is essential so that planners can intelligently engineer problem areas during the design phase before construction of retorting plants. It is hoped that, through careful application of enzyme histochemical procedures, changes in enzymological constituents culminating in disease will be elucidated.

Male Wistar/Lewis rats and Syrian hamsters of both sexes are to be used in the study. This group is subdivided into experimental animals, normal controls, and sham controls (saline instilled intratracheally). The experimental animals are to receive weekly 1 to 50 mg of Fischer assay spent shale (FASS) in saline. All animals will be euthanized for histochemical determination at systematic intervals. Main tissues of interest in this study are tracheal and bronchial epithelium and lung parenchyma. As the study progresses, enzymes from liver, kidney, and other tissues will likewise be examined.

After the tracheal, bronchial, and alveolar spaces are perfused with a matrix of OCT compound (Tissue-Tek) from Lab-Tek Products (via carefully

ligated tracheal intubation), the entire respiratory tract distal to the larynx is immersed in liquid nitrogen. Freezing continues until the tissue temperature has nearly equilibrated. The left lung lobe and trachea, including the bronchial bifurcation, are separated and retained. These tissues are stored in a freezer (-20°C) until sectioning. Each tissue is then mounted on a tissue cassette and sectioned with a microtome cryostat (-20°C) at 8-µm thickness (unless otherwise specified). The tissues are finally subjected to the following enzyme histochemical procedures.

Alkaline phosphatase.--Bancroft's technique for localization of this enzyme, incorporating simultaneous coupling with substituted naphthols, will be used because of the sharp localization and product it produces.¹ This enzyme is selected because of its inclusion in lysosomes.

Cytochrome oxidase.--Metal chelation techniques will be used to demonstrate this enzyme *in situ*.² This is a representative enzyme of the end respiratory process, oxidative phosphorylation.

5'-Nucleotidase.--This will be demonstrated using lead metal reduction for the indicator.³ Lead ions are chelated to the phosphate radical created when 5'-nucleotidase acts on its substrate, adenosine-5'-phosphate (AMP). 5'-Nucleotidase will be studied because of its role in energy mediation, levels of which might be changed during carcinogenesis.

Adenosinetriphosphatase (ATPase).--ATPase is a membrane-associated enzyme which will be studied using lead chelation. It is incorporated in the energy mediation pathway, resulting in formation of creatinine phosphate.⁴

Succinic dehydrogenase.--This converts succinate to fumarate in the tricarboxylic acid cycle (Kreb's cycle) and can be localized sharply using the ditetrazolium chloride-nitro-BT (NBT) method.⁵

Glucose-6-phosphatase.--Cryostat sections (16 µm) are required to demonstrate this enzyme by lead metal chelation techniques which were first described by Wachstein and Meisel.³ Glucose-6-phosphatase is intimately involved in the anaerobic glycolysis portion of glucose respiration which, in turn, leads into the tricarboxylic acid cycle.

After histochemical procedures are completed, coverslips are mounted using a water-soluble medium.

Slides are observed, graded, and photographed with light microscopy.

The techniques have already been developed and demonstrated in this Laboratory. More work is warranted in the application of histochemical negative controls (denaturation and specific enzyme inhibitors); however, some of these aforementioned techniques serve as positive controls in the study.

In the future, pure carcinogenic compounds will be substituted for oil shale products. It is hoped that, by using these compounds in tumor production, the true sequence of enzymatic change can be demonstrated for reliable base-line information.

Histochemistry has been used to study the biochemistry of diseases, including cancer.⁶ It is hoped that, through utilization of these techniques, certain conditions or combinations of conditions might establish a pattern during the onset of carcinogenesis. If these patterns are presented consistently in subsequent investigations of this type, a useful study and diagnostic tool could be developed.

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THE HOT PARTICLE PROGRAM

Comparative Tumorigenicity of Localized and Diffuse Lung Irradiation

[E. C. Anderson, L. M. Holland, J. R. Prine, D. M. Smith, and R. G. Thomas]

The primary objective of the "hot particle" program has been to measure directly the relative tumorigenicity of isolated, particulate alpha sources in the lung compared with diffuse irradiation of major fractions of the lung mass with

comparable amounts of radiation. The latter type of exposure corresponds with the usual experimental or epidemiological situation, whereas the former might result from inhalation of a few insoluble particles (e.g., of PuO_2). Criticism has been directed at the use of data derived from diffuse exposure for the estimation of particulate hazard. A straightforward response to such criticism would be experiments to establish relative toxicities of the two types of exposure, since extrapolation of this ratio to man would be less difficult than extrapolation of absolute tumorigenicity. (This is the approach adopted in the classic dog experiments at the University of Utah comparing the toxicity of radium, plutonium, and strontium.)

The model system chosen to represent the particulate case does not duplicate the complexities of a real inhalation exposure but provides a simplified approximation which gives better control and manipulability of the variables. Briefly, the alpha radioactivity is incorporated into very uniform, inert, and insoluble ceramic microspheres of high-fired ZrO_2 which are large enough (10 μm in real diameter) to lodge permanently in the capillary bed of the lung when injected into the jugular vein of small laboratory animals. The uniformity of the spheres ensures closeness in radiation intensity, their immobility simplifies the dosimetry and, coupled with their large size, permits ready identification of the microregion exposed. Previous annual reports have described the methodology and presented preliminary results.

The choice of the Syrian hamster as the primary experimental animal is justified by its

widespread use in chemical carcinogenesis studies. To obtain a reference measure of tumorigenicity of diffuse irradiation, we have exposed hamsters to the three different modalities summarized and compared in Table I. The first modality used was the method of Little¹ in which a colloidal sol of ^{210}Po in saline (without Fe_2O_3) was administered in weekly intratracheal (IT) instillations. As shown by Little^{2,3} and confirmed by our studies,⁴ the alpha activity is widely distributed throughout the entire lung. Clumping is minimal, and the mobility of polonium (effective mean residence time in the lung about 12 days) further distributes the alpha dose so that, to a first approximation, the entire lung is exposed in a diffuse but nonuniform manner. Using a dose of 120 nCi each week for 7 wk, the nominal exposure (total energy divided by total lung mass) is 3 krad. We have observed 24 lung tumors (12 adenomas and 12 malignancies) in 22 of the 65 hamsters exposed or $34 \pm 7\%$ -- in agreement with the results of Little.¹⁻³ Further experiments at other doses and with plutonium substituted for polonium are in progress.

In the second modality, ^{238}Pu was incorporated into ZrO_2 microspheres at a specific activity of 2.1 pCi/sphere, and 70 000 spheres were administered via the jugular vein. If the spheres were randomly distributed throughout 1 g of lung of density 0.2 g/cm^3 and if the alpha range in unit density material were 40 $\mu\text{g}/\mu\text{m}^2$ (4 mg/cm^2), then approximately 35% of the lung mass would be within alpha range of one or more of the spheres. [Nonrandom distribution will reduce this fraction, but the effect of lung structure⁵ will be to increase it.]

TABLE I
LUNG TUMOR INDUCTION BY DIFFUSE RADIATION EXPOSURE

Modality	Nuclide	Specific Activity (pCi/sphere)	Number of Spheres	Lung Burden (nCi)	Animals with Tumors/Total Animals	Tumor Incidence (% + S.D.)
Intratracheal sol	^{210}Po	N.A. ^a	N.A. ^a	120	22/65	34 ± 6
Intravenous spheres	^{238}Pu	2.1	70 000	140	17/153	11 ± 3
Intravenous spheres	^{147}Pm	450	50 000	22 000	20/54	37 ± 7
		70	300 000			
		450	10 000	4 000	17/94	18 ± 4
		70	50 000			

^aNot applicable.

The mean distance between randomly distributed spheres would be 140 μm compared with the alpha range of 180 μm in homogeneous material of density 0.2. It is clear, therefore, that, under these exposure conditions, the spheres are not producing isolated, noninteracting radiation fields and that tumorigenicity might be expected to resemble to some degree that of a truly diffuse irradiation. The nominal dose rate was 13 krad/yr, continuing for the life of the animal. We observed 19 lung tumors (14 adenomas and 5 malignancies) in 17 of 153 hamsters studied, or $11 \pm 3\%$. One-third of the exposures were supplemented by IT zymosan and some of these with 300 rad of whole-body x-irradiation. Since there was no significant difference in tumor incidence among the subgroups, they were combined for the present tabulation (see Table I). For the exposures to microspheres alone without supplements, the incidence was $10 \pm 3\%$. This is significantly lower than the $30 \pm 9\%$ observed for the ^{210}Po IT exposure. It is difficult to compare the radiation doses, since that from the ^{210}Po was essentially completed within 10 wk, whereas that from the microspheres continued for the life span of the animal. The first tumor from the microspheres was observed at 26 wk, after the animal had accumulated a dose of 6.5 krad. This is comparable with the 3-krad total dose from polonium.

In the third modality, ^{147}Pm (a soft beta emitter) was incorporated into microspheres. The beta spectrum has a maximum energy of 224 keV and an average energy of about 62 keV. The half-thickness of 5 mg/cm^2 is comparable to the 4- mg/cm^2 range of 5-MeV alphas, while the maximum beta range is 10-fold larger and, hence, exposes 10^3 -fold greater tissue volume. Thus, the promethium irradiation is effectively confined to the lung, but there is considerable overlap of the radiation fields of individual microspheres, and the exposure is more nearly uniform than either of the alpha exposures. The low specific ionization (LET) of the beta particles, of course, results in greater uniformity of energy deposition on the microscale as well. Spheres of specific activity 70 or 450 pCi/sphere were prepared and doses of 10 000, 50 000, and 300 000 administered via the jugular vein. Four experiments were done, one pair with lung burdens of

22 μCi (obtained with 50 000 and 300 000 spheres of the two specific activities, respectively) and one pair with lung burdens of 4 μCi (obtained with 10 000 and 50 000 spheres). There was no significant difference in tumor incidence between members of a pair; thus, they were combined in Table I. In the 4- μCi experimental groups, about half of the hamsters also received 1000 rad of x rays to the chest cavity. This did not significantly affect tumor incidence, and the data were pooled (see discussion of adjuncts in the following pages). The corresponding dose rates were 5 and 25 krad/yr. Both exposures enhanced the incidence of tumors. At the lower dose rate, 18 tumors (6 adenomas and 12 malignancies) were observed in 17 of 94 hamsters, for an incidence of $18 \pm 4\%$. At the higher dose rate, 31 tumors (8 adenomas and 23 malignancies) were observed in 30 of 89 animals, for an incidence of $34 \pm 6\%$. A fifth experiment using 6000 spheres at 0.07 nCi/sphere to give a lung burden of 0.4 μCi gave no tumors in 37 hamsters. All five results are plotted in Fig. 1.

The three diffuse modalities compared in Table I, of course, differ greatly among themselves in the characteristics and distribution of the radiation and in the duration, rate, and total intensity of exposure. For present purposes, it is encouraging, therefore, to note that they all induce significant numbers of tumors at frequencies of 10 to 30% for doses of the order of 10^3 to 10^4 rad. This proves the sensitivity of the model system and provides a rough index mark against which to compare the tumorigenicity of localized particle exposures.

Fig. 1. Dose-response curve for lung tumor induction by IV ceramic microspheres loaded with ^{147}Pm .

TABLE II
LUNG TUMOR INCIDENCE IN LOCALIZED RADIATION EXPOSURE

<u>Modality</u>	<u>Nuclide</u>	<u>Specific Activity (pCi/sphere)</u>	<u>Number of Spheres</u>	<u>Lung Burden (nCi)</u>	<u>Tumors/Animals</u>
Intravenous spheres	^{239}Pu	0.07	2000	0.14	0/45
		0.22	2000	0.44	0/54
		0.42	2000	0.84	2/57
		0.91	2000	1.8	0/58
	^{238}Pu	2.1	2000	4.2	1/66
		4.3	2000	8.6	0/54
		13.3	2000	26.6	0/53
		59.4	2000	119	0/52
	^{239}Pu	0.22	6000	1.32	0/17
		0.42	6000	2.5	0/37
	^{238}Pu	4.3	6000	26	0/141
		59.3	6000	356	2/158
Controls	None	0	Various	0	3/220

Table II summarizes the results of experiments in which plutonium-loaded microspheres with specific activities ranging from 0.07 to 59 pCi/sphere (see column 3) were administered intravenously (IV) at either 2000 or 6000 spheres/hamster (see column 4). These results have been reported and discussed previously.⁶ The fraction of lung irradiated is 1 or 3%, respectively, for these sphere numbers, and the individual particles are far enough apart (560 μm for 2000 spheres) that most of them can be considered isolated foci of irradiation. The total lung burdens are given in column 5 of the table and range from 0.14 to 356 nCi. The corresponding nominal dose rates, calculated as if the total mass of the lung were irradiated, range from 13 to 33 krad/yr. When the calculation is made on the basis of the fraction of lung mass theoretically exposed by 2000 or 6000 spheres, the corresponding range is 1.3 to 1000 krad/yr. The tumorigenic dose rate of the 70 000-sphere exposure, therefore, is included within the range of the localized exposures. The wide range of particle specific activities was studied because of possible dependence of tumorigenicity on this parameter.⁷ However, in none of these exposures is the resulting tumor incidence significantly different from background in control animals (last line of Table II).

While tumor induction by localized exposure was too low to quantitate, it is clearly small compared with the incidence in "diffuse" exposures (Table I). This result would seem to discredit hypotheses which suggest an increased hazard from localized irradiation and, in fact, demonstrates that the opposite is true: localized irradiation is less tumorigenic than diffuse exposure.

A reduction in tumor incidence would result from a localized dose if tumor production were proportional to mass of tissue at risk. Such a proportionality would result, for example, if the rate-determining process were the occurrence of some improbable local event such as the malignant transformation of a single cell. In this case, it is clear that the more cells which are irradiated, the greater is the probability that a tumor will be induced. Since the exposures to 2000 and 6000 spheres irradiate only about 1/35 and 1/12 as much tissue as the 70 000-exposure, one would expect the corresponding tumor incidences to be about 0.3% and 1%, respectively, for 2-pCi spheres by scaling down from the productive experiment. These numbers are below the limit of detection in these experiments. If tumor incidence were a strong function of dose rate, one might still expect a detectable yield at some more propitious specific activity

other than 2 pCi. This is not observed, suggesting that either 2-pCi spheres deliver the most effective dose rate (as has indeed been suggested) or the variation is not great enough to be detected. In fact, the available dose-response curves for lung cancer (as summarized in WASH-1320, Figs. V-1 through V-4) show tumor yield to be insensitive to dose,⁸ as was also the case in our ¹⁴⁷Pm study (Fig. 1).

It is premature to speculate further on mechanistic implications, pending results of further experiments now in progress. The qualitative conclusion that localized irradiation is less tumorigenic than diffuse irradiation follows from the data now at hand.

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Adjuncts

Ferric Oxide and Pu/ZrO₂-Induced Pulmonary Carcinogenesis [D. M. Smith].--The discovery that adhering chemical carcinogens to ferric oxide (Fe₂O₃, "hematite") particles dramatically increases the

respiratory tract tumor incidence laid the foundation for modern lung carcinogenesis studies.^{1,2} It was thought initially that Fe₂O₃ particles served merely as "inert" carriers facilitating carcinogen transport across bronchial epithelium. However, more recent work suggested that Fe₂O₃ is in itself cocarcinogenic.³ This is in agreement with the findings of Steinback *et al.*⁴ that several dusts, MgO, Al₂O₃, and C, given intratracheally (IT), acted synergistically with diethylnitrosamine injected subcutaneously in the induction of respiratory tract tumors in Syrian hamsters.

Relationships between amounts and varying dose schedules of Fe₂O₃ and benzo(a)pyrene (BAP) and subsequent respiratory tract tumor induction have been reported by Saffiotti *et al.*⁵ One possible mechanism for Fe₂O₃ tumor enhancement, other than concentrating carcinogens and delaying their clearance from the respiratory tract,³ is that, when it was administered alone IT, cell turnover as evidenced by ³H-thymidine incorporation into respiratory tract epithelium was increased by a factor of 16 over controls 24 h after injection.⁶ Anything that increases DNA synthesis has the proclivity of acting as a cocarcinogen. However, Little *et al.*⁷ found no marked difference in the tumorigenicity of IT ²¹⁰Po sols with and without Fe₂O₃, suggesting that alpha radiation given diffusely may induce respiratory tumors via different mechanisms than chemical carcinogens. With these ideas in mind, we initiated an experiment to study the effect of Fe₂O₃ instillations on tumor induction in Syrian hamsters by alpha-active PuO₂/ZrO₂ microspheres.

Materials and methods for animal care, microsphere production, and injection were similar to those given elsewhere.⁸ The ZrO₂ microspheres had a ²³⁹Pu content of 1.1 pCi/sphere. A lung burden of 33 nCi resulted from 30 000 spheres administered either by IV injection into the jugular vein or by IT instillation. Approximately 15% of the total lung mass was irradiated. Fe₂O₃ (certified anhydrous, Fisher Scientific Company, Fair Lawn, N. Y.) was suspended via sonification (50 W for 3 min) with a Sonifier cell disruptor (Heat Systems Company, Melville, N. Y.) in 0.15 M NaCl and administered by IT instillation in doses of either 0.3 or 3.0 mg in 0.2 ml saline. Three schedules of instillation were used, beginning 1 wk after

microsphere administration: no instillation, one instillation, and 10 instillations at weekly intervals. Hamsters were allowed to live out their life spans and necropsied as soon as possible after death. Tissues for microscopic examination were prepared by usual techniques, sectioned at 6 μ m, and stained routinely with hematoxylin-eosin.

Tumor incidences are summarized in Table I which gives the ratio of animals with tumors to total animals and the percent incidence $\pm$ the S. D. as calculated for binomial statistics ($\sigma^2 = npq$), where n = number of animals, p = fraction with tumors, and q = fraction without tumors). Approximately one-third of the tumors were adenocarcinomas and two-thirds adenomas with induction times of 25 to 82 wk. Control animals receiving Fe_2O_3 once a week for 10 wk did not develop tumors (0/18 = 0 $\pm$ 5%) -- in agreement with the negative results of others.^{1,2,5,7}

Animals receiving IV spheres alone had a primary lung tumor incidence of 2 $\pm$ 2% and those given IV spheres and a single instillation of Fe_2O_3 0 $\pm$ 3%. Ten instillations of Fe_2O_3 increased the lung tumor incidence to 7/41, 17 $\pm$ 6% for 0.3 mg/wk, and 1/24, 4 $\pm$ 4% for 3.0 mg/wk.

TABLE I

EFFECT OF FERRIC OXIDE ON TUMOR INDUCTION BY
MICROSPHERES

	Animals with Tumors/Total Animals IV Spheres	IT Spheres
<u>No Ferric Oxide</u>		
	1/66 = 2 $\pm$ 2% ^a	4/73 = 5 $\pm$ 3% ^b
<u>Ferric Oxide (3 mg once)</u>		
	0/27 = 0 $\pm$ 3%	8/21 = 38 $\pm$ 11%
<u>Ferric Oxide (0.3 mg 10 times, weekly; 3 mg total)</u>		
	7/41 = 17 $\pm$ 6%	13/33 = 39 $\pm$ 9%
<u>Ferric Oxide (3.0 mg 10 times, weekly; 30 mg total)</u>		
	1/24 = 4 $\pm$ 4%	18/35 = 51 $\pm$ 8%

^aTotal burdens: 33 nCi (30 000 spheres $\times$ 1.1 pCi/sphere), 17 animals; 45 nCi (50 000 spheres $\times$ 0.9 pCi/sphere), 21 animals; and 54 nCi (60 000 spheres $\times$ 0.9 pCi/sphere), 28 animals.

^bTotal burdens: 33 nCi (30 000 spheres $\times$ 1.1 pCi/sphere), 17 animals; and 76 nCi (36 000 spheres $\times$ 2.1 pCi/sphere), 56 animals.

When the microspheres were given IT, 4/73 (5 $\pm$ 3%) hamsters developed lung tumors. Even a single Fe_2O_3 instillation raised the tumor incidence to 8/21 (38 $\pm$ 11%) -- in marked contrast to the lack of effect with IV spheres. Ten repeated instillations did not result in a significant additional increase in tumor incidence (13/33, 39 $\pm$ 9% for 0.3 mg/wk, and 18/35, 51 $\pm$ 8% for 3.0 mg/wk). Whether 0.3 or 3.0 mg Fe_2O_3 was instilled did not appear significant. These calculated incidences have not been corrected for intercurrent deaths but, since the survival curves were similar for the experimental groups, results should be comparable on a relative basis. It is concluded from these results that IT instillation of Fe_2O_3 enhanced tumor induction by both IV and IT Pu/ZrO₂ microspheres, that enhancement is greater for IT spheres, and that IT spheres may be somewhat more tumorigenic than IV spheres in the absence of hematite enhancement.

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Combined Inhalation and Injection [R. G. Thomas,

D. M. Smith, M. I. Tillery (Group H-5), and J. R. Prine].--Several postulates have been voiced for the reason why plutonium-containing particles lodged in the hamster lung by intravenous (IV) injection do not produce statistically significant numbers of tumors except in large numbers and at lung burdens on the order of 100 nCi/g. One such premise is that the isolated static particle does not irradiate a sufficient fraction of the lung mass and that the initiation of cancerous growth requires an insult to be delivered to a sizable volume of tissue. Another possibility (supported by the ferric oxide instillation results given previously in this report) is that some essential insult is lacking in a pure microsphere exposure and can be supplied by other (smaller) particulates. These possibilities can be tested by experiments now in progress which combine exposures to both static IV microspheres and inhaled aerosols in various proportions. By using polydispersed aerosols, it is possible to increase greatly the number of particles administered and to reduce the size and specific activity per particle while maintaining a given total burden of radioactivity.

Syrian golden hamsters (of both sexes) were used in these experiments to facilitate a direct

comparison with past and ongoing "hot particle" studies. Nine groups of animals were divided as shown in Table I. Because of accidental deaths during the injection or inhalation (INH) process, the numbers of animals at $t = 0$ in each group differed. The animals with combined INH and IV administrations received the injected particles 1 wk prior to the inhalation. The inhalation chamber and aerosol generation system were essentially the same as those used for small-animal experiments at the Lovelace Foundation.¹ The starting material in the nebulizer² was zirconium dioxide sol similar to that used in the process of manufacturing particles for IV injection.³ The desired amounts of plutonium and ⁵⁷Co were added to the sol in the nebulizer prior to generation of the droplets. The ⁵⁷Co was used as a tracer because of its pure gamma emission. Sampling was accomplished with a cascade impactor⁴ and electrostatic precipitator.⁵ A Zeiss particle analyzer was used to obtain the necessary values for computing the aerosol parameters. The median aerodynamic diameters were approximately 2 μ m, with a geometric standard deviation of approximately 2. Sacrifices for tissue distribution of the label were performed on 2 hamsters each at 1, 2, 4, 8, 16, and 32 days post-exposure in groups A-D. It was established that the label (⁵⁷Co) remains with them through the intramuscular (IM) injection of a resuspension of

TABLE I

APPROXIMATE NUMBER OF NANOCURIES OF PLUTONIUM IN THE WHOLE BODY

Group	Number of Hamsters	Number of Spheres	Injection (nCi)	Inhalation ^a (nCi)	Total (nCi)
A ^b	63	60 000	100	0	100
C ^b	63	30 000	50	20	70
H ^c	71	20 000	55	100	155
G ^c	62	20 000	55	65	120
B ^b	48	0	0	25	25
I ^c	61	0	0	140	140
Controls	57	IV + INH ⁵⁷ Co/ZrO ₂ , no plutonium			
	56	IV + INH ZrO ₂ , no ⁵⁷ Co or plutonium			
	80	No dosage			

^aValues of total body burden at 6 days.

^b²³⁹Pu incorporated into the ZrO₂ particles.

^c²³⁸Pu incorporated into the ZrO₂ particles.

aerosolized particles. In following IM-injected hamsters for 371 days, the best-fit first-order kinetic equation indicated a half-life in the body of approximately 250 yr. Tissue data from these IM animals indicated that essentially all label was in muscle. It is a general phenomenon that the label from such particles is solubilized to the extent of about 5 to 10% in the period shortly after entering the body, either by injection or inhalation. This finding was confirmed in these studies in that essentially no loss from the injection site occurred after a few days. A least-squares line through all whole-body data through 371 days indicated an intercept of about 96% of the injected dose; the average standard deviation about each point was calculated to be 5.5% of the mean.

Data from the tissue and whole-body measurements are still being analyzed. Upon death, histological examination is being carried out, and indications to date are that the tumor incidence is increased in groups H and I. Total tumor incidence in these groups is approximately 25 and 35%, respectively, with about equal numbers of carcinomas and adenomas. Group I (inhalation only) has shown 4 carcinomas, with about two-thirds of the animals yet to be processed for histopathological examination.

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Interaction between Systemic Alpha Exposure and Lung Exposure [L. M. Holland, J. R. Prine, D. A. Smith, and E. C. Anderson].--One of the principal characteristics of the use of ZrO_2/PuO_2 ceramic microspheres is that the delivered radiation dose is restricted to the lung. This is true whether the microspheres are given intravenously (IV) or intratracheally (IT)* and even if the number of microspheres is sufficient to expose most of the lung. There has been no evidence of solubility of isotope from the microspheres nor of transport of the spheres to other organs.

Intratracheally administered ^{210}Po differs from IV microspheres in several notable ways, including mobility within the lung, diffuse irradiation of the lung, brief residence time in the lung, and early translocation to other principal organs. The question of whether this exposure of other organs (liver, kidneys, spleen, lymph nodes, circulating blood) enhances the genesis of lung tumors by ^{210}Po has not been answered.

If ^{210}Po is administered by a parenteral route other than the trachea, such as intramuscular (IM), the alpha exposure of the lung is minimal and plutonium microspheres can provide an independent exposure of the lung. By varying the combinations of these two modalities, their interaction can be established. Three groups of experimental hamsters were exposed as shown in Table I. The first group, which received no lung dose, served as a treated control to identify the effects of polonium alone and for organ distribution studies. The second group received a lung exposure from IV ^{238}Pu microspheres in addition to the IM polonium, and the third group was the same as the second except that the lung exposure was from IT microspheres.

The total lung burden of ^{238}Pu (110 nCi) was chosen because our experience has shown this to be the most productive level for tumors with IV microspheres. The total body burden of ^{210}Po (500 nCi) was chosen because it approximates the liver dose in hamsters which received IT ^{210}Po and produced a 50% tumor yield.¹ Thomas and Stannard reported a rapid clearance from the lung after a single IT instillation of ^{210}Po in rats, followed by a gradual

* Any passage through the gastrointestinal tract following lung clearance is being neglected as a source of tissue irradiation.

TABLE 1

LUNG ONLY vs SYSTEMIC EXPOSURE

Lung Exposure ^a Route/Dose	Parenteral Exposure ^b Route/Dose	Number of Animals ^c
<u>Experiment 1</u>		
None	IM/500 nCi	100
<u>Experiment 2</u>		
IV/110 nCi	IM/500 nCi	100
<u>Experiment 3</u>		
IT/110 nCi	IM/500 nCi	100

^a2.8 pCi ²³⁸Pu/sphere x 40 000 spheres.

^b500 nCi ²¹⁰Po sol/0.2 ml saline.

^cSyrian hamsters.

build-up period of about 10 days in the liver, spleen, and kidneys.² They also found a slower lung clearance time after multiple instillations. They report that the liver of rats contained 0.7%/g of the delivered dose on the day of a single IT instillation with a 72-day half time. Stannard found that the rat liver contained 5%/g of the delivered dose on the day of a single IV injection with a 30-day half time.³ Our IM injections probably resemble more closely the IT results.

Since nearly all animals in the three experimental groups are still alive, no definitive results can be reported. These experiments may help answer the question of whether the irradiation of other principal organs is in any way connected with the increased tumorigenic efficiency of IT administered ²¹⁰Po vs the IV administered "hot particle."

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Zymosan and Zymosan Plus X Irradiation [L. M. Holland, R. G. Thomas, J. R. Prine, and D. A. Smith].--The reticuloendothelial system (RES) plays

a role in the defenses against invasion by foreign entities. An overt response by the RES is often observed when particulate foreign material enters the body. Zymosan (active ingredient glucan) is a 1-3 linked polysaccharide that acts as a powerful stimulant of the RES when introduced parenterally. This stimulation of RES cells or tissue macrophages could be a factor in tumor production by radioactive materials, since these cells play a role in localization and attempts at elimination by the host organism. When zymosan is administered by intratracheal intubation, there is a brief but reasonably profound response by pulmonary RE cells.

Two groups of hamsters were used to study the possible adjunctive effect of zymosan on irradiation of the lung by intravenously administered plutonium-bearing microspheres. The first group of 61 hamsters received a lung burden of 126 nCi (2.1 pCi x 60 000 spheres), and the second group of 35 hamsters received 6 nCi (0.2 pCi x 30 000 spheres). Both groups were given an initial zymosan treatment of 0.3 mg/day for 3 consecutive days, followed by a regime of 0.6 mg biweekly for 6 total treatments, as shown in Table 1. Eighteen of the animals in each group were also exposed to 300 rad of whole-body x irradiation 6 months after the last zymosan treatment.

Of the 61 animals in the first group (126-nCi lung burden), 8 died with primary lung tumors and, of these, 4 had received the x-ray exposure as well as zymosan. Two of the tumors were carcinomas, and the others were described as adenomas. One animal developed a systemic reticulum cell sarcoma, as well as a pulmonary adenoma, and two others exhibited only systemic reticulum cell sarcomas. Only 4 tumors of any type have been observed thus

TABLE I

MICROSPHERE/ZYMOBAN EXPERIMENT

Lung Burden (nCi)	Number of Animals Zymosan Alone	Number of Animals Zymosan + X-Ray
<u>Group 1</u>		
126	42	19
<u>Group 2</u>		
6	17	18

far in the second group (6-nCi lung burden), and only 2 of these, a squamous carcinoma and an adenoma, were primary in the lung. The other 2 tumors included a reticulum cell sarcoma and an undifferentiated carcinoma involving the adrenal gland.

The incidence of tumors (8 in 61 hamsters) in animals that received zymosan and 126-nCi microspheres (with or with x irradiation) averages 13% and has 95% binomial probability limits of 5.9 to 24.6%. This is not significantly different from the results of another experiment without zymosan in which hamsters received 168 nCi (2.1 pCi x 80 000 spheres). Tumor incidence in this experiment was 9 in 91, averaging 10% with 95% limits of 4.7 to 18.1%. Thus, it cannot be concluded that the zymosan treatment had a significant effect on tumor incidence. X-ray treatment was similarly without significant effect, the 95% limits being 2.7 to 22.4% for no x irradiation and 6.1 to 45.% with x irradiation. For the group with the 6-nCi lung burden, the incidence of 2 tumors in 35 animals has 95% limits of 0.7 to 19.2%, which is not significantly different from the incidence in control groups (no zymosan) of 3 tumors in 220 animals with limits of 0.3 to 4.3%.

These studies were begun to see if the limited tumorigenicity of alpha radioactive microspheres was due to the lack of some essential injury not supplied by the radiation. Zymosan does not indicate a positive effect, but this lack of significant enhancement is of considerable importance when taken in conjunction with the strikingly positive results with Fe_2O_3 reported above. A limit is imposed on the possible adjunctives which operate in a synergistic or additive manner.

Pathology

The MUMPS Animal Data System [J. R. Prine, C. A. Goad, D. M. Smith, and D. E. Jackson].--Reliable, accurate storage and retrieval of data from large numbers of animals subjected to gross and microscopic necropsy examination are a challenging endeavor and most efficiently managed by use of electronic data processing. The MUMPS (Massachusetts General Hospital Utility Multi-Programming System) language¹⁻⁴ has been adapted utilizing a DEC (Digital Equipment Corporation, Maynard, Mass.) PDP-11/40 computer. The programs in the system are

all designed for use by nonprogrammers, and all communication with users is interactive in a narrative mode.

There are 3 groups of data files presently: (1) experimental animal colony; (2) documentation of each experiment; and (3) pathology files. Data files for clinical chemistries and microbiology will be integrated with the system in the not too distant future. The animal colony files record information concerning sources and shipments of animals and usage to which animals in a shipment are designated, while the experimental files are currently used to store information about "hot particle," external irradiation, and toxicology experiments. Pathology files contain complete descriptions of gross and microscopic necropsy findings. As of January 1977, there are over 7500 cases in the system, with cross-reference identification and narrative comments for each case. The narrative comments entered for each case are checked word-wise against the master thesaurus. If a match is not found, the user is asked to confirm the correct spelling of the term which is then added to the thesaurus. This list provides a quick reference for terms which will yield data on a vocabulary search.

The data base is organized to accommodate search programs that will retrieve all information on the following: (1) A single experiment or group of experiments; (2) a search of the pathology files for a specific word or group of words such as "adenocarcinoma" or "liver focal necrosis with regeneration" (this type of search may be confined to a specific experiment(s), control group or all control groups or the entire pathology file encompassing a multitude of diverse experiments and species); and (3) a sort of any collection of data within any one of the 15 categorized items enumerated on the veterinary necropsy protocol such as weight, serial number, or availability of photomicrographs.

The experiment file is designed to extract automatically the date of death from the pathology file and, on request, generate and print out survival tables and curves. Search programs allow the retrieval of experiments by number, date of experiment, species used, route of administration, or material given.

TABLE I

VETERINARY NECROPSY PROTOCOL

NECROPSY NUMBER?

1. PROGRAM?
2. SPECIES?
3. SERIAL NUMBER?
4. PROJECT NUMBER?
5. EXPERIMENT NUMBER?
6. SEX?
7. SHIPMENT NUMBER?
8. WEIGHT?
9. DATE OF DEATH?
10. TYPE OF DEATH?
11. TISSUE SAVED?
12. GROSS PHOTOS?
13. PHOTOMICROGRAPHS?
14. RADIOGRAPHS?
15. MICROSCOPIC FINDINGS?

GROSS FINDINGS:

MICROSCOPIC FINDINGS:

The animal colony file provides documentation of the necessary acquisition records and experiments to which the animals are assigned. This ensures trace-back to each shipment of animals in the event of any disease problem. A clinical notes file is employed which is used to document any observations made during the course of an experiment. This file is indexed by date.

All animals committed to experiments are entered in the system with the information accorded in Table I, with the date of death, type of death, and facts relevant to any necropsy procedure held in abeyance until the animal dies or the experiment is completed.

A typical description of an experiment from the experimental file is given as Table II. In this study, Wistar/Lewis rats were exposed to retorted oil shale via intratracheal instillation. A description of this nature, almost instantly available from the system, is an invaluable adjunct to evaluation of data and any problems that arise during the experimental course.

Examples of the ability of the system to handle life span data are given in Figs. 1 and 2. In this experiment (program 1, project 12, experiment 42), hamsters were given hot particles, followed by

weekly administrations of intratracheal Fe_2O_3 . An excerpt from the complete pathology file printout for this same group of animals is given in Fig. 3.

In conclusion, a system has been assembled to store quickly and accurately and to retrieve and process large quantities of data from animal experiments. Advantages of this system include input and retrieval by random access of information in a narrative form, complete usage by noncomputer programmers, and cross-referencing between the major data files so that an animal or groups of animals can be followed completely from the time of arrival in the laboratory until complete analysis of the experimental data.

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Lesions Induced by Adjuncts to the Hot Particle Program [D. M. Smith and J. R. Prine].--As described in a previous section of this report, administration of "hot particles" has been supplemented with a variety of agents to help delineate the role of particulate radiation in tumorigenesis. Ferric oxide given intratracheally beginning 1 wk after either IV or IT injection of Pu/ZrO_2 microspheres dramatically increased the incidence of pulmonary neoplasms. The tumors morphologically were classic examples of either adenomas, adenocarcinomas, or mixed adeno-squamous carcinomas. As expected, Fe_2O_3 elicited prominent reactions in peripheral portions of the bronchial tree [i.e., proliferations of terminal bronchiolar epithelium (bronchiolar adenomatoid lesions or BAL) when given alone or in conjunction with hot particles]. These lesions were indistinguishable from those reported by Saffiotti *et al.*^{1,2}

When inhalation of radioactive particles was combined with IV injection of hot particles, a spectrum of lesions resulted including increased frequencies of neoplasia. The series of morphological changes (granulomas, fibrosis, etc.) known

7-1-11

14DEC76 1 EXPERIMENT TERMINATED AND ALL ANIMALS KILLED

Fig. 1. Tabulation of survival information.

Fig. 2. Survival curve of animals on a specific experiment.

29 76 1 SYR 12 42 3941 F 6874 83 8 JAN 76 DTY N N N Y

GROSS FINDINGS:
NGL

MICROSCOPIC FINDINGS:
LUNG-L MUCINOUS ADENOCARCINOMA, MICROSPHERES PRESENT, FE203 3+. TRACHEA LYMPH NODE-HEAVY DEPOSIT FE203, THYROID-STROMAL FIBROSIS.

23 76 1 SYR 12 42 3942 F 6874 121 7 JAN 76 D N H N N H

GROSS FINDINGS:
POSTMORTEM AUTOLYSIS NO TISSUE SAVED.

143 76 1 SYR 12 42 3927 F 6874 75 31 JAN 76 DTY N H N H Y

GROSS FINDINGS:
NGL

MICROSCOPIC FINDINGS:
LUNG-FE203, MUCINOUS ADENOCARCINOMA, THYROID-STROMAL FIBROSIS.

285 76 1 SYR 12 42 3940 F 6874 105 11 FEB 76 D Y N H N H Y

GROSS FINDINGS:
NGL

MICROSCOPIC FINDINGS:
LUNG-BAL 2+, FE203 3+, MICROSPHERES NOT FOUND POSSIBLY MASK BY FE203, TRACHEA, THYROID-NSL.

205 76 1 SYR 12 42 3924 F 6874 94 11 FEB 76 D Y N H N H Y

GROSS FINDINGS:
ADRENAL-LEFT, MASS 2 X 4 CM.

MICROSCOPIC FINDINGS:
LUNG-MICROSPHERES NOT FOUND, FE203, BAL 1+, HEMOSIDEROCYTES.
ADRENAL-POORLY DIFFERENTIATED CARCINOMA OF CORTEX.
HEART, KIDNEY, SPLEEN, INTESTINE-NSL.

257 76 1 SYR 12 42 3910 F 6874 56 20 FEB 76 DTY N H N H Y

GROSS FINDINGS:
NGL

MICROSCOPIC FINDINGS:
LUNG- R MICROSPHERES PRESENT OFTEN MASKED BY 3+ FE203, BAL 1+, L MUCINOUS ADENOMA, TRACHEA, THYROID-NSL.

291 76 1 SYR 12 42 3944 F 6874 131 23 FEB 76 DTY N H N H Y

Fig. 3. Printout of pathology file for the same group of animals.

collectively as "radiation pneumonitis" was seen not infrequently, whereas it is an extremely rare finding when hot particles are given by themselves.³

Zymosan instilled IT into hamsters with hot particles elicited an acute inflammatory reaction that was replaced in 3 to 5 days by a pronounced migration of macrophages into the bronchial tree. After zymosan instillations ceased, these lesions abated and respiratory tracts took on the appearance of those containing hot particles only, with no discernible changes morphologically.

Based on Stanton and Blackwell's⁴ observation that the incidence of primary lung tumors induced by methylcholanthrene was enhanced by administering fluorocarbons which resulted in areas of the lung being infarcted, hamsters were injected with difluorotetrachlorethane (DFTC) after they had received hot particles. Shortly after administration of DFTC, pulmonary infarcts resulted which were characterized by acute inflammation and necrosis distal to the infarcted vessels. As time elapsed, fibrous connective tissue was elaborated by fibroblasts in the infarcted areas. These scars gradually underwent contraction so that only a small portion of the lung was involved.

In this presentation, we have discussed only those changes that occur with supplemental insults to hot particles, as the primary lesions resultant from particulate radiation have been described in previous annual reports.

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Chromosome Aberrations in Syrian Hamster Lung Cells by Inhaled ²³⁸PuO₂/ZrO₂ Particles

[A. N. Stroud]

A cytological investigation of the chromosome damage to epithelial lung cells following inhalation of PuO₂/ZrO₂ aerosols was undertaken¹ to supplement other studies of the "hot particle" program. The experiment was designed to observe the induction of chromosome aberrations at different time intervals following inhalation exposure. This was accomplished by culturing explants of lung tissue. The experiment also examines the possibility that the high degree of radioresistance which has characterized the response of hamster lung to exposure to alpha-active microspheres may be due, in part, to the relatively quiescent state of the cells *in vivo*. That is, cells may have sustained potentially fatal damage from the irradiation of the genetic material, but this damage may not interfere with a viable degree of somatic function and is not manifest until the cells attempt to divide. If this hypothesis is true, then stimulating the cells into mitosis might activate damage and precipitate cell death. Any damage to the chromatin material concurrently would become apparent as chromosome aberrations. Such a situation would help explain the apparent anomaly of lack of histologically visible damage or loss of function in lung sections which clearly have static microspheres of high specific activity surrounded by normal lung tissue which must have absorbed krad doses of radiation.

In this study, the aerosol particles of ZrO₂ containing ²³⁸Pu were inhaled for 22 min by healthy 4-month-old Syrian hamsters of either sex. Initial lung burdens averaged 140 nCi, or about 1.4 µCi/kg body weight. The inhalation chamber and aerosol generation system were essentially the same as those used for small-animal experiments at the Lovelace Foundation.² Starting material in the nebulizer was the same zirconium dioxide sol used to manufacture spheres for intravenous injection. The desired amounts of ²³⁸Pu were added to the sol in the nebulizer prior to generation of the droplets. The aerosolized material had an approximate median aerodynamic diameter of 2 µm with a σ_g of 2.

Conventional aseptic techniques were used for growing lung epithelial cells *in vitro* for chromosome

analysis. After removal of one or both lungs of an animal at different time periods following exposure (24 h, 1 and 3 wk), they were fragmented into single cells. The cells were cultured in Leighton tubes on glass coverslips for 63 or 138 h. The media used for promoting rapid cell proliferation and early mitotic stimulation (after 63 h of cell cultivation) were formulated in our Laboratory. Chromosome analyses of presumably epithelial cells were performed after blocking cells in metaphase with Velban. Although one cannot distinguish between a fibroblast and an epithelial cell when in mitosis, many of the mitoses were seen among clones of epithelial cells which were identified as polygonal cells. For this reason, it was assumed that most, if not all, mitotic cells were epithelial in origin.

Table I gives a summary of the chromosome aberrations produced by ^{238}Pu microspheres after different times of cell cultivation. The mean of aberrations per cell was 0.18 ± 0.07 after 63 h of cultivation (87 h after a 22-min inhalation exposure). All aberrations were of the chromosome type (Figs. 1A and 1B), indicating that these cells were undergoing their first mitotic division. After 138 h of cultivation, the aberration frequency was 0.26 ± 0.035 per cell, with chromatid-type aberrations prevailing (Fig. 1C). The difference ($8 \pm 8\%$) is not statistically significant between the two irradiated groups. Translocations and exchanges were observed following mitosis, indicating that some chromosome repair was occurring.

During the explant operation, radioactive aerosol particles were transferred along with the

lung cells, as shown by the high density of alpha tracks in autoradiographs of typical areas of cell proliferation (Fig. 1D). It is not known whether the radioactivity was depleted or concentrated in the process; therefore, the relative dose rates in vivo and in vitro are unknown. This uncertainty will be eliminated in future experiments.

Chromosome analyses of lung cultures were not possible when the animals were sacrificed 1 and 2 wk following exposure, since the cultures had an extremely low mitotic index. The observed mitoses had extensive chromosome damage, and cell populations were found to be significantly smaller than at the earlier time of sacrifice (24 h). Cell death was apparent, and many dead cells had become dislodged from the coverslips on which they were growing. Retention of the microspheres in the cultures was demonstrated by the many alpha tracks produced with autoradiography, indicating that many cells were being irradiated continuously both in vivo and in vitro.

Relatively few lung tumors have been produced by microspheres lodged in the lung by intravenous injection and inhalation,³ yet at the cellular level one sees chromosome damage and cell death. The data suggest that (1) chromosome lesions are a combination of damage produced in vivo and in vitro; (2) repair of some sublethal damage can occur, but apparent residual damage remains after short-term exposures (87 to 162 h) and possibly after a longer exposure to microspheres; and (3) when cells are explanted, retention of ^{238}Pu microspheres in vivo for extended periods (1 wk or longer) resulted in irreparable chromosome damage, loss of proliferative

TABLE I

CHROMOSOME ABERRATIONS OF LUNG EPITHELIAL CELLS IN VITRO
FOLLOWING ^{238}Pu MICROSPHERE (140-nCi) INHALATION BY THE SYRIAN HAMSTER

Experimental Group	Cell Cultivation ^a (h)	Total Exposure Time (h)	Metaphases ^b		
			Number Scored	Number Abnormal	Frequency of Abnormal (%)
Control	63	87	36	0 ± 1	
Irradiated			28	5 ± 2	18 ± 7.0
Control	138	162	44	0 ± 1	
Irradiated			160	42 ± 5.6	26 ± 3.5

^a Inhalation of the microspheres was for a period of 22 min at 24 h before initiation of lung cell cultures.

^b Two slides from duplicate cultures were scored for each value. No mitotic figures were seen before 60 h after culture.

Fig. 1. Chromosome aberrations of Syrian hamster lung epithelial cells in culture 24 h after inhalation of 3.6 μCi of aerosolized ^{238}Pu microspheres. (A) Metaphase spread with a dicentric (arrow) after 63 h in culture (polyploid cell). (B) Metaphase spread with an isochromatid break (arrow) after 63 h in culture (the full complement of 44 chromosomes not present). (C) Metaphase spread with a chromatid break after 135 h in culture (arrow). (D) Plutonium alpha tracks in a 63-h lung culture exposed 3 days (round objects are resting cells). Magnification of (A), (B), and (C) $\times 2000$ and (D) $\times 1000$.

ability, suppression of mitosis, and cell death.

It appears from these observations that most cells with irreparable chromosome damage are affected in their normal growth and function and are unable to survive for more than one division; therefore, they would not be classified as potential cancer cells. Cells with reparable chromosome lesions (translocations, exchanges, etc.) survive and function with this residual damage. Nevertheless, after several divisions, the damage may be expressed as resulting in the cell becoming

genetically predisposed to malignancy. Cells that are being continuously exposed to sublethal amounts of radiation may become potential cancer cells in the late adult life of the animal.

In light of these early findings, it is postulated that, if the alpha particles were delivered locally to the lung by intravenous injection, as opposed to diffuse irradiation from inhalation, the type and frequency of chromosome damage would be different. Experiments to compare radiation-induced chromosome aberrations by the two different

administration routes will center on relating the degree of damage to the number and specific activity of intravenously administered particles.

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Dosimetry of ^{210}Po Instillations

[E. C. Anderson, G. A. Drake, J. E. London, and J. S. Wilson]

Colloidal sols of ^{210}Po have been shown to be effective tumorigens when instilled intratracheally into hamster lungs.¹ This method of administration permits accurate control of the injected dose, and the resulting wide distribution of alpha activity throughout the lung parenchyma^{2,3} results in virtually total (although not uniform) lung irradiation. Some aggregation of the colloid occurs so that the source is "particulate" in nature. However, the specific activity of the particles is very small (as judged by the resulting "stars" in autoradiographs), and their mobility (as evidenced by the short retention time in the lung) serves to disperse the radiation dose *in vivo*. Little has used absorption onto Fe_2O_3 particles to vary the degree of dispersion and has found no strong effect on tumorigenicity.⁴

This modality of exposure provides an important reference point against which to evaluate the tumorigenicity of "hot particle" microsphere exposures. It demonstrates the susceptibility of hamster lung to alpha-induced tumors and provides a measure of this susceptibility for nonlocalized irradiation of the entire lung. However, two complications must be kept in mind: the rapid translocation of polonium from the lung results in irradiation of other organs, especially the liver and kidneys, and the repeated weekly instillations necessary to maintain the lung

burden are an additional trauma which may be significant.

Our experimental protocol is similar to that of Little.² A colloidal suspension of ^{210}Po is freshly prepared immediately before each instillation by adding 10 μl of a $\text{Po}(\text{NO}_3)_2$ solution (acidity 1 M HNO_3) to 10 ml physiological saline. No Fe_2O_3 carrier is used. The final pH is estimated to be about 3.0. At 0.5 h after dilution, the instillations are begun. A group of 50 to 100 hamsters requires 1 to 2 h, and "dummy" doses are taken for assay before, during, and after the series. These show some decline in activity over the series, usually amounting to 10 to 30%. The average value is taken as the nominal dose. The volume administered is 0.2 ml/animal under sodium methohexital anesthesia.

The dummy dose (generally 20 μl) is assayed by injection into 10 ml of liquid scintillation counting solution (New England Nuclear Corp. Aquasol or Amersham-Searle PCS). Using high-gain low-threshold settings on a Packard Tri-Carb counting system, efficiency has been shown to approach 100% and to be insensitive to electronic changes and chemical quenching.

To determine polonium retention as a function of time, hamsters were serially sacrificed (4 animals per point) and the lungs dissolved in a commercial tissue solubilizer (New England Nuclear Protocol). The lungs were separated into 3 components to obtain information on the distribution of the activity: (1) the entire left lung; (2) the apical lobe and lateral lobule of the right lung; and (3) the remainder. Solution was accomplished by heating to 55°C overnight. Decolorization with benzoyl peroxide was unnecessary. Counting efficiency was determined by adding a known quantity of ^{210}Po and recounting and was generally greater than 90%. The slow decay of chemiluminescence required that the samples be refrigerated in the dark for 12 to 15 h before the count rate stabilized. No significant change was noted when counting was continued for another 24 h.

The results for lung retention following a single instillation are shown in Fig. 1. Each point is the average of 4 hamster values, and the bars give the estimated standard error of the mean.

Fig. 1. Retention in hamster lungs of ^{210}Po following single IT instillation (see text and Table I for discussion of the curves).

Fig. 2. Results of assay of 7 doses of ^{210}Po given at weekly intervals (the line has the expected decay half-time of 138.4 days).

Fig. 3. Retention in hamster lungs of ^{210}Po following the last of 7 weekly IT instillations (see text and Table I for discussion of the curves).

calculated from the scatter of the individual values. About 20% of the dose leaves the lung within 3 h (previous measurements showed 95 to 102% recovery at 1.5 h). The curve is clearly multicomponent, but the data are inadequate by themselves for precise resolution.

In another experiment, polonium was administered intratracheally 7 times at weekly intervals. For preparation of the instillation solutions, a constant volume was diluted from stock with the result that the administered dose declined with the decay rate of ^{210}Po . This is demonstrated in Fig. 2 in which the measured dummy doses are plotted as a function of time, and the visual "best-fit" line is drawn with a slope equivalent to a half-time of 138.4 days. The scatter of the data reflects the variation in dummy dose measurements (4 per point) resulting from the instabilities of hydrolyzing polonium sol. The dose schedule can be described adequately as decreasing with the ^{210}Po physical half-life from an initial value of 84 nCi. The observed lung retention as a function of time after the last of these 7 instillations is plotted in Fig. 3, in which it is arbitrarily expressed as fraction of a single dose of 84 nCi. [This normalization was chosen rather than setting the initial retention at unity to quantitate the accumulation of the 7 x 84-nCi (minus physical decay) total administered dose. Both biological elimination and physical decay are involved in determining the magnitude of the initial retention.]

To determine the total radiation dose to the lung, it is necessary to resolve these data into biological components so that the dose can be integrated over the preceding period during which the 7 doses were being accumulated and also over the period beyond 100 days by extrapolation. The relative values of retention are not precise enough to give a unique decomposition, but the absolute value of retention following the last instillation provides the needed resolution, as will now be demonstrated. Assuming independence between the components and among the several instillations, then for a single component, i , leaving the lung with a biological rate constant, λ_i , in weeks, the accumulation, s_i , immediately after the last of n instillations, expressed as fraction of a single

dose retained in that compartment, is given by the sum:

$$s_i = 1 + e^{-\lambda_1 i} + e^{-2\lambda_1 i} + \dots + e^{-(n-1)\lambda_1 i}, \quad (1)$$

since the successive times since instillation are 0, 1, 2, ..., (n-1) wk. The sum of a geometric series is:

$$S = a(1 - r^n)/(1 - r), \quad (2)$$

where a is the first term (in this case = 1), r is the common ratio between terms ($e^{-\lambda_1 i}$), and n is the number of terms. In this case,

$$s_i = (1 - e^{-n\lambda_1 i})/(1 - e^{-\lambda_1 i}). \quad (3)$$

Physical decay is taken into account by suitable adjustment of the sum of administered doses, since it operates over the entire duration of the experiment, whereas biological elimination operates only for the period over which a given instillation is in the hamster.

To calculate R , the total retention immediately after the last instillation, s_i must be summed over all biological components, with the weighting factors, f_i , accounting for the fraction allotted to each component:

$$R = \sum_i f_i (1 - e^{-n\lambda_i})/(1 - e^{-\lambda_i}). \quad (4)$$

The procedure to optimize the estimation of biological elimination rates uses the dependence of R on the f_i and λ_i given in Equation 4. We select an arbitrary but reasonable estimate for the rate constant (biological and physical) of the longer component (the data do not justify more than 2 components), make a graphical fit to the data of Fig. 3 at longer times, subtract this from the data, and fit the shorter component to the residuals. An estimate of the retention in each compartment at zero time is obtained from the intercepts of the 2 lines. The ratio of this extrapolated retention to the value of s_i calculated from Equation 3 gives the corresponding value of f_i . A number of such arbitrary pairs of components are acceptable in terms of their fit to the retention vs time curve when no restriction is placed on the sum of $f_1 + f_2$. This sum varies considerably with the assumed half-time of the longer component, as shown in Fig. 4.

Fig. 4. Total ^{210}Po retention calculated from Eq. (4) as a function of the assumed biological half time of the longer component (see text).

In fact, the sum cannot exceed unity, and the data from a single instillation (Fig. 1) show that, for the two components considered here, it lies between 0.8 and 0.9 (the missing activity being lost in the first few minutes after instillation). The only portion of the curve in Fig. 4 which is compatible with this restriction is that for which the biological half-time of the second component lies between 40 and 70 days. The corresponding f_2 obtained by extrapolation in Fig. 3 is between 0.04 and 0.02. The associated first component has a half-time between 8 and 10 days with $f_1 = 0.70$ to 0.90.

Within this range, parameters can be found which provide fairly good fits to both the single and 7 instillation results, but there are systematic differences between the two. The two curves drawn in Figs. 1 and 3 and labeled "C" and "D" have the parameters shown in Table I. Curve G is the better fit to the single instillation, while D is superior for the 7 instillation exposure. The difference is outside the estimated errors of the determinations and may represent a true difference in retention resulting from the different

TABLE I
PARAMETERS FOR RETENTION FUNCTIONS

Curve	Component 1		Component 2		Sum
	Half-Time (days)	Fraction	Half-Time (days)	Fraction	
D	8.5	0.832	47	0.036	0.868
G	9.8	0.720	47	0.037	0.757

experimental conditions. However, the two sets of parameters yield nearly identical radiation doses.

For dosimetric purposes, the area under the retention curve can be expressed as the equivalent exposure time, $\bar{t}$, over which the total administered actual dose (corrected for physical decay to the time of administration) would deliver the same integrated radiation exposure if the dose were invariant in time. [This equivalence is derived from the fact that

$$\int_0^{\infty} e^{-\lambda t} dt = 1/\lambda.]$$

Thus,

$$\bar{t} = \sum_i f_i / \lambda_i, \quad (5)$$

where λ_i is the total (physical plus biological) decay rate of the i th component. For the several acceptable fits, the value of $\bar{t}$ lies between 12 and 13 days.

Dividing the rate of alpha energy deposition by the total mass of hamster lung (including blood, which absorbs about half the energy), one calculates a dose rate of 0.27 rad/day per nCi/g. Using an average lung mass of 1 g, a $\bar{t}$ of 12.5 days corresponds to an integrated total dose of 3.4 rad/nCi.

This conversion factor is within the range of those used by Little¹ which appear to range from 1.4 to 4.0 rad/nCi, depending on the details of administration. In view of the experimental variation possible in both the instillation process and the preparation of polonium sol, a real variation in this factor would not be surprising. The appropriate dosimetric factor, therefore, should be based on the retention curve actually measured for a given experiment.

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EXTERNAL IRRADIATION

Chromosome Aberrations of Mouse Blood Lymphocytes following Protracted Irradiation with ⁶⁰Co

[A. N. Stroud and J. F. Spalding]

There is great concern about the environmental hazards of the cumulative effects of ionizing radiations and other pollutants, especially at low doses, on future generations of man. Chromosome aberration production is one of the most dramatic effects of ionizing radiation at the cellular level. Damage to the cell nucleus which contains the genetic material essential for life may result in aberrations of the chromosomal strands. New chromosome configurations may be produced and, in many cases, lead to mutations. True gene mutations induced by radiation may have far-reaching consequences for future generations if they occur in germinal tissue. Attempts to measure radiation-induced chromosome aberrations in human populations in terms of dose-effect relationships have been made on lymphocytes of humans primarily after exposure to diagnostic and therapeutic applications of x rays and radioisotopes in medicine; however, the biological effects of dose protraction by different dose rates and by the acute fractionation method on man have not been feasible, with the exception of a few nuclear accidents.

In the experiments reported here, chromosome damage to blood lymphocytes is being assessed for an ongoing life-shortening study with protracted ⁶⁰Co irradiation of C57Bl/6J mice.¹ At this time, lethality in the first replicate of the life-shortening study is approximately 10% of the animals exposed; thus, insufficient data are available to report on this phase. Chromosome studies using mice have been impeded because a reliable method for producing mitotic yields for statistical analysis has not been available. We have developed a reproducible in vitro method whereby 200 to 5000

lymphocytic mitotic cells can be analyzed in metaphase with a small amount of mouse blood (0.1 ml).² The mouse has 40 chromosomes, all of which are telocentric in the normal configuration and which are only recognized as distinct entities in metaphase. For these studies, 100 to 200 well-spread metaphase chromosomes on 2 to 5 slides were analyzed for radiation-induced chromosome aberrations. Controls were scored in the same manner.

Animals for this study were irradiated at selected distances from a gamma-ray point source. The wide range of doses and dose rates involved in the experimental design are described in a previous report.¹ Five doses and 5 dose rates were used for each exposure. The total whole-body doses ranged from 20 to 1620 rad, delivered at dose rates ranging from 0.7 to 25 rad/min. The first chromosome analysis on blood lymphocytes of controls and irradiated animals was made within 6 h of completion of a protracted dose regime. Thereafter chromosome analyses were made at 24 h and 1, 2, 4, 8, and 16 wk following exposure.

Preliminary results indicate that (1) mouse blood lymphocytes are very radiosensitive since chromosome damage is seen with doses as low as 20 rad delivered at 0.7 rad/day (Fig. 1); (2) chromosome aberration frequency is related to both dose and dose rate and is inversely related to the length of time required to give a unit dose of irradiation; (3) extending the time required to deliver a dose of irradiation results in fewer aberrations, probably because over a longer period of time cells with irreparable lesions are incapable of a second division; (4) the types of chromosome aberrations observed indicate that repair and cell death are also taking place; and (5) aberration frequency does not approach control values 16 wk after irradiation, probably because the cell renewal systems which contain cells with lesions are still reacting against cell loss.

It is anticipated that this study will be completed at the end of fiscal year 1977 and that the results of this experiment will lead to the study of

Fig. 1. Mouse blood lymphocyte metaphase chromosomes. (A) Normal chromosomes with a complement of 40. (B) Radiation-induced chromosome aberrations following ⁶⁰Co, 20 rads at 0.7 rad/day (24 h after exposure ended). Arrows point to a chromatid break and a centromere break.

the effects of other environmental pollutants on chromosome damage.

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Partial-Body Irradiation of Mice

[J. F. Spalding, J. E. London, J. R. Prine, D. M. Smith, and R. F. Archuleta]

Studies have been done on the late effects or persistent radiation injury in partially exposed animals;¹⁻⁵ however, life shortening and response to general stress of mammals in which all osseous tissue was either exposed or shielded from conditioning x-ray doses have not been reported. Delayed radiation injury in gut-shielded and gut-exposed mice as measured by a reduction in mean after-survival (MAS) in a continuous gamma-ray field (dose rate of approximately 50 rad/day) was reported earlier.⁶ In these earlier studies, persistent injury reflected as a bone marrow decrement in radiation resistance was observed in animals with x-ray conditioning doses to the gut tissue only and with gut tissue shielded. The injury was observed to be approximately linear with conditioning dose given; based on a unit weight of tissue exposed (g/rad dose), the gut injury (as a reduction in MAS) was equivalent to that of whole-body and osseous tissue-exposed animals. This communication reports the preliminary results of a study designed to determine the amount and nature of long-term radiation-induced injury in gut-exposed and gut-shielded animals in terms of diseases which result in life shortening. The following observations suggest that radiation injury which results in life shortening is provoked by the irradiation of gut tissue, as well as osseous tissue.

Eleven groups (24 mice/group) of strain RF/J female mice about 90 days of age were x-ray conditioned as shown in Table I. Conditioning doses of x rays were given at approximately 200 rad/min in 200-rad fractions at 7-day intervals. The last fraction in each dose group was given on the same

TABLE I

TOTAL X-RAY CONDITIONING DOSES TO WHOLE-BODY AND AREA-EXPOSED MICE

Group ^a	X-Ray Treatment	
1	0 dose	Control
2	1200 rad	Whole-body
3	800 rad	Whole-body
4	400 rad	Whole-body
5	1200 rad	Gut only
6	800 rad	Gut only
7	400 rad	Gut only
8	1200 rad	Gut-shielded
9	800 rad	Gut-shielded
10	400 rad	Gut-shielded

^aAll mice were anesthetized for the same number of times whether or not they were exposed to x irradiation.

day. Thus, 800-rad group exposures were started 2 wk after the 1200-rad group and 400-rad groups 2 wk after that. Restraint techniques used to make selective *in situ* body area x-ray exposures were reported previously.⁶ Thermoluminescent dosimeters (TLD) were used to determine the doses attributable to scatter under the 6.35-mm lead shield. These TLD readings were insignificant and were assumed to be representative of scatter in the exposed animal. Twenty-four mice were quick-frozen while under anesthesia in the exposure jigs, exposed, and shielded areas dissected at the delineation of the lead shield to determine the weight of the specific fraction of the body exposed under the two shielding conditions (gut only and gut shielded). These observations are shown in Table II. Following the last conditioning exposure, control and experimental mice were housed in box-type cages where they remained until death. Complete necropsies, including gross and microscopic examination of tissues, were performed as the animals succumbed.

Life shortening in the various treatment groups (Table II) is shown in Table III. The mean life span of the sham control group was 446 days. This compares well with 452 days for strain RF/J reported at Bar Harbor,⁷ which was the source of the mice used in this investigation. Mean life span was shorter in all x-ray conditioned mice including the whole-body, gut only, and gut-shielded groups. Life

TABLE II

BODY, ORGAN, OR TISSUE EXPOSED TO X-RAY CONDITIONING DOSES (WEIGHT IN PERCENT)

Group	Whole-Body	Stomach	Gut	Bone	Kidney		Liver	Spleen	Pelt
					Right	Left			
Gut-shielded	74	5	2	100	100	77	53	15	66
Gut-exposed	26	95	98	0	0	23	47	85	34

TABLE III

MEAN LIFE SPAN OF X-RAY CONDITIONED RF/J STRAIN MICE

Conditioning Treatment	Life Span	Reduction in Life Span	
		(%/100 rad of exposure)	Corrected to Whole-Body ^a Exposure
Sham Control	446	-	-
Whole-Body			
1200 rad	261	3	-
800 rad	259	5	-
400 rad	334	6	-
Gut Only			
1200 rad	328	2	9
800 rad	400	1	5
400 rad	426	1	5
Gut-Shielded			
1200 rad	282	3	4
800 rad	328	3	4
400 rad	333	6	8

^aGut-exposed x 4.00; gut-shielded x 1.33.

span was usually shorter in mice with larger conditioning doses within each exposure condition. However, when percent life shortening per 100 rad of conditioning dose is tabulated, whole-body and gut-shielded conditioned groups showed decreasing effects per rad with increasing total doses, while gut-exposed animals showed an increase in effects per rad as the total dose was increased. This difference in response may be more closely related to total tissue exposed than to type of tissue exposed.

Death distributions for x-ray conditioned groups are plotted in Figs. 1A, 1B, and 1C as cumulative deaths in percent vs survival time in days. Lethality curves of whole-body and gut-shielded conditioned animals are generally steeper than that of control mice. However, slopes for gut-conditioned

Fig. 1. Comparative survival curves of control and x-ray conditioned strain RF/J mice. (A) Control and whole-body conditioning doses of 400, 800, and 1200 rad. (B) Control and gut-shielded conditioning doses of 400, 800, and 1200 rad. (C) Control and gut conditioning doses of 400, 800, and 1200 rad.

mice were more nearly similar to that of control mice, showing shallower slopes and greater variances.

The mouse strain used (RF/J) was obtained from the Jackson Laboratory, and the incidence of leukemia at death in untreated mice at that laboratory is reported to be 43%.⁷ The frequency of lymphoproliferative disease in control and x-ray conditioned mice in this study is tabulated in Table IV. The incidence of lymphoma in the sham control group (38%) is in close agreement with the findings of Yuhas.⁷ Tumor incidence at death is shown in Table IV in percent, the age range of deaths at which the tumors were observed, and the mean age at death

TABLE IV
LYMPHOPROLIFERATIVE TUMOR INCIDENCE IN X-RAY
CONDITIONED RF/J STRAIN FEMALE MICE

Conditioning Treatment	Tumor Incidence			Mean Life Span
	(%)	(age range)	(mean)	
<u>Sham Control</u>	38	270-483	403	446
<u>Whole-Body</u>				
1200 rad	61	228-409	287	261
800 rad	79	226-426	260	259
400 rad	43	290-476	340	334
<u>Gut-Exposed</u>				
1200 rad	63	243-407	325	328
800 rad	50	238-489	385	400
400 rad	47	260-534	379	426
<u>Gut-Shielded</u>				
1200 rad	56	228-315	273	282
800 rad	47	264-434	352	328
400 rad	67	227-487	342	333

for animals in each group which were observed to have lymphoma at time of death. Tumor incidence in all x-ray conditioned groups was greater than in the control group but was not consistently dose-related. The mean age at death of control and conditioned animals with lymphoma is compared with mean life span of each respective group. A comparison of these mean values points to lymphoproliferative disease as the major cause of death (life shortening) in conditioned animals (Table IV).

The above observations have led to the conclusion that, in a mammalian species with a normally high incidence of lymphoproliferative disease, (1) persistent radiation-induced lesions (or changes) are intimately associated with development of neoplastic diseases; (2) the incidence of the disease may be somewhat dose-dependent when most of the body is exposed; and (3) protective shielding of all osseous tissue does not prevent radiation induction of the life-shortening lesion. It is of interest to note that conditioning doses to the gut only (25% of body weight) resulted in a percent tumor incidence at death similar to whole-body conditioned and bone tissue (75% of body weight) conditioned animals (Table IV, column 2).

Some mice involved in this study are still alive. However, the number is small and should not significantly affect the findings of this report. The above experimental regime is also being performed on strain C57Bl/7J mice which normally have a zero incidence of leukemia at death.

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PION RADIOBIOLOGY

Effects of Negative Pions and X Irradiation on Testes Weight Loss and Spermatogenic Stem Cell Renewal in Mice

[L. S. Gomez, D. M. Smith, J. F. Dicello, H. I. Amols, and E. C. Rivera]

Two of the many end points which have been used to study radiation effects on the mammalian testis include testes weight loss¹⁻⁴ and spermatogenic stem cell regeneration.⁵⁻⁷ In this study, these end points were used to investigate the comparative effects of x rays and Bragg peak negative pions in C₃H mice. The mouse was selected because testes weight loss data from this Laboratory and others^{1,3,4} have shown response to be dose-rate-independent over the range used in this study. This was important

because of the low dose rates of negative pions available at the time of the experiments (e.g., 10 rad/min or less). High x-ray dose rates were used to simulate clinical conditions.

Testes of adult C_3H mice were irradiated with single doses of x rays or negative pions in the Bragg peak region at the Clinton P. Anderson Meson Physics Facility (LAMPF). The mice were exposed 4 at a time in a Lucite container which was arranged so that the testes of all 4 mice were within 1.5 cm of the radiation beam. The mice were anesthetized 10 min before irradiation by intraperitoneal injection of sodium pentobarbital, 70 mg/kg body weight. Because the testes descend into the scrotum during anesthesia, irradiation was confined to the testes, scrotum, and small portions of the pelvis and tail. The testes of x-irradiated mice received doses of 25 to 1600 rad at either 185 or 914 rad/min; negative pion-irradiated mice received doses of 30 to 800 rad at either 3 or 10 rad/min. Thirty-five days after irradiation, the mice were killed, testes weight loss was determined, and spermatogenic epithelium activity was examined microscopically.

No discernible differences in testes weight loss were seen between x-irradiated and negative pion-irradiated mice given comparable doses (see

Fig. 1. Change in testes weight loss following a single exposure to either x rays or Bragg peak negative pions. The average weight of all controls was taken as 100%. The number of animals per point varied from 4 to 8.

Fig. 2. Single dose-survival curves for spermatogenic stem cells of mouse testes exposed to either x rays or Bragg peak negative pions.

Fig. 1). At doses greater than 400 rad, spermatogenic epithelium regeneration occurred in discrete foci. Regeneration is assumed to occur if a single spermatogenic stem cell survives irradiation. Stem cell survival was assayed by determining the fraction of seminiferous tubule cross sections containing spermatogenic clones. The mean D_0 value of the single-dose survival curve for x rays is 250 rad and for negative pions 143 rad. Comparison of the stem cell survival curves yielded an RBE of about 1.7 at 10% survival (Fig. 2).

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Late Effects of Radiation on the Rat Rectum

[W. C. Black, L. S. Gomez, M. M. Klígerman, and E. C. Rivera]

These studies, which consist of 3 separate experiments, were designed to measure the late effects of x rays on the rat rectum for later comparison with the effects of negative pions on the same tissue. The grading scheme for radiation-induced rectal lesions is based on the comparison of experimental lesions with clinically resected radiation-associated bowel lesions. The 6 parameters of abnormality are (1) ulceration; (2) colitis cystica profunda; (3) mucosal atypia; (4) fibrosis; (5) hypertrophy of the muscle coat; and (6) vascular damage.

In the first experiment, 36 adult male Sprague-Dawley rats were irradiated one-at-a-time in a Lucite exposure chamber with single doses of 300-kV x rays at a rate of 359 rad/min. The quantitative histopathologic technique developed for this study was used to analyze rats exposed to 2000 to 6000 rad of x rays and killed 1 to 6 months later. Between these times, epithelial ulceration and glandular atypia diminished in severity, whereas vascular and connective tissue lesions increased in severity (Fig. 1). Dose-response curves were constructed at 1 month. For epithelial lesions, the slope decreased or slowly increased with dose (Fig. 2). For connective tissue and vascular lesions using the 6-month data, the slope increased with dose (Fig. 3). While the slope is determined, in part, by the numerical scale of effect assigned each end point, the scales themselves have been derived from clinical experience with similar lesions in patients and

Fig. 1. Late effect end points of the rat rectum based on histological comparison of experimentally produced lesions. Six parameters were scored in each rectum examined.

Fig. 2. Dose-response curves for epithelial lesions in the rat rectum to single doses of 300-kV x rays. Lesions were scored 1 month after irradiation.

Fig. 3. Dose-response curves for connective tissue and vascular lesions in the rat rectum to single doses of 300-kV x rays. Lesions were scored 6 months after irradiation.

are weighted to express their contribution to the clinical problem.

The second experiment consisted of 200 adult male Sprague-Dawley rats exposed one-at-a-time to fractionated doses of 300-kV x rays at a rate of 310 rad/min. The rats were randomly divided into 2 major groups: (1) 4-month sacrifice, 6 rats/dose; and (2) life span, 5 rats/dose. In addition, there were 18 control animals sham-irradiated. Rats were assigned as in Table I.

Rats in the 4-month group were killed and the rectal tissue examined histologically. The same parameters were scored in this experiment as in the first experiment, and dose-response curves were constructed which showed the tissue-sparing afforded by a fractionated irradiation scheme when the fractions are separated by 24 h (Fig. 4). Withers and Mason¹ have shown that repair of sublethal cellular injury in colonic mucosal cells of the mouse is essentially complete 3 h after a dose of radiation. Regeneration of surviving cells during the time between fractions also contributes to the sparing of tissues when a dose is divided into multiple fractions. Withers and Mason¹ have also shown that extending the fractionation interval beyond 3 h does not significantly increase the contribution of repair of sublethal damage to the sparing of the colonic

Fig. 4. Dose-response curves for the rat rectum to fractionated doses of 300-kV x rays given at 24-h intervals. Each curve consists of the total score from the six parameters examined. Six rats were examined at each dose point.

mucosa, but it does increase the time during which cells may regenerate. A 24-h fractionation interval was used in this study for x irradiation because this interval was the shortest anticipated when performing negative pion irradiations. As shown in Fig. 4, increasing the number of dose fractions increased the opportunity for surviving cells to repair sublethal injury.

In the third experiment, 60 adult male Sprague-Dawley rats were exposed one-at-a-time to fractionated doses of negative pions at a dose rate of 24 rad/min. The pion-irradiated rats are scheduled to be killed 4 months after irradiation. The tissue taken at that time will be graded using the same technique used with the x-irradiated rats.

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TABLE I

DOSAGE REGIME FOR X-IRRADIATED RATS

1 (rad)	Number of Fractions ^a			10 (rad)
	2 (rad)	5 (rad)		
2000	2000	3500		4000
3000	3000	5000		6000
4000	4000	6500		8000
5000	5000	8000		10 000

^a Each dose level includes 11 irradiated rats: 6 each for 4-month sacrifice and 5 each for life span observation.

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INTRODUCTION

The Division of Biomedical and Environmental Research funds several projects in the Industrial Hygiene Group (H-5) related to evaluation and control of health hazards associated with the working environment. These include applied studies on the sampling, characterization, and control of fine particles; analysis of tissues from autopsies of individuals from the general public and those potentially exposed to plutonium; evaluation of glove permeability for selected highly toxic materials; and analysis of bioassay samples from previous LASL employees who were potentially exposed to plutonium.

Primary efforts in the applied aerosol program have been directed toward evaluating the performance of filters used in air cleaning engineering control systems for facilities handling plutonium. The efficiency of high-efficiency particulate aerosol (HEPA) filters is being defined for high-density ultra-fine aerosols ($< 0.2\text{-}\mu\text{m}$ aerodynamic diameter) to simulate the performance of these air cleaners against plutonium aerosols which may be released under normal or accident conditions. Studies are continuing to quantitate the effects of saltation on particle resuspension and to provide a basis for extending preliminary controlled laboratory experiments to simulate environmental conditions of concern. In cooperation with the Mammalian Biology

Group (H-4), test aerosols from raw shale and spent shale from the proposed commercial process have been developed for small-animal inhalation exposure experiments. A new program is evaluating the standard EPA stack sampling method to quantitate existing limitations and eventually to develop new techniques or instrumentation to monitor better the particulate emissions.

Analysis of autopsy tissue samples for plutonium has continued. Samples from individuals in the general population exposed only to worldwide fallout are providing base-line data from several geographic locations in the United States. Data are also included for areas close to major plutonium facilities. Detailed chemical and autoradiographic analyses of samples from an occupationally exposed individual indicate the levels of exposure associated with the individual, the concentration and size distribution of plutonium within the lung, and the absence of histological changes. Both the general population and occupational exposure samples have defined those organs having the highest concentration and largest amount of plutonium.

In work jointly sponsored by ERDA and NIOSH, the permeability of protective gloves for selected highly toxic materials has been determined. Urine, blood, and fecal samples from former LASL employees, potentially exposed to plutonium, have also been analyzed as part of the plutonium workers study.

FINE PARTICLE STUDIES RELATED TO HEALTH AND AIR CLEANING REQUIREMENTS FOR ENERGY SYSTEMS

[M. I. Tillery, H. J. Ettinger, J. C. Elder, C. I. Fairchild, and M. Gonzales]

HEPA Filter Collection Efficiency for Ultra-Fine Particles

The performance of high-efficiency particulate air (HEPA) filters against high-density submicron particles must be well defined so that air cleaning systems used in nuclear facilities can be designed properly. Performance of HEPA filters for particles having aerodynamic diameter (D_{ae}) greater than $1\text{ }\mu\text{m}$ is well established; however, collection efficiency for ultra-fine ($D_{ae} < 0.2\text{ }\mu\text{m}$) particles collected predominantly by diffusion is not well defined. In this size range, collection of relatively dense ($\rho \geq 9\text{ g/cm}^3$) particulates due to overlapping of inertial and diffusional collection mechanisms needs to be quantitated. Initial studies were carried out with polydispersed ^{238}PuO aerosols having a density of approximately 10 g/cm^3 and a median $D_{ae} > 0.2\text{ }\mu\text{m}$.¹ Studies are now in progress to generate high-density metal or metal oxide aerosols to simulate plutonium having a median $D_{ae} < 0.2\text{ }\mu\text{m}$. The initial efforts were directed toward materials which provide adequate analytical sensitivity by neutron activation and can produce aerosols by compressed air atomization of dilute colloidal suspensions. Six "sols" have been investigated to date.

Aerosols were initially generated from a ZrO_2 sol to evaluate the feasibility of producing aerosols $< 0.2\text{ }\mu\text{m}$ (D_{ae}). Good quality aerosols were produced that had activity median aerodynamic

diameters (AMAD) $< 0.2\text{ }\mu\text{m}$ as determined by neutron activation of Andersen cascade impactor samples. However, HEPA filter efficiency measurements could not be made because of the small cross section of zirconium for neutron activation. Subsequent studies were carried out with 5 different metal sols having a larger neutron activation cross section. Electron microscopy showed that the aerosols produced from the lanthanum oxide sol were composed of two markedly different particles, and further work with this material was discontinued. The dysprosium oxide sol produced a good aerosol, but the half-life of its neutron activation products was too short to permit an accurate determination of particle size from cascade impactor samples, and this work was also terminated.

Holmium oxide, silver oxide, and gold appear to provide adequate aerosols for testing a single-stage HEPA filter. The only difficulty encountered has been a slight build-up of charge in the colloidal suspension of gold, resulting in the loss of material from the generator solution. The addition of surfactants to the generator solution reduced this problem, and studies are now under way to determine the optimum surfactant and solution concentration.

Most of the effort has been directed toward generation and characterization of submicron aerosols over a range of densities with materials having sufficient analytical sensitivity to measure accurately the performance of the HEPA filters. Only a limited number of HEPA filter efficiency measurements have been made, and these are summarized in Table I. All results indicate that HEPA filters

TABLE I

HEPA FILTER EFFICIENCIES

<u>Colloidal Suspension Material</u>	<u>Particle Size</u>		<u>Collection Efficiency</u> (%)	<u>Number of</u> <u>Runs</u>
	<u>AMAD^a</u> (μm)	<u>σ_g^b</u>		
Gold	0.29	2.41	99.98 ^c	1
Silver oxide	0.155 ± 0.007	1.84 ± 0.09	99.993 ± 0.004^c	4
Holmium oxide	0.140 ± 0.000	1.78 ± 0.26	99.983 ± 0.005^d	3

^aAMAD is activity median aerodynamic diameter determined by neutron activation of cascade impactor samples.

^b σ_g is geometric standard deviation determined as in a.

^cMass efficiency determined by neutron activation of upstream and downstream filter samples.

^dParticle number collection efficiency determined by condensation nuclei counter.

perform better against dense submicron particulates than indicated by their performance against 0.3- μ m dioctylphthalate (DOP) particles used for routine quality control.

Future studies will include accurate determination of particle densities using the spiral centrifuge aerosol spectrometer and determination of HEPA filter performance against submicron particles as a function of particle size and density.

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Generation of Aerosols for Inhalation Experiments

[In cooperation with the Mammalian Biology Group (H-4)]

The potential utilization of oil shale to provide combustible fuels and other chemicals has resulted in initiation of an investigation of the potential toxicity of materials that workers involved in the oil shale industry may be exposed to. This information will also help to determine the necessary controls to limit environmental releases. Initial studies have been directed toward the production of reproducible inhalable aerosols of raw shale from the Green River shale formation and from spent shale from two different retorts (Laramie Energy Research Center above-ground simulated in situ and the TOSCO II process). Aerosols have been produced by crushing the shale in a jaw crusher to a maximum particle size of about 1 cm, then ball-milling for periods from 0.5 to 6 h. The powdered material is then packed in the feed tube of a Wright¹ dust feed apparatus at a pressure of approximately 1000 psi for subsequent aerosol generation. Using these techniques, exposure atmospheres have been produced with concentrations of approximately 70 mg/m³ with a particle size distribution that resulted in more than 70% respirable according to the ACGIH respirable dust curve.² Chronic inhalation exposure studies are currently being carried out by the Mammalian Biology Group (H-4) using these aerosols.

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Optimization of Filters and Stack Probe for Aerosol Stack Sampling (EPA Pass-Through Funding)

The U. S. Environmental Protection Agency stack sampling methods are being evaluated, and improved methods of extracting particulate samples from plant stacks are being developed. Method 5 is generally applied to the determination of total particulate mass emitted from stationary sources and is subject to a number of problems which affect its accuracy, convenience, and versatility. These include alteration of the aerosol in the sampling apparatus or during sample handling, formation of a "false" aerosol from gaseous precursors in the sampling apparatus, deposition in the sampling probe, breakage and leakage of the glassware comprising the sampler, lack of particle size measurement capabilities, and variation of collection efficiency among glass fiber filter media used in the method. To date, evaluation work has included measurement of probe deposition of several monodispersed dye aerosols and polydispersed fly ash and glass bead aerosols in sizes ranging from 1.2- to 14- μ m mass median diameter (mmd) and collection efficiency of two glass fiber filter media using monodispersed dye aerosols with a 1.2- to 4.1- μ m mmd.

Deposition studies have been conducted with the probe at ambient temperature (20°C) and with the probe heated (160°C) at full flow of 28 l/min and at half-flow. The 160°C upper limit was adopted to include a proposed change from the 120°C operating temperature in the original Method 5. Although this change may not be adopted by the EPA, 160°C represents a conservative condition for measurement of deposition (and of filter efficiency). Experiments indicate up to 95% of the 13.4- μ m mmd aerosol deposited in the probe prior to the filter. Figure 1 gives deposition profiles of the 13.4- μ m mmd glass bead aerosol and of 2.0- and 3.8- μ m mmd fly ash aerosols for a sampling rate of 30 ± 2 l/min. The geometric standard deviation (σ_g) of these aerosols indicated that the aerosols were polydispersed but not broadly distributed. About 53% of the 3.8- μ m mmd fly ash and 7% of the 2.0- μ m mmd fly ash are deposited in the nozzle (positions 1 and 2) which acts

Fig. 1. Loss in stack sampling probe.

as an impaction jet. Although relatively low deposition by the 13.5- μm mmd aerosol is indicated in Fig. 1, the larger particles in this aerosol undoubtedly impinge on the tube wall, rebound, or are reentrained from that surface and travel to the liner before deposition by sedimentation.

A similar effect of particle size was also observed at half-flow (14 l/min) in ambient temperature runs. Deposition of the 3.8- μm mmd aerosol dropped to 46% in the nozzle (71% overall in the train) at the lower flow; the 2.0- μm mmd aerosol deposited 4.6% in the nozzle (10.5% overall in the train).

Changes in deposition caused by heating the probe to 160°C were minor except at the exit elbow (position 17). Deposition here was 2% at ambient temperatures and 10% at 160°C. This can be attributed to a 1.6 times higher velocity at this point under heated conditions.

Enlarging the nozzle diameter from 0.64- to 0.99-cm reduced deposition of the 3.8- μm fly ash aerosol by 50%. The deposition studies emphasized the need for uniform and thorough component washing and mass analyzing techniques, improved

transition between components of the train, use of larger probe nozzle size where compatible with maintaining the required isokinetic sampling, and elimination of cyclone pre-separators and the unnecessary bends which accompany them.

Efficiency measurements of two glass fiber filters commonly used in EPA Method 5, MSA 1106 BH, and Whatman GF/A, are in progress. Gelman A glass fiber filters are used as reference filters and exhibit efficiencies consistently above 99.9%. Fluorescent dye aerosols generated from a Berglund-Lui vibrating orifice monodispersed aerosol generator were collected on the test filter over a range of flow rates and at temperatures near 160°C. Aerosol penetrating the test filter was collected on the reference filter which was located in the same holder. Filter collection efficiency was determined by fluorometric measurement of the aerosol mass deposited on each filter. Experimental problems experienced with this technique early in the program (other reference filters containing varying amounts of spurious fluorescent material) caused many of the early data to be suspect. However, after a conservative correction was applied, numerous data showed efficiencies of 94 to 98%. These experiments are being repeated with a satisfactory reference filter to confirm these efficiencies and to include an aerosol in the submicron size range.

Resuspension of Particles from Surfaces

Saltation movement of large particles is the main driving mechanism in the wind-driven resuspension of fine particles.¹ Particles in the size range 80 to 700 μm are more easily suspended than finer dust. Because of their high settling velocity, they rapidly return to the surface, striking and resuspending the smaller particles. Resuspension resulting from momentum transfer from saltation to deposited particles is being studied in a 20-cm diameter wind tunnel where fully developed turbulent air flow at controlled velocities simulates atmospheric winds. Fine particle beds on a smooth, flat plate are bombarded by large saltation particles introduced into the wind tunnel 3 m upstream of the bed. Resuspension rates and vertical flux (upward transport of particles) are defined by this experimental work.

Resuspension of two sizes of spherical aluminum

bed particles has been studied with no saltation particles, 0.125 g of 100- μm glass diameter mono-dispersed spheres as saltation particles, and 1 g of 200- μm -diameter monodispersed glass spheres as saltation particles. The polydispersed bed particles had count median diameters (CMD) of 3.7 and 7.0 μm and geometric standard deviations (σ_g) of 1.7 and 1.6, respectively.

Replicate experiments were made with each bed particle size at a wind velocity of 10 m/sec. These experiments indicated that saltation particles increase the resuspension rate, with resuspension of the finer particle bed influenced more by saltating particles. Resuspension rates (fraction of initial bed resuspended/sec) are given in Table I. Average coefficients of variation (approximately 40% for the 7- μm bed particles and 75% for the 3.7- μm bed particles) indicate that resuspension rates can be quantitatively reproduced within a factor of 2 in the wind tunnel. The coefficients of variation suggest that there is no significant difference between the no saltation and 100- μm saltation runs; however, runs at high wind velocities indicate a definite increase in resuspension due to both sizes of saltation particles.

Vertical flux (particles/cm² sec) determined from particle size distributions at two different heights increase by approximately 1 order of magnitude between no saltation, 100- μm saltation, and 200- μm saltation conditions. Positive vertical flux (upward particle transport) was exhibited for particles ranging in size from 0.4 to almost 40 μm in diameter. The increase in vertical flux with saltation particles was about 3 orders of magnitude greater (10 particles/cm² sec) for the 3.7- μm CMD particle bed than for the 7- μm CMD particle bed. Present data indicate that the reproducibility of

Fig. 1. Pulse runs of 8-in.-tunnel velocity profiles (Z = distance above tunnel floor).

vertical flux measurements is approximately 1 order of magnitude.

Natural resuspension is often the result of gusts significantly greater than the average wind velocity. This is because resuspension is proportional to friction velocity (a function of surface shear stress) raised to an exponent reported to be between 3 and 5, depending upon soil properties. Experiments simulating gusty winds indicated that resuspension rates determined by measurements of horizontal flux (g/cm² sec) near the surface were much lower than for a steady wind, whereas horizontal flux measurements at the tunnel mid-height were greater for gusty wind than for a steady wind. The anomalous results were found to be caused by eddy (vortex) currents near the surface. This eddy flow was generated as the wind velocity changed rapidly. However, fluid mechanics considerations² suggest that eddy flow was not created by fluid acceleration as the velocity increased but by the decreasing pressure gradient as the wind velocity reached the higher gust level. Figure 1 shows velocity profiles measured at different times during wind excursion from 11 to 16 m/sec. The velocity was increased in

TABLE I
RESUSPENSION RATES (FRACTION OF INITIAL BED RESUSPENDED/SEC)

Resuspension Bed Particle Size		Saltation Particles		Resuspension Rate	Number of Runs
CMD (μm)	σ_g	Size (μm)	Total Mass (g)		
7	1.6	-	-	0.15 ± 0.02	3
7	1.6	100	0.125	0.20 ± 0.11	3
7	1.6	200	1.000	0.31 ± 0.11	3
3.7	1.7	-	-	0.04 ± 0.04	3
3.7	1.7	100	0.125	0.06 ± 0.03	3
3.7	1.7	200	1.000	0.27 ± 0.13	3

0.6 sec and held constant for the time shown at the bottom of the curve. The average velocity is shown above the steady-state profiles (10.8 and 15.9 m/sec), but the maximum velocity is shown above the gust profiles. All data points were for maximum velocity occurring at each height measured down to 0.5 mm above the surface. The profiles at 1 and 2 sec show a normal change as the average velocity increased, but profiles at 3 to 10 sec indicate a reverse flow at 0.1 cm above the plate, probably caused by eddy flow. Eddy currents evidently did not appear earlier because the decreasing pressure gradient did not generate an eddy near the surface until well after the maximum flow far from the wall was established.

A new wind tunnel with a 51-by 71-cm rectangular cross section by 30 m long has been designed and is in the procurement and fabrication stage. This tunnel will provide better simulation of the microscale and integral scale of atmospheric turbulence than the present tunnel. In addition, the new tunnel will permit further experiments with spheres, soil resuspension, complex topography, gusty wind effects, and reentrainment of particles from duct surfaces and filters.

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DETERMINATION OF PLUTONIUM IN MAN

[J. F. McInroy, H. A. Boyd, B. C. Eutsler, M. W. Stewart, M.D. (consultant), and G. L. Tietjen (Group T-13)]

Particle Size Distribution in the Lung of an Occupationally Exposed Individual

Many questions have arisen concerning the size distribution of inhaled PuO_2 particles undergoing long-term retention in the human lung. Studies were completed of the particle size distribution in the lung of a former LASL employee who was 46 yr old and had 26 yr of potential exposure to plutonium while working as a metal fabrication technician. As reported previously,¹ portions of the lung from this

individual were fixed in 10% formalin and thin-section slides prepared for histological and autoradiographic examination. The tracks associated with the plutonium "stars" were counted and estimates made of the equivalent plutonium particle sizes, assuming that the particles were $^{239}\text{PuO}_2$. The techniques of this calculation have been previously reported.^{1,2} The remaining portions of the lung were analyzed chemically for their total plutonium content. Estimates have been made on the activity associated with the different size particles and the number of particles of the various size ranges present in the lung, and these are summarized in Table I.

Chemical analysis indicated that this lung had a total burden of 35.7 nCi and, on the basis of the autographically determined size distribution, contained an estimated 4×10^7 particles. The activity median diameter (amd) of the particles was calculated to be 0.16 μm with a geometric standard deviation of 1.4. The largest particles observed had a diameter of 0.3 μm and contained 9 fCi of ^{239}Pu . Histological examination of the above lung by a consulting pathologist revealed no microscopic changes that were inconsistent with the normal aging process.

Lungs from 2 other occupationally exposed cases have been studied similarly. However, in each case, the total lung burden determined by chemical analysis was only 4 nCi, and several weeks exposure of the autoradiographs failed to show significant plutonium stars for evaluation. Alternate techniques of particle size determination are being investigated.

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TABLE I
DISTRIBUTION OF $^{239}\text{PuO}_2$ PARTICLES IN LUNG OF CASE 7-138

$^{239}\text{PuO}_2$ Particle Diameters (μm)	Observations Number	Total (%)	Cumulative Frequency (%)	^{239}Pu Concentration (fCi/particle)	Activity/Particle Size Interval ^a (fCi)	^{239}Pu Activity in Lung ^b (fCi/lung)	Particles per Lung ^c (number)
< 0.038	1	0.8	0.8	0.02	0.02	6.66×10^3	3.33×10^5
0.048	5	4.1	4.9	0.04	0.20	6.66×10^4	1.67×10^6
0.055	12	9.9	14.8	0.06	0.72	2.40×10^5	4.00×10^6
0.065	9	7.4	22.2	0.09	0.81	2.70×10^5	3.00×10^6
0.082	11	9.1	31.3	0.19	2.09	6.96×10^5	3.66×10^6
0.094	5	4.1	35.4	0.28	1.40	4.46×10^5	1.59×10^6
0.103	15	12.4	47.8	0.37	5.55	1.85×10^6	5.00×10^6
0.111	2	1.7	49.5	0.46	0.92	3.06×10^5	6.65×10^5
0.118	10	8.3	57.8	0.55	5.50	1.83×10^6	3.33×10^6
0.130	2	1.7	59.5	0.74	1.48	4.93×10^5	6.66×10^5
0.140	23	19.0	78.5	0.92	21.16	7.05×10^6	7.66×10^6
0.164	2	1.7	80.2	1.49	2.98	9.92×10^5	6.66×10^5
0.177	15	12.4	92.6	1.87	28.05	9.94×10^6	4.99×10^6
0.202	5	4.1	96.7	2.78	13.90	4.63×10^6	1.67×10^6
0.223	2	1.7	98.4	3.73	7.46	2.48×10^6	6.65×10^5
0.255	1	0.8	99.2	5.58	5.58	1.86×10^6	3.33×10^5
> 0.303	1	0.8	100.0	9.37	9.37	3.12×10^6	4.02×10^7
Totals	121	100			107.19	3.57×10^7	4.02×10^7

^aActivity observed/particle size interval = number of particles observed x ^{239}Pu concentration (fCi/particle).

^bCalculated on the basis that analysis of the lung indicated a total lung activity of 35.7 nCi (35.7×10^6 fCi). Therefore, the lung activity is $35.7 \times 10^6 / 107.2$ or 3.33×10^5 times the activity measured by counting tracks in a small portion of the lung and tabulated in the preceding column.

^cNumber of particles/lung = ^{239}Pu activity in lung (fCi/lung) : ^{239}Pu concentration (fCi/particle).

Plutonium in the Tissues of the General Population

Data are being accumulated on the concentrations of plutonium in the tissues of the nonoccupationally exposed general population. These data will provide information on the distribution of plutonium in the body as a function of time, establish base-line levels of plutonium, and provide useful control information for comparison with the studies of plutonium effects and distribution in occupationally exposed individuals.

Tissues have been collected from 6 geographic locations over a period of 17 yr. The number of autopsy cases received to date is shown in Table I. The year the first sample was collected from each area is also indicated, with tissues continuously collected from that date to the present except for

TABLE I
AUTOPSY CASES RECEIVED FROM
THROUGHOUT THE UNITED STATES

Area	Year First Sampled	Number of Cases Received	
		CY 1976	1959-1976
Colorado	1970	16	323
Georgia-South Carolina	1972	58	158
Illinois	1973	12	53
Pennsylvania	1974	83	304
New York	1968	0	36
New Mexico (including LASL)	1959	30	406
Totals		199	1280

New York City. All autopsy cases from New York City were collected in 1968, and none have been received since that time.

In identifying the median concentrations of plutonium in the samples from the general population, we have previously not extrapolated counting results for any sample that was below our minimum detectable level (MDL) of 0.01 pCi. The data were included in determining the total sample size (i.e., counted in the cumulative frequency), but the data points were not plotted on the log-normal graphs. In many cases, where more than 50% of the analytical results were < MDL, the median value consequently had to be extrapolated from a least-squares fit of the data, or the median was represented simply as being less than the minimum reporting level (< MRL). It has been recommended^{1,2} that all measured count rates and counting errors be reported since "negative or very small positive numbers contain information about the total variability associated with a given counting system." The reporting

of "less than" numbers results in a loss of information, and the simplest and most direct approach would be to report all results even though the counting error may be large relative to the net count. We have consequently reexamined our data and utilized all count data in estimating the parameters that describe their distribution.

The All Population data in Table II include the additional analyses performed this year of 170 liver, 200 lung, 62 lymph node, 169 kidney, 51 gonadal, 13 vertebral, 162 spleen, and 81 thyroid samples. The numbers in parentheses below the median values indicate the number of samples analyzed at the time of this writing. No known occupationally exposed cases are included in this table. A backlog of several hundred tissues is in various stages of analysis.

The median values indicated were estimated from a least-squares fit on a cumulative logarithmic probability curve of all measured results greater than 0. The results of previous gonadal specimens

TABLE II
50TH PERCENTILE DISTRIBUTION OF ²³⁹Pu IN HUMAN TISSUE^a

Location	Lung	Liver	TBLN ^b	Kidney	Spleen	Thyroid	Gonad	Vertebra	Rib	Bone ^c
Los Alamos	0.63 ^d (66)	0.5 (66)	4.20 (65)	0.11 (74)	e	e	e	0.55 (43)	e	e
New Mexico and U. S.	0.40 (106)	0.53 (102)	3.19 (102)	0.07 (111)	e	e	e	0.54 (73)	e	e
Colorado	0.20 (171)	0.52 (165)	1.84 (110)	0.08 (125)	0.03 (35)	0.17 (13)	0.05 (64)	0.40 (45)	0.20 (32)	0.53 (48)
New York	0.30 (36)	0.74 (32)	f	f	f	f	0.46 (33)	0.84 (32)	f	f
Georgia-South Carolina	0.21 (39)	0.61 (49)	e	0.02 (42)	0.06 (26)	e	0.11 (10)	0.30 (12)	e	e
Pennsylvania	0.12 (125)	0.51 (107)	1.14 (40)	0.06 (98)	0.06 (89)	0.28 (59)	0.15 (55)	e	0.34 (19)	e
All Populations ^g	0.24 (543)	0.58 (521)	2.42 (322)	0.06 (450)	0.05 (162)	0.25 (81)	0.13 (172)	0.50 (210)	0.25 (58)	0.50 (56)

^aPlutonium-239 pCi/kg wet tissue.

^bTBLN = tracheobronchial lymph nodes.

^cBone = bone sample site unidentified by pathologist.

^dNumber of samples analyzed.

^eInsufficient number analyzed for statistical significance.

^fSamples not requested and/or not received.

^gAll population numbers of samples include results noted under c above. The total number of tissues analyzed = 2575.

that were based upon analyses of 10% aliquots of the total tissue sample were deleted, and the results from reanalysis of these tissues using 50% aliquots are included in Table II. By using larger aliquots, the sensitivity of measurement was increased and the errors associated with extrapolation of the analytical results to the desired units of concentration (pCi/kg) were reduced. The lung and liver median values from each geographic area are basically unchanged from last year's summary.³ The lymph node and vertebra median values have increased by approximately a factor of 2, indicating that normalizing the small count rates (< 0.01 pCi/sample analyzed) to activity/kg of tissue shifted some of these values upward. The small soft tissues (kidney, gonads, spleen, and thyroid) that usually do not contain sufficient amounts of plutonium greater than the 0.01-pCi level per sample analyzed and which, under the "old method" of data reporting, would have been listed as having median values "< MRL" now have calculatable medians.

Using the median concentrations for the All Population data, the highest concentrations observed are tracheobronchial lymph nodes > liver > vertebrae > thyroid > lung > gonad > kidney > spleen. This ranking is somewhat different than that previously reported,³ partly because of the availability of analyses of more kinds of tissues (spleen, thyroid, etc.) and also because of the change in determining the median values.

Caution must be exercised in the formulation of inferences about apparent differences in tissue concentrations between geographic locations, since the summarized results in Table II do not account for the time spread over which the samples were collected. As noted in Table I, this time span varies from 17 yr (New Mexico) to 2 yr (Pennsylvania). The variation in fallout with time may account for much of the observed differences, especially in the lung and tracheobronchial lymph nodes.

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Analysis of Special Samples

At the request of the ERDA Rocky Flats Area Office in Golden, Colorado, several animal tissues from a farm near that facility were analyzed for plutonium. The frozen samples were forwarded to LASL by the local county health department and were analyzed using our routine plutonium analytical procedure for human tissues. Since the sample sizes were relatively small, all tissues less than 50 g were each analyzed in toto to take advantage of the maximum sensitivity of the procedure. Samples greater than 50 g were analyzed in duplicate. The ²⁴²Pu was added to each sample prior to muffling as an internal tracer for determining the overall chemical recovery of plutonium.

The analyses for ²³⁹Pu shows that duck gonads, piglet brain, kidney, liver and lung, lamb ovaries, and duck femur were not statistically different from 0 at the 5% level of significance. The thyroid of the farmer, surgically removed because of a benign adenoma, was also analyzed with these tissues and contained no plutonium. Three horse testicles were analyzed in duplicate and, of these, only one sample contained a measurable quantity of ²³⁹Pu, which was consistent with our findings in human tissues in the general population.

During evaluation of the plutonium spectra, it was noticed that other alpha energies were present. These were identified, on the basis of their energy, as thorium isotopes (²²⁸Th, ²³⁰Th, and ²³²Th) and their daughter products (²²⁴Ra, ²¹²Bi, and ²²⁰Rn). The analytical procedure used was quantitative only for plutonium, not for thorium; therefore, the thorium results are inconclusive. The finding of thorium in these tissues was not unexpected, since the earth's crust in this area contains uranium and thorium. Additional analyses are being performed using ²³⁴Th as a tracer to quantify the thorium in the sample.

Health of Los Alamos Plutonium Workers

[G. L. Voelz, M.D. and L. H. Hempelmann, M.D.]

Medical reexaminations of 18 persons in the follow-up study of 24 Manhattan Project plutonium workers have been completed. The last time this group was studied completely was in 1971-1972.¹ Figure 1 is a photograph of some of the group during their visit to Los Alamos in October 1976. In 1975, one of the study subjects, age 50 yr, died as a result of injuries suffered when struck by an automobile. Another person in the study group died in 1959 at age 36 yr due to a coronary occlusion (heart attack). These two deaths have occurred among the original 26 subjects selected for study in 1952. All 24 living persons have been contacted during 1976 and are known to be alive and active. The remaining examinations will be completed early in 1977.

The examinations included complete medical history, physical examination, blood chemistry profiles, hematological studies, including chromosome analysis of peripheral lymphocytes, radioanalysis of peripheral lymphocytes, radioanalysis for plutonium in biological samples (urine, blood, and feces), sputum cytology, and x-ray studies (chest, pelvis, femur, and dental). All persons were counted with phoswich detectors for plutonium (chest, liver, and hands) and by large NaI (Tl) detector for other radionuclides (whole-body).

The ages of these persons in December 1976 ranged from 50 to 65 yr, with 56 yr as the average. The current examinations have shown that they continue to be active people with diseases that are not unusual for their ages such as coronary artery disease (1), hypertension (1), gout (1), history of ulcer (1) and kidney stones (2), bronchitis and

Fig. 1. Former Manhattan Project plutonium workers (1943 to 1945) visit the new plutonium facility under construction at Los Alamos.

emphysema (1, heavy smoker). No cases of cancer were detected in these examinations. A previously reported melanoma of the skin and a small skin cancer on the back of a hand showed no evidence of further activity or recurrence. A new diagnosis of interest was asymptomatic Paget's disease (osteitis deformans) of the bone which appeared as characteristic roentgenographic changes in the pelvis associated with a high alkaline phosphatase value. Reexamination of previous bone roentgenograms showed that changes were

present in 1971 and 1965 and have only progressed slightly during the intervening period.

Sputum samples were sent to Dr. Geno Saccomanno for examination of bronchial epithelial cells. Lung cytology of all 18 persons was normal. Three individuals were classified as having moderate cellular atypia -- a normal classification for this age group; two of the three are current cigarette smokers. Findings of severe cellular dysplastic changes on one individual 5 yr ago have not been confirmed on the

TABLE I

CURRENT BODY BURDEN ESTIMATES AND PLUTONIUM CONCENTRATION IN URINE SAMPLES
COLLECTED FROM THE MANHATTAN PROJECT WORKERS

Employee Identification ^a	Estimated Body Burden (μCi)		Year of Collection (pCi/24 h sampling period)					
	1972	1976	1971	1972	1973	1974	1975	1976
3	0.42	0.20	2.28	2.22	--	2.05	--	2.17
4 ^b	0.26	0.18	1.18	1.09	0.82	1.14	1.05	0.95
1	0.21	0.12	0.90	--	--	0.79	--	0.54
9	0.11	0.10	0.69	--	--	0.45	--	0.72
6	0.14	0.083	0.41	--	0.36	0.38	--	--
7	0.15	0.081	0.71	--	--	0.85	--	0.87
5	0.18	0.079	0.69	--	--	0.74	--	0.75
17	0.13	0.070	0.41	--	--	0.35	--	0.38
18	0.10	0.061	0.49	0.77	0.46	0.32	--	--
8	0.11	0.053	0.47	--	--	0.50	--	0.51
10 ^b	0.10	0.044	0.26	--	--	0.50	0.26	--
11	0.05	0.034	--	--	--	--	--	--
20	0.05	0.033	0.22	--	--	0.20	--	--
23	0.04	0.032	0.16	--	--	0.09	--	0.05
27 ^b	0.05	0.027	0.14	0.13	0.12	0.09	0.10	0.10
12	0.12	0.025	0.28	--	--	0.24	--	0.16
21	0.04	0.025	0.10	--	--	--	0.19	--
22	0.05	0.023	0.20	--	--	0.08	--	0.15
15 ^c	0.03	0.023	--	--	--	--	--	--
16 ^d	0.03	0.018	0.24	--	--	0.25	0.11	--
24	0.03	0.018	0.10	--	0.11	0.20	--	0.30
19	0.02	0.017	--	--	--	0.22	--	0.20
25 ^b	0.01	0.014	0.05	0.06	--	0.03	0.02	0.05
2	0.03	0.009	0.23	--	--	0.05	--	0.10
13	0.005	0.007	0.05	--	--	0.03	--	0.02
26	0.006	0.006	0.04	0.08	--	0.03	--	--

^aList ordered by descending 1976 body burden estimates.

^bPresently employed at LASL.

^cDeceased in 1959.

^dDeceased in 1975.

recent examinations, including bronchoscopic brushings.

Studies of chromosomes of peripheral lymphocytes are under way, and the results will be entered in the next annual report.

All in vivo measurements for plutonium in the chest and liver were below our detection limit. No activity was found on the hand surveys except for one individual with a known plutonium-contaminated wound of the right index finger. It is estimated again that the plutonium content of the wound is $5 \pm 2 \text{ nCi } ^{239}\text{Pu}$. No effect due to this deposit was apparent on physical examination.

Samples of urine, feces, and blood were collected in order to determine, if possible, the plutonium content and ratios between them 30 yr after exposure. The completed urine values are shown in Table I, column 1976. Analyses of blood and fecal samples are in progress. From the data obtained during 1971 to 1976, it is apparent that the rate of change in plutonium excretion in urine as a function of time for this group is very small: less than the precision of the analytical technique.

Plutonium body burden estimates on these persons have been revised from those given in earlier publications in order to reduce discrepancies noted between estimates made from urine excretion values and those based on post-mortem tissue analyses. Table I lists both our latest estimates and the 1972 estimates. The new estimates, which are believed to have greater accuracy, include corrections that are dependent on the number of urine samples for the individual and time intervals between samples.

Autopsy tissue samples were obtained on Employee No. 16 who was killed accidentally in 1975. Table II shows the estimated content of the various organs. The liver sample was below our detection limit and did not correspond with other values in this case. It appears that the liver sample received with this set of tissues is not from this case. A liver burden estimate consequently has been made from liver-to-bone concentration ratios observed in other occupationally exposed cases similar to this. The total plutonium content in tissues (22.7 nCi) and the 1976 estimate from urinary excretion (18 nCi) was made independently and is in excellent agreement.

TABLE II

PLUTONIUM BODY BURDEN ESTIMATES FROM TISSUE ANALYSES

<u>Tissue</u>	<u>Content (nCi)</u>
Lung	10.4
Lymph nodes (tracheobronchial)	3.5 ^a
Skeleton	3.4 ^b
Liver (estimated)	5.0
Other soft tissue	0.4 ^c
Total	22.7

^a Extrapolated to 15 g.

^b Skeletal burden extrapolated from rib concentration.

^c Other soft tissues included blood, brain, gonad, kidney, muscle, skin, spleen, and thyroid.

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Epidemiologic Study of Plutonium Workers

[G. L. Voelz, M.D. and L. H. Hempelmann, M.D.]

A research protocol was written to describe this new study on plutonium workers at 6 ERDA contractor locations: Los Alamos, New Mexico; Rocky Flats, Colorado; Mound, Ohio; Hanford, Washington; Savannah River, South Carolina; and Oak Ridge, Tennessee. A mortality study is planned in which a records search will develop ultimately the causes of death by death certificate for an estimated 7200 workers exposed to plutonium during the past 33 yr and another estimated 6000 workers who worked in and around plutonium areas and are considered potentially exposed. An unexposed cohort of workers at each location will be selected to include appropriate ages, sex, occupation, starting year of employment, and race characteristics for comparison with the exposed cohort.

A subset of mortality groups will be selected for a morbidity study. This will be conducted by health history questionnaires from each individual in this part of the study. It is planned that all persons with higher exposures will be studied this way but that the less exposed and nonexposed will

constitute about one-third of those in the larger mortality study. The morbidity study should provide better information on health history, occupational work history, smoking data, and mortality information on these subjects.

The protocol plan was reviewed in March 1976 by an expert committee of epidemiologists and biostatisticians selected by DBER/ERDA and in October 1976 by a LASL Advisory Committee. Suggestions of these committees are being incorporated into the study design.

The most important effort in 1976 has been in planning and testing procedures to be used in the study. This effort has included data base design, questionnaire design, and procedures for data collection, editing, storage, and retrieval. The data are stored in a flexible data base management system, a commercial software program called System 2000, which permits both practical and efficient management of data storage as well as providing easy, rapid assessment and analysis.

The first phase of data base design was completed and tested. This phase includes approximately 40 components of information about each subject. Persons in the morbidity study are estimated ultimately to have about 65 components of information in the data base. The characteristics of the system make it possible to use the computer to assist in selection of a suitable population of nonexposed subjects from the total number of employees available. This process was also tested in 1976, and further investigations are being carried out to automate the control selection procedures as much as possible.

Extensive record work was done to identify all past and present plutonium workers at Los Alamos. Records of known or potentially exposed persons at the Los Alamos Scientific Laboratory (3400) and Zia Company (1370) are now available for entry into the computerized data base. In addition, personnel records of other persons not exposed to plutonium were abstracted for 5200 Zia workers. Similar data on about 15 000 LASL employees were obtained through records previously abstracted for the ERDA health and mortality study at the University of Pittsburgh. These persons will be used as potential subjects for the nonexposed comparison group.

Entry of initial data on subjects into the developing data base was started with LASL and Zia employees. Information on persons in the exposed category is placed in the permanent data base as available. Information on nonexposed persons is being retained in a separate temporary data base until after selection of nonexposed (control) persons is completed; then the persons to be studied will be transferred to the permanent data base.

Questionnaires to be used in the morbidity study were designed, and a small sample distribution of Los Alamos subjects was queried to test the questionnaire design. Further testing on an independent group of subjects is planned before further distribution to study subjects is made. After morbidity subjects are located, questionnaires will be sent requesting information regarding personal characteristics, occupational history, health experience, and smoking history.

Meetings were held with health physics and management personnel at Rocky Flats and Hanford as preparation to the start-up of data collection in 1977 and to assist in planning the study.

In cooperation with the New Mexico Tumor Registry, a study of cancer mortality rates in Los Alamos for the period 1950 to 1969 has been conducted. Preliminary results suggest that the age-adjusted mortality rates in Los Alamos (male 142.6; female 127.3) do not differ significantly from those of the state of New Mexico (male 136.3; female 115.1) or those in the United States as a whole (male 174.0; female 130.1). The small population size of Los Alamos prevents any significance from being placed on these values or on evaluation of individual tumor types. The significantly low lung tumor mortality rate in males (12.0) at Los Alamos compared to the United States rate (38.0) for the period 1950 to 1969 is not reflected in the incidence rates for the years of 1969 through 1974. Although such a study will permit only gross comparison with other populations, it is of interest since Los Alamos citizens in this isolated community have been closely associated with the nuclear industry, especially the plutonium industry, as employees and as neighbors to major plutonium facilities.

Analysis of the Human Health and Ecological Consequences of Plutonium and Other Transuranics

[J. W. Healy, J. R. Rodgers, and R. L. Thomas]

A review of current literature on the uptake of americium, neptunium, and curium in plants was completed. This review emphasized once again the lack of detailed data necessary to assess this pathway to man, as well as the difficulty of interpreting much of the information in the literature because of incomplete descriptions of techniques and results and differing bases for reporting. While there are few values and representative types of plants have not been studied, it appears that both americium and neptunium are accumulated in plants to a much greater degree (perhaps 100 to 1000 times) than plutonium. It is concluded from this that ingestion may be of much greater importance for these elements than it is for plutonium. Additional work is badly needed, particularly for ^{241}Am which is a daughter of ^{241}Pu .

A study of the upper limits for transuranic waste shallow earth burial was started with a goal of completion shortly after the end of the calendar year. This work is aimed at providing a value based upon health and ecological considerations rather than economics. A task group consisting of members from DBER, ORNL, BPNWL, SRL, Dow Chemical Corporation, and Northwestern University with observers from NCR, NRC, and EPA has been established to guide and assist in this project; however, LASL has the main responsibility.

Detailed comments were provided to ERDA on several studies from EPA aimed at guidance for plutonium in soils. A first draft of this guidance was reviewed, with comments submitted to Battelle-Northwest for inclusion in their overall compilation.

GLOVE PERMEABILITY STUDIES (work supported jointly by the National Institute for Occupational Safety and Health and DBER/ERDA)

[R. W. Weeks]

Permeation of Methanolic Aromatic Amine Solutions through Commercially Available Glove Materials

The principal mode of worker contact with chemicals is frequently via the hands and forearms. To minimize contact by this route, the worker will frequently don protective gloves to afford himself

some degree of protection. There is little quantitative information at the present time concerning the rate at which various chemicals or combinations of chemicals permeate commercially available protective gloves. To permit a more objective evaluation of the best protective glove for one particular situation, tests were performed to determine the rate at which various commercially available glove materials were permeated by methanolic solutions of aromatic amines. Because of our special interest in the OSHA-designated carcinogenic compounds,¹ we have limited these studies to the general category of aromatic amines.

A section of a commercially available glove material was used as a semipermeable membrane between two chambers of a permeation test cell. One side of this cell was filled with the challenge solution and the other with physiological saline solution. Aliquots of the physiological saline solution were monitored as a function of time until chemical breakthrough occurred. This monitoring was effected by measurement of the absorption maxima (437 nm) of the Schiff base formed from p-dimethylaminobenzaldehyde and the amine in the challenge solution and included appropriate corrections for dilution effects.

Results from studies of dilute methanolic solutions of certain aromatic amines are given in Table I. Excluding MOCA, for each of the glove materials tested (except PVC), the breakthrough time for all amines was relatively constant for a given glove material. However, the breakthrough time for a given amine did vary from one glove material to another and, thus, was a function of the particular glove material under consideration. Table I also shows that the breakthrough time was affected by factors other than glove thickness. An example of this was that 8-mil natural latex surgeon's gloves afforded a breakthrough time considerably greater than did the 30-mil neoprene material.

Somewhat surprising, in view of its recommended superiority,² was the rapid breakthrough experienced with 30-mil butyl rubber gloves. The butyl gloves examined in these experiments had the most rapid breakthrough of any gloves tested except the nitrile and polyethylene samples. Even under the conditions of a 500- $\mu\text{g}/\text{ml}$ challenge concentration (Table I) and assuming continuous contact with the challenge

TABLE I

GLOVE MATERIAL PERMEATION BY AROMATIC AMINES

		Aniline ^a	p-Chloroaniline ^a	p-Toluidine ^a	Methylenedianiline ^a	MOCA ^{a,b}
Lower limit of detection ($\mu\text{g}/\text{ml}$) ^c		1.1	1.7	1.6	1.8	3.5
Material	Glove Thickness (mil)	Breakthrough ^d (h)				
Natural latex	8	49 $\pm$ 2	47 $\pm$ 1	45 $\pm$ 1	45 $\pm$ 1	35 $\pm$ 2
Hypalon-neoprene ^e	30	26 $\pm$ 1	26 $\pm$ 1	26 $\pm$ 1	25 $\pm$ 1	-
Hypalon	30	21 $\pm$ 1	21 $\pm$ 1	21 $\pm$ 1	20 $\pm$ 1	-
Neoprene	30	15 $\pm$ 2	15 $\pm$ 2	14 $\pm$ 2	15 $\pm$ 1	-
Butyl	30	4 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 1	-
Polyethylene	2	4 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 1	-
Nitrile	11	1 $\pm$ 0.5	1 $\pm$ 0.5	1 $\pm$ 0.5	1 $\pm$ 0.5	-
Polyvinyl chloride	9	21 $\pm$ 1	21 $\pm$ 1	55 $\pm$ 4	80 $\pm$ 5	51 $\pm$ 1

^aChallenge concentration was 500 μg of aromatic amine/ml of methanolic solution.

^bMethylenebis(2-chloroaniline).

^cLower limit of detection or breakthrough concentration is defined as that value (ppm) obtained from the Beer's law plot wherein $A = (0.000 + 2\sigma_{A,0})$. $\sigma_{A,0}$ is the standard deviation of the absorbance at zero concentration. It was determined individually for each compound studied.

^dBreakthrough given in hours with estimated standard deviation..

^eNeoprene in contact with physiological saline solution and Hypalon in contact with the methanolic solution of the amine.

TABLE II

BREAKTHROUGH TIME OF METHANOLIC MOCA^a SOLUTIONS AS A FUNCTION OF GLOVE MATERIAL

Glove Material	Measured Amine Concentration at Breakthrough ($\mu\text{g}/\text{ml}$)	Breakthrough Time (h) ^b
Natural latex, surgeon's gloves	37	2.00 $\pm$ 1.00
Hypalon/neoprene ^c	28	1.00 $\pm$ 0.50
Hypalon	29	1.00 $\pm$ 0.50
Neoprene	29	1.00 $\pm$ 0.50
Butyl	30	1.00 $\pm$ 0.50
Polyethylene	30	1.25 $\pm$ 0.50
Nitrile	28	0.70 $\pm$ 0.25

^aMethanolic methylenebis(2-chloroaniline) at a concentration of 9.8 mg/ml.

^bSee footnote d in Table I.

^cSee footnote e in Table I.

solution, these gloves would not afford complete worker protection for more than 4 h.

An additional set of experiments was run in which glove materials were challenged by 1.0 wt % concentration of MOCA in methanol (OSHA-excluded concentration).¹ Table II shows that, for each of the materials tested, the observed breakthrough time was less than 2 h.

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INTRODUCTION

The primary focus of this group is on environmental studies designed to gain information on the transport, deposition, and mobility in natural systems of a variety of pollutants derived from energy development activities. The behavior of energy system-associated waste materials is documented and evaluated. The direct effects of energy resource development are researched via ecological, geological, and atmospheric descriptions, measurements, and assessments. The information thus obtained may be employed in selecting engineering alternatives and in mitigating adverse environmental conditions.

As indicated in the summary reports which follow, most project activities are field-oriented with analytical and experimental laboratory backup. The approach is generally a descriptive one with the geological and hydrological systems, the

atmospheric conditions, and the ecosystems characterized and sampled by an interdisciplinary team of scientists. This in situ emphasis in environmental research is believed to enhance validity and applicability of the information obtained.

Applied environmental science is emphasized at LASL as indicated by program work on fossil, geothermal, and nuclear energy systems. Responsible development of the potential Rocky Mountain Region energy resources of coal, uranium, and shale oil will depend on increased understanding in the areas of complex terrain-influenced atmospheric transport and dispersion of pollutants; effects of trace-level contaminants on hydrologic systems in arid environments; and ecological behavior and mobility of waste materials in the ecosystems surrounding these resources. Environmental field studies summarized herein include work on stabilization of tailings at uranium processing areas in

New Mexico; ecological fate of waste materials generated from the coal processing operations at the Four Corners area; effects of a dry geothermal energy demonstration plant situated in a fir-spruce forest setting; studies on the behavior of environmentally aged radionuclides, principally plutonium, in a wide variety of ecosystems; and ecological aspects of the development of the trans-Alaska oil pipeline.

Most but not all of the short environmental reports included herein are on tasks associated with projects supported by ERDA's Division of Biomedical and Environmental Research. Some additional topics have been included in the interest of completeness. In each case, reference is made to detailed reports with only general objectives and principal accomplishment features portrayed within these project overview reports.

ECOLOGICAL STUDIES

Ecological Investigation of Dry Geothermal Energy Demonstration

[K. H. Rea, D. A. Dahl, and A. K. Stoker]

The depletion of nonrenewable fossil fuel resources has made it necessary to seek alternate energy forms. One form currently under study by the Los Alamos Scientific Laboratory involves heat extraction from hot dry rock geothermal reservoirs, with potential application to the production of electricity.^{1,2}

The Laboratory's geothermal project presents a unique opportunity to study the environmental impact of a resource extraction technology during both the development and production phases. There are few indicators of the types of environmental problems that may be encountered with the dry hot rock (DHR) heat extraction technique, since the Laboratory's geothermal program is the only one of its kind in the world. Our approach in initiating this study has been to describe quantitatively the ecosystem surrounding the DHR site and to identify the types and amounts of chemicals and/or materials that will be released during the electrical production phase. Specific objectives are to (a) develop an environmental resource data base at the site; (b) determine differences in composition and quantity of ecosystem components near the facility and at adjacent control sites; and (c) identify solubilized chemicals and other materials in effluent waste water.

Water quality monitoring was initiated in 1973 to establish base-line concentrations of 13 elements in surface and ground waters prior to development of the DHR site. Water sampling has continued throughout development of the site and currently includes 9 surface and 14 ground water stations. Results of the chemical analyses are summarized annually in Los Alamos Scientific Laboratory reports.^{3,4} To date, variations observed in the chemical constituents of both surface and ground waters are insignificant.

The DHR site is drained by the Jemez and Rio Guadalupe River systems. Stream flow characteristics of both these systems are well defined with over 16 yr of continuous records at 3 gauging stations. Stations upstream on the Jemez and Rio Guadalupe Rivers were abandoned in June 1976. Regression analyses of the mean annual discharge at

the permanent U. S. Geological Survey station and the 2 abandoned stations indicate good correlation ($r^2 = 0.98$ $P < 0.01$, $r^2 = 0.99$ $P < 0.01$) and allow an accurate means of estimating stream flow from the one set of records.

Weather instrumentation (a mechanical weather station and weighing bucket rain gauge) was installed about 150 m west of the DHR facility to record continuously temperature, wind speed, wind direction, and precipitation. Large variations in these climatic parameters can cause severe changes within the ecosystem which might be accidentally considered an effect of the DHR project if knowledge of the climatic regimes did not exist. Furthermore, information on the temperature regimes and prevailing wind patterns will aid in determining where the thermal plume and any associated contaminants might go. The precipitation data may be utilized also to make crude estimates of run-off from the watershed and thereby allow predictions of transport of materials from the DHR site.

Weather data are reduced by computer into tabular and graphical displays on a monthly basis. The graphical analyses are designed to allow the reader a quick multifaceted view of the data set. Figure 1 (the precipitation summary for July 1976) shows a strong afternoon maximum for precipitation events due to summer convective activity.

The primary concern of this project is the quantification of the DHR energy extraction facility's effects upon the local environs. Environmental impacts, if present, will be most detectable as changes in the natural populations as a function of distance from the DHR site. These impacts should be reflected as changes in composition and densities of small- and large-animal populations and as changes in densities and cover values in plant populations. Base-line data on natural pre-existing gradients within all communities surrounding the DHR site are necessary before statements can be made about the impact of this facility upon the ecosystem.

Three major vegetative complexes exist at the DHR site. A grass-forb complex, which was seeded following a wildfire in 1971, closely surrounds the DHR site and extends westward across the mesa. This grass-forb complex is surrounded by a mixed coniferous forest and is interspersed with closed

Fig. 1. Precipitation summary at the DHR site.

stands of aspen. Before comparisons between areas adjacent to and areas away from the DHR site can be made, the biological components of these complexes must be quantified. Therefore, 4 sets of permanently marked line transects were established to characterize representative portions of each complex. Each set contains at least 3 line transects which are spaced at 50-m intervals, are perpendicular to the DHR site, and consist of 10 contiguous 1-m² plots.

Measurements on cover and density of the dominant species are summarized by complex in Table I. The 15 species shown dominate the 3 complexes; however, due to the great variability exhibited between complexes, selection of 1 or 2 key species for intensive study across all vegetative types is impractical.

Small-mammal populations were studied in each complex as a further quantification of the biological components of the ecosystem. Permanently marked lines consisting of 20 stations with 10 m between stations were trapped on 3 consecutive days in June and again in September. *Peromyscus maniculatus* and *Eutamias minimus* comprised over 90% of

TABLE I

PERCENT COVER AND DENSITY (NUMBER/m²) OF THE DOMINANT SPECIES AT THE LASL DHR SITE

Species	Grass-Forb Site		Vegetative Complex			
	Cover	Density	Conifer		Aspen	
			Cover	Density	Cover	Density
<i>Agropyron spicatum</i>	0.27 ± 0.42 ^a	0.45 ± 0.65	-	-	-	-
<i>Agropyron trachycaulum</i>	38 ± 73	1.2 ± 2.0	-	-	-	-
<i>Blepharoneuron tricholepis</i>	-	-	0.22 ± 0.35	0.67 ± 1.0	-	-
<i>Dactylis glomerata</i>	3.5 ± 2.1	3.5 ± 3.3	-	-	-	-
<i>Koeleria cristata</i>	0.68 ± 0.59	1.6 ± 1.3	-	-	0.46 ± 1.7	2.8 ± 9.0
<i>Phleum pratense</i>	8.0 ± 3.3	11 ± 3.7	-	-	1.6 ± 2.2	5.0 ± 5.0
<i>Sitanion hystrix</i>	0.06 ± 0.13	0.11 ± 0.26	0.53 ± 0.37	3.3 ± 1.8	-	-
<i>Berberis repens</i>	0.94 ± 0.81	2.6 ± 1.9	0.45 ± 0.88	1.6 ± 3.4	1.2 ± 2.7	3.7 ± 8.1
<i>Arctostaphylos uva-ursi</i>	-	-	5.1 ± 6.0	19 ± 26	-	-
<i>Chimaphila umbellata</i>	-	-	0.15 ± 0.39	3.5 ± 4.0	-	-
<i>Quercus gambelii</i>	0.14 ± 0.18	0.07 ± 0.10	1.7 ± 1.7	0.62 ± 0.54	0.8 ± 3.4	1.2 ± 5.0
<i>Pinus ponderosa</i>	0.11 ± 0.12	0.07 ± 0.07	11 ± 16	0.15 ± 0.25	-	-
<i>Populus tremuloides</i>	-	-	2.5 ± 4.1	0.03 ± 0.06	17 ± 5.5	4.9 ± 3.7
Fir (<i>Pseudotsuga menziesii</i> and <i>Abies concolor</i>)	-	-	27 ± 33	0.32 ± 0.47	-	-

^a Confidence levels are expressed as $\bar{x} \pm t_{0.05} s_{\bar{x}}$.

the catch in all communities during both trapping sessions. Capture data for P. maniculatus were sufficient to estimate roughly population density.⁵ The density estimate in the aspen complex was 17 ± 9.2 (S.D.) animals per ha. The highest density was found in the grass-forb area with 23 ± 9.4 animals per ha, and the lowest density was in the mixed coniferous forest with 11 ± 15 animals per ha.

The DHR site environs are a major wintering ground for one of New Mexico's big game species, the Rocky Mountain elk (Cervus canadensis). This animal is easily disturbed and tends to avoid human activity. A unique opportunity exists at the DHR site to quantify the effect of this form of human activity upon the wintering habits of elk. These data will be of great value in predicting the fate of the Jemez Mountain Range elk herd before full development of the known geothermal area occurs. Therefore, pellet group plots were established throughout the grass-forb complex as a means of estimating elk population densities. A total of 112 plots were established utilizing a stratified random design which allows for comparison of

population estimates in an area near the DHR site to those of an area farther removed from the facility. Initial readings of the plots show similar variance structures between outlying and close-in plots, indicating similar frequency distributions around the sample means. The means were not significantly different, indicating uniform elk usage on the mesa in the past.

Two avian population study areas were established in the vicinity of the DHR site to determine the distribution and numbers of birds within the various habitat types before operation of the energy extraction system. These data can be compared to data taken after commencement of the energy extraction operations to determine what effects the system has upon the avian component of the ecosystem. One study area (the well site) is a 20-ha area located on the north side of the DHR site; however, due to encroachment by the DHR facility, this study area has been reduced to an effective area of 17.5 ha. The other area (the open mesa site) is an 18-ha area located on the mesa approximately 0.5 km west of the DHR site. The well site

TABLE II

NESTING POPULATION ESTIMATES OF VARIOUS BIRD SPECIES ON THE DHR SITE
AND GRASS-FORB SITE DURING THE SUMMER OF 1976

Species	Well Site		Grass-Forb Site	
	Total Pairs	Pairs/40.5 ha	Total Pairs	Pairs/40.5 ha
Black-chinned hummingbird	2	5	2	4
Common flicker	2	5	3	7
Yellow-bellied sapsucker	1	2	2	4
Hairy woodpecker	2	5	1	2
Traill's flycatcher	1	2	0	0
Western wood pewee	2	5	0	0
Violet-green swallow	3	7	0	0
White-breasted nuthatch	1	2	0	0
Pygmy nuthatch	2	5	0	0
Pause wren	2	5	1	2
American robin	1	2	0	0
Western bluebird	3	7	1	2
Warbling vireo	1	2	0	0
Yellow-rumped warbler	2	5	0	0
Western tanager	5	12	0	0
Cassin's finch	1	2	0	0
Green-tailed towhee	0	0	5	11
Gray-headed junco	3	7	2	4
Totals	34	80	17	36

encompasses parts of 3 ridges and 2 small gullies which are well balanced in composition between the 3 vegetative complexes. In contrast, the open mesa site is dominated by the grass-forb complex. Censuses were conducted on 2 consecutive days during the spring (March), summer (June), and autumn (November) months to determine seasonal changes of the populations in both numbers and utilization of habitats.

Spring and autumn bird populations were very modest in both numbers and species because of elevation, exposure, and severe weather conditions of the area. A total of 41 species were noted during the census periods, but only 18 of those were found to nest within the study areas (Table II). Twice as many species nested within the well site as within the open mesa site due to a more diverse habitat and the "edge effect" represented by the coniferous forest islands.

All data collected during the 1976 field season will be subjected to statistical analysis and the results published. From the brief analyses done to date, it is apparent that these communities possess large natural variability. Therefore, extensive data will need to be collected on an annual basis to detect changes in these communities which can be attributed to the DHR energy extraction project.

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Ecological Consequences of North Slope Oil Development

[W. C. Hanson and L. E. Eberhardt]

Forty arctic foxes (*Alopex lagopus*) were ear-tagged at Prudhoe Bay during the spring and summer months, bringing to 125 the total number of animals marked in that area since studies began in March 1974. Several tag returns have been received from animals killed in the immediate Prudhoe Bay area, usually by vehicle traffic; in addition, 2 long-range reports were received from native Canadian Eskimo trappers that represented movements of 720 and 1100 km.

Population densities of arctic foxes in the Prudhoe Bay area appeared to be greater than during 1975. However, since this study began, there has not been any evidence of the dramatic cyclic fluctuations in population densities that typify arctic fox populations in other circumpolar locations. A resident trapper on the Colville River delta, some 80 km west of Prudhoe Bay, indicated that fox populations in his area have been unusually high during early periods of the trapping season over the past few years but that nearly all animals disappear by late December and reappear in early March. This suggests that the foxes moved to Prudhoe Bay to scavenge for food in the oil camps when natural food became scarce and that this resulted in moderation of the normal cyclic population fluctuations. In an effort to document the suspected mass movement of arctic foxes to Prudhoe Bay during winter months, 16 foxes were tagged on the Colville River delta adjacent to U. S. Naval Petroleum Reserve Number 4, and efforts will be made to resight or retrap these animals at Prudhoe Bay during January 1977.

Blood samples have been drawn from 69 animals during this study for purposes of disease analysis, particularly rabies, in cooperation with the National Center for Disease Control and the Alaska Division of Public Health. No detectable levels of rabies antibodies were found in 40 samples collected during last winter; results are pending on the 29 samples collected this summer. Emphasis is being placed on rabies research because the disease represents a potential serious human health hazard should it become epizootic, as it was prior to 1960. There has currently been an outbreak in western and

northwestern Alaska that might be magnified by the unusual concentrations of foxes at Prudhoe Bay.

Research was also conducted at the Alaska Division of Public Health laboratory to evaluate corneal smears and skin biopsies as a diagnostic tool in rabies epidemiology. The results indicated that several drawbacks to these methods may be present and that reliance on blood samples will be required.

Sixteen foxes were radio-collared and tracked this field season to determine local movement patterns of the Prudhoe Bay foxes, particularly in response to oil field development activities. A detailed analysis of the telemetry data awaits computer reduction; however, preliminary observations indicate that the home ranges of denning adult foxes varied from 13 to 26 km² (Fig. 1). Visits to oil camps and garbage dumps by adult and juvenile foxes were quite limited during early periods of the denning season but became much more frequent during the summer as the pups became mobile and independent (Fig. 2). By late summer, some families had moved entirely into the camps and were living on food

Fig. 2. Home range of juvenile arctic fox.

scavenged from oil field employees and the garbage dumps.

Studies were continued on both arctic and red foxes (*Vulpes fulva*) in the vicinity of the trans-Alaska pipeline between Prudhoe Bay and the Happy Valley construction camp, a distance of about 100 km. This entailed periodically visiting all known red and arctic fox dens discovered during 1974 and 1975 to determine their 1976 status, conducting searches for new dens, and gaining additional information from pipeline camp personnel that will allow an evaluation of the possible ecological competition of these closely related species. Observations of fox behavior were made at selected dens placed under 24-h surveillance once a week to record 5-min intervals of duration and activity of adult fox visits to dens, number and types of prey carried to the den, directions of travel, adult/pup interactions, pup/pup interactions, expansion of pup activity areas, and other behavioral data.

Ecological studies of birds and small mammals in the vicinity of an experimental natural gas pipeline installation were continued for the second year. Data obtained during the summers of 1971, 1972, and 1973 under industry-sponsored research programs were made available for publication and

Fig. 1. Home range of adult arctic foxes.

TABLE I

NESTING BIRDS IN VARIOUS SECTORS OF THE PRUDHOE BAY STUDY AREA

Sector	Area (ha)	Semipalmated Sandpiper				Lapland Longspur			
		1972	1973	1975	1976	1972	1973	1975	1976
Arctic Gas	6	12	10	10	7	1	1	2	5
North	9	1	8	5	5	0	2	2	1
West	20	4	5	5	4	5	4	2	2
Southwest	25	3	5	1	3	2	3	2	1
Southeast	15	3	5	2	2	5	5	2	2

comparison with current research results; this will not only extend the data base but will also facilitate predictions about long-term effects and recovery of tundra ecosystem components from petroleum resource developments.

The 1976 nesting season for tundra birds was considerably less successful than during 1975 because of inclement weather that maintained a lingering snow cover and large expanses of standing water in low-center polygon and Carex meadow habitats and a high degree of predation. Numbers of nests of semipalmated sandpipers and Lapland longspurs found on the 82-ha study area at Prudhoe Bay are shown in Table I to illustrate the shifting of these species in occupation of available nesting habitat and increased nesting during the 1973 optimum conditions. A total of 92 nesting birds of 4 species were banded during the 1976 season, and 12 birds banded in previous years were recaptured, providing new data on nest site and pairing fidelity.

Small-mammal population studies were again conducted by live-trapping within 17 grids of 0.116 ha each within the Alaskan arctic gas study area and its periphery. A population density of 7.1 collared (or varying) lemmings per ha was estimated from 8200 trap-nights of effort during July and August; this was a decrease from the 19.2 lemmings per ha estimated last year. Greater mobility of the lemmings, particularly adult males, was noted from observations of tagged individuals that repeatedly crossed pipeline berms and made journeys of 300 to 500 m straight-line between points of capture.

Study areas were established during early May near Franklin Bluffs, 50 km south of Prudhoe Bay, and near Happy Valley, 100 km south of Prudhoe Bay,

for purposes of determining the impact of the installation and operation of the trans-Alaska pipeline and haul road upon small mammals and tundra-nesting birds. The Happy Valley site was abandoned on May 29 after repeated damage to the personnel tent by a grizzly bear that had been pauperized by pipeline construction activities. The Franklin Bluffs study area consisted of a 400-x 2500-m gridded bird study plot and 32 small-mammal trapping grids of 0.116 ha each, situated in such a manner to measure changes in animal populations that would be affected by pipeline and road activities, if such occur. The inclement weather conditions that prevailed over most of the North Slope were apparently responsible for the very low nesting densities of the bird species (15 nests/100 ha) compared to those at Prudhoe Bay (60 nests/100 ha); although the Franklin Bluffs site contained a greater diversity of species. Forty-three birds representing 6 species were banded and color-marked for continuing studies of population dynamics of those species.

Live-trapping revealed that the collared (or varying) lemming, Dicrostonyx groenlandicus, was the most abundant small mammal in the Franklin Bluffs habitats, and a population density of about 4.3 animals/ha was estimated from tagging and recapturing the animals. Next in importance was the tundra vole, Microtus oeconomus, followed by the singing vole, Microtus miurus, and the brown lemming, Lemmus trimucronatus.

A Survey of Plutonium Concentration Variability in Soil Samples from Various Locations

[T. E. Hakonson]

The large variability associated with environmental plutonium data is currently recognized as a major problem in designing field studies of this

element. The large sample sizes required for acceptable statistical control of an experiment place severe restrictions on the kinds of research questions that can be addressed within the limits of time and money.

The purpose of this review is to summarize some of the available data on plutonium concentration variability in terrestrial soil components and to identify potential sources of this variability, to serve as guidance in designing studies that are both efficient and effective in achieving desired goals. Plutonium data from 7 geographical regions representing 15 terrestrial study sites were selected for review based on the availability of data, source of study area plutonium, sampling methodology, and regional climate. The intent in selecting specific study areas was to present plutonium concentration variability estimates from a diverse array of study-related factors.

Description of Some Environmental Plutonium Study Areas.--The 7 sites chosen to represent the varied conditions under which plutonium can exist in the environment are listed in Table I. Sources of plutonium in these areas include industrial liquid effluents (Los Alamos), accidental releases from industrial and military sources (Rocky Flats; Thule, Greenland), fallout from both single and multiple weapons tests (Trinity Site; Glenn and Janet Islands, Eniwetok Atoll), and nonfission explosive tests with plutonium devices (Nevada Test Site).

Plutonium Concentration Variability in Study Area Soils.--The variability in soil plutonium concentrations, expressed as the coefficient of variation (CV, standard deviation/mean), ranged from 0.21 to 3.2, while plutonium concentrations varied from 0.15 to 460 000 pCi/g (Table II). The pattern was similar in whole soil samples to various depths in

TABLE I

SOME CHARACTERISTICS OF ENVIRONMENTAL PLUTONIUM STUDY AREAS

<u>Location</u>	<u>Source of Plutonium</u>	<u>Mean Annual Precipitation (cm)</u>	<u>Reference</u>
<u>Los Alamos</u>			
Mortandad Canyon	Industrial liquid effluent	46	1-3
DP-Los Alamos Canyon		46	1-3
Acid-Pueblo Canyon		46	1-3
<u>Trinity Site</u>	Single weapons test		
Ground Zero	1 km from Ground Zero	15	4,5
Area 21	44 km from Ground Zero	20	4,5
<u>Eniwetok Atoll</u>	Weapons test		
Janet	Multiple ground zeros	145	6
Glenn	Multiple fallout	145	6
<u>Rocky Flats</u>	Unintentional		
Macroplot 1	Release from leaking drums	40	7,8
Macroplot 2		40	7,8
<u>Nevada Test Site</u>			
Area 13	Safety test shots		
Strata 1		8	9,10
Strata 6		8	9,10
Area 5 (GMX)			
Strata 1		8	9,10
Strata 4		8	9,10
<u>Thule, Greenland</u>	Aircraft accident	13	11

TABLE II
PLUTONIUM CONCENTRATION AND VARIABILITY ESTIMATES IN SOME STUDY AREA SOILS

Location	n	0 to 2.5 cm		n (depth, cm)	To Depth	
		mean (pCi/g)	CV		mean (pCi/g)	CV
<u>Los Alamos</u>						
Mortandad Canyon	5	140	0.52	15 (30)	90	0.79
DP-Los Alamos Canyon	6	0.42	0.63	21 (30)	0.73	1.6
Acid-Pueblo Canyon	7	10	0.48	21 (30)	21	1.7
<u>Trinity Site</u>						
Ground Zero	8	0.44	0.82	8 (25)	0.07	0.68
Area 21	8	2	0.48	8 (33)	0.14	0.68
<u>Eniwetok Atoll</u>						
Janet	12	18	1.9	138 (15)	16	1.3
Glenn	3	0.15	0.21	29 (15)	0.11	0.62
<u>Nevada Test Site</u>						
Area 13						
Strata 1	4	880	1.2	39 (5)	36	1.4
Strata 6	3	460 000	0.67	47 (5)	14 000	3.1
Area 5						
Strata 1	5	2 200	1.5	41 (5)	59	1.4
Strata 4	2	150 000	0.8	23 (5)	7 300	1.1
<u>Thule, Greenland</u>				6 (5)	0.16	1.5
<u>Rocky Flats</u>						
Macroplot 2	12	116 ^a	3.2			

^aConcentration in $\leq 45\text{-}\mu\text{m}$ size fraction to depth of 3 cm.

TABLE III

PLUTONIUM CONCENTRATION COEFFICIENT OF VARIATION
IN SURFACE SOILS

Location	Plutonium Source	CV
Glenn	Fallout	0.21
Trinity, Area 21	Fallout	0.48
Mortandad	Liquid effluent	0.52
DP-Los Alamos	Liquid effluent	0.63
NTS-A-13, Strata 6	Safety shot	0.67
NTS-A-5, Strata 4	Safety shot	0.80
Trinity, GZ	Ground zero	0.82
Acid-Pueblo	Liquid effluent	0.87
NTS-A-13, Strata 1	Safety shot	1.2
NTS-A-5, Strata 1	Safety shot	1.5
Thule, Greenland	Aircraft accident	1.5
Janet	Multiple ground zeros	1.9
Rocky Flats		
Macroplot 2	Industrial accident	3.2

that the range in CVs (0.62 to 3.1) was relatively small compared to the range in plutonium concentrations (0.11 to 14 000 pCi/g).

There were no significant linear relationships between the magnitude of the CV and the corresponding plutonium concentration within the ranges of the data. Generally lower variability was associated with fallout (Trinity Site) and liquid effluent (Los Alamos) sources of plutonium (Table III), while higher CVs were associated with safety shot (NTS) and Ground Zero areas (Trinity, Janet). This pattern seems reasonable based on suspected physical forms of the plutonium in the respective study areas. However, other site-related factors such as study area climate (and its effects on plutonium weathering), topography, and sampling methodology contribute to the total variability and confound any attempts to

TABLE IV

SOIL SAMPLING AND ANALYTICAL METHODOLOGY AT VARIOUS STUDY SITES

Location	Sampling Technique	Sampling Area (cm ²)	Sample Pretreatment	Amount Analyzed (g)
Los Alamos	Core	4.5	None	Whole sample ~ 25
Trinity	Core	4.5	None	Whole sample ~ 25
Eniwetok	Core	30	Ball-milled	10-50
Nevada Test Site	Core-excavation	100-127	Ball-milled	10
Thule, Greenland	Excavation	100	Remove > 0.6 cm 5 samples composited	~ 100
Rocky Flats	Excavation	25	Remove > 0.5 cm	5-10

TABLE V

SPATIAL RELATIONSHIPS OF ²³⁸Pu CONCENTRATION VARIABILITY IN MORTANDAD CANYON SOILS

	Overall	Within Strata		Within Plot	
		0 to 160	160 to 2560	0 to 160	160 to 2560
Coefficient of variation	1.2	0.79	0.94	0.75 (0.31)	0.85 (0.60)
n	27	15	12	5	4

relate variability to any specific factor. The difficulties in comparing soil plutonium data between sites can be appreciated by examining some of the potential components of variability at the various sites.

Soil sampling and analytical methodology employed at several sites are summarized in Table IV to emphasize the potential for these factors as components of the total variability. Although chemical procedures for plutonium analysis between sites are similar in that they involve HNO₃-HF digestion, followed by column separation and alpha spectrometry, considerable differences exist in sampling techniques, sample preparation, and in the size of soil aliquot analyzed for plutonium.

One of the sources of field sampling variability for soil plutonium is the heterogeneous distribution of the element within the study areas. An example of the effect of spatial heterogeneity on variability is given in Table V for Mortandad Canyon at Los Alamos where a plutonium concentration gradient exists with distance down the canyon. The CVs in Table V were calculated for all samples (n = 27) collected within a 2560-m segment of stream channel, for two strata (0 to 160 and 160 to 2560 m) within that segment, and for triplicate samples taken at each of 9 plots within that segment.

The variability was considerably reduced by examining the data from smaller spatial units as achieved through stratification. The concentration CV was calculated as 1.2 when all the data from the contaminated portion of the canyon were used, whereas the data from two segments of stream channel reduced the CV to 0.79 and 0.94 for the respective strata. Within-plot variability based on triplicate samples from each sampling location within the 2560-m segment averaged 0.75 and 0.85 for the two strata, indicating that closely spaced replicate samples did not greatly reduce the variability as measured over much larger areas as represented by the two strata.

The relationships between soil plutonium concentration variability, soil particle size fraction, and soil depth were investigated at those sites where data were available (i.e., Los Alamos and Rocky Flats) to examine further the components of plutonium concentration variability. The data in Table VI illustrate the relationship of the CV with soil particle size fraction and soil depth for soils from Mortandad Canyon at Los Alamos. There were no significant relationships between CV and soil particle size fractions in either study area as inferred by analysis of variance. However, in all cases, there was a significant change ($p \leq 0.05$) in CV with soil depth.

TABLE VI

RELATIONSHIP OF PLUTONIUM CONCENTRATION VARIABILITY (COEFFICIENT OF VARIATION)
WITH SOIL PARTICLE SIZE FRACTION AND SOIL DEPTH IN MORTANDAD CANYON

Depth Profile (cm)	Soil Particle Size Fraction					
	(< 53 μ m)	(53 to 105 μ m)	(105 to 500 μ m)	(500 to 1000 μ m)	(1 to 2 mm)	(2 to 23 mm)
0-2.5	0.84	0.49	0.79	0.65	0.64	0.48
2.5-7.5	0.58	0.54	0.62	0.58	0.93	0.62
7.5-12.5	0.38	0.49	0.44	0.65	0.46	0.55
12.5-22	0.44	0.35	0.13	0.11	0.16	0.50

All available data were examined using linear least-squares techniques to determine the significance of plutonium concentration vs depth relationships (Table VII). Only four comparisons were significant ($p \leq 0.05$): those from Glenn, Trinity Site (Ground Zero and Area 21), and macroplot 1 at Rocky Flats. Regression slopes for the first three areas were positive, whereas the slope was negative for the Rocky Flats area. It would appear that a clear definition of such relationships would be instrumental in efficient study design and would provide insight as to the mechanisms of soil plutonium mobility.

Conclusions.--Plutonium concentration variability in soils from 7 geographical regions ranged from 0.48 to 3.2. The CV was relatively consistent, considering that plutonium concentrations varied through 6 orders of magnitude and appeared to be

independent of the magnitude of plutonium concentration within the ranges of observed data. Vertical and horizontal inhomogeneity in plutonium contamination within study areas comprises a part of the total observed variability, while soil particle size fractions do not appear to contribute significantly to the overall variability at those sites examined.

The relationship between CV and soil depth was statistically significant in the study areas at Trinity and Glenn and at macroplot 1 at Rocky Flats. The CV increased with depth at the Trinity Site and Glenn study areas and decreased with depth in macroplot 1 at Rocky Flats.

Lower CV values were generally associated with fallout and effluent sources of plutonium. However, the diversity of environments and "age" of the plutonium at the various study sites likely contribute to overall variability and certainly complicate interpretation of data between sites.

Highest CVs were associated with accidental releases of plutonium. This type of release would be the most likely source of plutonium to presently uncontaminated ecosystems.

Differences in methodologies at the various study sites make comparison of the data difficult and emphasize the need for coordination of effort between sites to improve the utility and comparability of the data.

Studies should be designed to look specifically at the components of overall variability. Data from Rocky Flats and Los Alamos indicate that the analytical component of overall variability contributes less than 35% to the total. Other factors which might be considered include variability in mass of soil particle size fractions within study plots and the relationship of plutonium CV to the variability in other soil physical-chemical properties.

TABLE VII

LINEAR REGRESSION OF SOIL COEFFICIENT
OF VARIATION WITH DEPTH

Location	a_0^a	a_1	r^2
Glenn	0.38	0.02	0.59 ^a
Trinity Ground Zero	0.93	0.05	0.83 ^a
Trinity Area 21	0.75	0.04	0.82 ^a
NTS-A-13, Strata 1	1.8	-0.02	0.17
NTS-A-13, Strata 6	0.63	0.02	0.16
NTS-A-5, Strata 1	0.98	0.01	0.06
NTS-A-5, Strata 4	1.1	-0.02	0.28
Janet	1.7	0.02	0.27
Rocky Flats			
Macroplot 1	1.6	-0.06	0.53 ^a
Macroplot 2	2.2	-0.05	0.22

^aEquation of the form $y = a_0 + a_1 x$, where $y = CV$ and $x = \text{soil depth (cm)}$.

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Rainout Collateral Damage Study

[S. Barr and J. D. Klett]

Precipitation Morphology.--Studies of precip-
itation structure in the atmosphere have been
initiated in connection with an assessment of col-
lateral damage due to precipitation scavenging of
nuclear weapon debris. The insights gained from
these studies and the computational model developed
as the vehicle for expressing these insights promise
to be valuable additions to the assessment tech-
nology in a wide variety of practical problems
involving precipitation effects.

The fundamental premise is that precipitation
exhibits a high degree of variability in time and
space from less than 60 sec and 100 m out to the
scales of large-scale synoptic weather systems. In
applications which depend on the interaction of
several physical processes, each of which have
small-scale variations, the preliminary smoothing
of one process prior to estimating its covariant
interactions with the other processes can lead to
errors in the result. For example, the scavenging
effects on pollutant clouds which have dimensions
of less than a few kilometers depend on the inter-
action of that pollutant with a precipitation cell.
If the precipitation field also has variability on
the same scale, it is incorrect to assume that the
field is uniform. The data bases typically avail-
able for assessment projects have a spacing between
observation points of 10 to 100 km. In problems
that are driven by higher resolution effects (e.g.,
some scavenging problems, drainage basin runoff,
air-frame damage, electromagnetic energy trans-
mission), a subgrid-scale simulation is necessary.

The simulation of fine-scale precipitation
variability has been addressed in a computer code
(TEMPEST) which uses Monte Carlo techniques to esti-
mate the fundamentally stochastic aspects of rain-
fall morphology. The basic unit adopted in the
model is a cell, and the current representation of
the major elements of cell morphology are given in
Table I.

TEMPEST incorporates a sophisticated geometry
package, MCN,² which allows a very flexible specifi-
cation of number and shapes of precipitating zones.
The input parameters for rainfall character are zone-
dependent so that it is a straightforward matter to
construct a synoptic storm with its regions of
stratiform precipitation, drizzle frontal showers,
and post-frontal showers.

In preliminary tests of the code, we have
simulated the time history of rainfall collection at
a stationary rain gauge with a resolution of 0.1 h.
These were compared with high resolution exper-
iments^{3,4} to select the first round of input param-
eters. The ability of the model to produce integral
statistics on 1-, 3-, and 6-h accumulations was then
tested against traditional climatological data to
identify the spectrum of rain-area-fractions and inten-
sity distributions for a selected geographic site.

TABLE I
THE MAJOR ELEMENTS OF CELL MORPHOLOGY

1. Cell Size Distribution

Cell areas are log normally distributed according to $f(A_m) = \frac{1}{A_m \sqrt{2\pi\sigma_m^2}} \exp \left[-\frac{1}{2\sigma_m^2} (\ln A_m - m)^2 \right]$,

where A_m = cell area at maximum value during lifetime, $m = 2.5$ to 3.2 , $\sigma_m^2 = 0.3$ to 1.0 .

2. Rain Area Fraction

A number which reflects the fractional area coverage by precipitation in an identified precipitation zone.

3. Internal Cell Intensity Distribution

Maximum cell intensity R is distributed according to a specified probability distribution function. For location and time other than the maximum, intensity is adjusted through the function: $R(s, y, z, t) = R_m f(x, y) g(z) h(t)$.

- a. The horizontal dependence of R is determined by $f(x, y)$ which is presently in the form of a Gaussian function, $f(x, y) = \exp \left\{ -\gamma \left[\left(\frac{x}{x_m} \right)^2 + \left(\frac{y}{y_m} \right)^2 \right] \right\}$, where x_m and y_m are cell dimensions along principal axes of the elliptical cell. The parameter γ determines the extent of low-rate "background" rain and the peakiness of showers. Note γ choice interacts with area and rain area fraction.
- b. Cells undergo life cycles described by $h(t) = 1 - \alpha (t - t_0)^2$, where t_0 is the time of peak rainfall and α is a lifetime duration parameter.
- c. Vertical structure is given by $g(z)$, currently a normalized empirical function derived from O'Reilly.¹

4. Shape and Orientation

Shape and orientation with respect to the mean wind are two other parameters which have random variations. The shape is currently an ellipse with the ratio of principal axes determined randomly from a Gaussian distribution. Orientation is also treated as a Gaussian.

5. Movement

Movement is selected to be parallel to the mid-tropospheric wind.

It is important to note that TEMPEST is a research model in a developing stage. As new insights are gained into each of the component elements, they are updated.

Precipitation Scavenging.--A reasonable estimate of the net scavenging coefficient A (the fractional depletion rate of debris aerosol concentration in a precipitating cloud) is a basic requirement for any quantitative assessment of the rainout problem. However, since only approximate descriptions exist for most of the important scavenging processes, even when they occur in isolation and involve only idealized particles (e.g., spheres or infinite cylinders of homogeneous properties), it is clear that a truly definitive calculation of A will be unattainable for some time to come.

Nevertheless, in spite of the complexities of the problem, an important conclusion, making

possible a simple estimate of A , can be drawn from the many idealized case studies which have been carried out in recent years.³⁻⁷ These imply that in-cloud scavenging by convective, precipitating systems may be regarded basically as a two-stage process. In the first, all the scavenging mechanisms, and especially nucleation, Brownian and turbulent diffusion, and diffusion and thermophoresis, serve typically to transfer a major function of the debris particles (assumed here to be characterized by radius $r \leq 1 \mu m$) to the cloud water, predominantly the fraction comprised of small droplets and/or ice crystals which together possess most of the total absorbing surface of the cloud water, within a relatively brief time period of the order of 20 min or less. In the second stage, this polluted cloud water is scavenged primarily through inertial capture by relatively large precipitating

cloud particles (raindrops, snowflakes, and graupel or soft hail particles). This accretion process can cause a major fraction of the polluted water to fall out of the cloud in a time period similar to, though generally somewhat larger than, that for stage one. Thus, net fractional depletions of debris particle concentrations of the order of unity may occur within the span of 1 h, corresponding to an overall scavenging rate of 10^{-3} to 10^{-4} sec $^{-1}$. This overly theoretical conclusion is quite consistent with the observation, based on experiments in the field, that in-cloud scavenging operates with approximately the same efficiency as the processes which convert water vapor to precipitation.⁸

This description of the nature of in-cloud scavenging, in which the second stage is generally rate-limiting for the entire process, suggests that a reasonable upper bound for the effective scavenging rate is given by the accretion rate of small cloud particles by precipitation, with the former assumed to have already absorbed the debris aerosol through stage one processes. On the basis of this physical picture, we have obtained the following estimate for A in terms of the local precipitation rate R, expressed in units of mm rain/h:

$$A \text{ (sec}^{-1}\text{)} = 1.3 R^{0.79}. \quad (1)$$

To obtain this result, we have assumed a steady-state rain spectrum in accordance with the empirical description of Marshall and Palmer,⁹ namely:

$$n(a) = n_0 e^{-\alpha a}, \quad (2)$$

where $n(a) da$ is the number of drops/m 3 of radii (in cm) on the interval $(a, a + da)$, $n_0 = 1.6 \times 10^5 \text{ m}^{-3} \text{ cm}^{-1}$, and $\alpha = 82 R^{-0.21} \text{ cm}^{-1}$. Equation (1) is in good agreement with a numerical computation by Crandall *et al.*³ of A for contaminated droplets of radii $\geq 10 \text{ cm}$. From Equation (2) we can also calculate the effective local transport velocity $\bar{v}$ of the debris-laden rain:

$$\bar{v} \text{ (msec}^{-1}\text{)} = 3.1 R^{0.16}. \quad (3)$$

This mass-average velocity is consistent with data reported by Fullerton and Wilson.¹⁰ Given R, Equations (1) and (3) enable one to predict the deposition pattern to be expected from precipitation scavenging.

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Vegetative Stabilization of Uranium Spoils Areas

[E. Kelley and L. D. Potter (Biology Department, University of New Mexico, Albuquerque) and C. L. Wienke]

Physical and chemical properties of uranium mill tailings and mine spoils in conjunction with the harsh climatic factors of the semi-arid Southwest often preclude effective vegetative stabilization without the extreme costs of added amendments and continued maintenance. This phase of the "Ecological Investigations of Rehabilitation of Uranium Mill Tailings of the Southwestern United States" was undertaken to determine those environmental factors favoring natural plant succession on uranium spoils in the Grants mineral belt of New

Mexico. Studies are being conducted at two inactive carbonate and one acid mill tailings piles.

Observations at tailings piles indicated that (1) vegetation is sparse; (2) several species of plants are able to become rooted and continue growth in the carbonate tailings material; (3) germination occurs most frequently on deposits of blow sand; and (4) species rooted in the tailings material are as healthy and vigorous in appearance as those in the native soils of the area. Detrimental effects noted were (1) erosion of the blow sand from precipitation runoff which washed away seedlings;

(2) sandblast damage causing seedling death during periods of high winds; (3) a hard, dry, compacted surface which prevents root penetration; (4) a low (~25 cm) annual precipitation average; and (5) a rapid loss of moisture through evaporation from the surfaces of the tailings material.

Table I presents a summary of the analyses for pH, cation exchange capacity (CEC), and electroconductivity. The low pH of the acid tailings, low CEC in all the tailings material, and high conductivity readings encountered in one carbonate tailings pile could be a limiting factor in allowing

TABLE I
ANALYSIS FOR pH, CATION EXCHANGE CAPACITY (CEC), AND ELECTROCONDUCTIVITY (Ece)^a

	<u>Site A Acid</u>		<u>Site B Carbonate</u>		<u>Site C Carbonate</u>	
	<u>$\bar{x}$</u>	<u>S. D.</u>	<u>$\bar{x}$</u>	<u>S. D.</u>	<u>$\bar{x}$</u>	<u>S. D.</u>
pH (extract)	3.87	0.37	9.05	0.35	8.27	0.20
pH (paste)	3.28	0.15	9.35	0.41	8.42	0.17
Ece (mmhos)	4.90	2.04	4.87	2.37	9.86	4.65
CEC (meq/100 g)	1.96	0.89	6.98	3.04	5.27	2.97

^aNumber of samples = 12.

TABLE II
ANALYSES OF ELEMENTS EXTRACTED FROM MILL TAILINGS SAMPLES (in ppm)^a

<u>Element</u>	<u>Site A Acid</u>		<u>Site B Carbonate</u>		<u>Site C Carbonate</u>	
	<u>$\bar{x}$</u>	<u>S. D.</u>	<u>$\bar{x}$</u>	<u>S. D.</u>	<u>$\bar{x}$</u>	<u>S. D.</u>
Calcium	1400	920	86 500	10 600	32 000	8100
Magnesium	91	77	1100	280	140	50
Sodium	140	110	1800	900	2200	1000
Potassium	11	5	100	27	98	42
Iron	47	21	22	9	37	13
Zinc	0.9	0.7	0.4	0.2	3.1	3.7
Cadmium *	0.17	0.24	0.03	0.02	0.05	0.04
Copper	0.70	0.60	0.50	--	0.20	0.36
Manganese	86	71	120	15	220	43
Lead *	3.9	3.0	19	4.6	10	2.6
Mercury	0.01	0.01	0.01	0.004	< 0.005	--
Cobalt *	0.68	0.44	0.25	0.07	0.42	0.45
Chromium *	0.11	0.04	0.22	0.06	0.09	0.03
Nickel *	0.45	0.36	0.39	0.14	0.74	1.0
Arsenic	1.1	0.35	1.8	0.5	2.9	2.1
Selenium	0.41	0.27	0.03	0.02	14	5.1
NO ₃	2.0	0.59	8.0	5.4	0.88	0.68
Chlorine	160	190	77	16	58	32

^aNumber of samples = 12, except where noted by * where n = 4.

plant establishment on the raw tailings material.

Table II presents results of the chemical analyses of the tailings material. Elements identified as potential problems in the mill tailings are sodium in both carbonate piles and selenium in one carbonate pile. The acid tailings did not have excessive amounts of any element and were considerably lower in available calcium than the carbonate tailings. This may indicate that liming will be necessary for revegetation of acid process mill wastes.

Nineteen revegetation test plots have been established for one growing season. Local soils and mine spoil material were used as cover for 16 of these test plots. Composites of each cover material have been analyzed for the same elements as were the tailings samples. Plots were covered to depths of 5, 10, 20, and 30 cm with these materials. One plot at each site was established on the raw tailings material as a control. A mixture of native grasses and one shrub species was used to seed the plots.

Grass seedlings were observed growing on the carbonate and acid tailings material along the edges of the covered test plots. This edge area is influenced by runoff from the test plots and/or from continual disturbances of the surface by foot traffic or the investigators working around the test sites.

The results of these test plots are inconclusive at this time, but preliminary indications are that (1) a specific geologic member of the mine spoil material (Tres Hermanos sandstone) is of superior quality in allowing plant establishment and growth than either the raw tailings material or the local soils when used as a covering material; (2) the soil-covered plots had more seeds germinate than on the control plots; (3) survival of the seedlings was best on the Tres Hermanos sandstone mine spoil material; and (4) plant species can grow on the raw tailings material if there is a slight modification of the surface.

Although there are chemical factors that could be limiting to the establishment of vegetation on mill tailings, the preliminary conclusions, based on observation and testing, indicate that the physical factor of surface drying may be the most serious in preventing plant growth on these tailings piles.

Future research will concentrate on both chemical and physical aspects of the problem. Determination of chemical limitations will be tested by seed germination experiments using leachate from the mill tailings, greenhouse experiments for germination and growth response on raw tailings material, and field test plots utilizing straw mulch, burlap, and soil as surface modification on the tailings material. These plots will be seeded with native grass species to determine which surface modification gives the best results.

Terrain Influence on Low-Level Meteorological Transport

[S. Barr and W. E. Clements]

The objectives of the project are to define and document the major effects of terrain irregularities on the transport, dispersion, and deposition of effluents released to the atmosphere. These goals are pursued through an integrated program of problem definition, theory, field experiments, and computational modeling. Initial efforts have been directed toward studying the processes in a single canyon and their interactions with the mesa-level wind. This local problem has been addressed with simple slope-flow theory, wind and temperature data analysis, visual tracers, fluorescent particle tracers, and hydrodynamic codes.

A previous annual report¹ established the rationale for intensive field studies in Los Alamos Canyon and offered preliminary observations of wind, temperature, and turbulence modifications introduced by the canyon topography. Continued efforts since the last progress report have focused on (1) extending the background meteorological measurements to cover sample periods from all seasons of the year; (2) a series of visual tracer experiments to characterize the diffusive properties of the nocturnal downslope flow; and (3) initiation of a series of quantitative tracer experiments run under nighttime drainage conditions to document the diffusive character over travel distances of 6 km.

Wind and temperature measurements from an array of sensors covering both rims and the floor of Los Alamos Canyon have provided climatological statistics of canyon winds. Some of these gross features are summarized in Table I. They have also yielded a number of case studies of physical processes. One

TABLE I

COMPARISON OF DOWNSLOPE FLOW PROPERTIES IN LOS ALAMOS CANYON AND THE NORTH FORK OF PAJARITO CANYON

Property	Mean Value $\pm$ Standard Deviation			Units
	Los Alamos Canyon (Fall 1974)	Los Alamos Canyon (Summer 1975)	Pajarito Canyon (Summer 1974)	
Onset time	3.4 ± 1.4	1.8 ± 1.1	3.6 ± 3.0	Hours after the temperature begins to drop in the canyon
End time	0.6 ± 1.3	0.8 ± 0.6	0.0 ± 1.0	Hours after the temperature begins to rise in the canyon
Flow duration	15	13	9	Hours
Flow speed	1.5 ± 0.3	0.6 ± 0.2	0.14 ± 0.07	m s^{-1}

phenomenon of practical interest is an eddy caused by flow separation under cross-canyon winds.

The onset of the canyon rotor is related to wind speed at mesa level. A more detailed look at the onset of a rotor in Los Alamos Canyon is shown in Fig. 1. Wind and temperature are given at both canyon and mesa level from 1800 to 2400 MST on November 19, 1975. From 1800 to 2100 MST, the mesa winds were less than 2 m s^{-1} from the south-southwest (cross-canyon), while the canyon wind remained less than 1 m s^{-1} from the west-southwest. The more westerly component of the canyon wind is attributable to a westerly drainage component in the canyon. During this time, the canyon temperature remained some 8°C lower than the mesa, again indicating poor mixing. At about 2030 MST, the mesa winds began to increase and reached 4 m s^{-1} at 2200 MST with a slight shift to the west.

Fig. 1. Hourly average wind and temperature 1.5 m above the ground in Los Alamos Canyon and on adjacent mesa.

Meanwhile, the canyon winds shifted to north-northwest, picking up a northerly component from the induced rotor. The canyon temperature began to rise in this period, reaching the mesa-level temperature by 2215 MST and remaining there, indicating complete mixing with the mesa. It appears from this and other data that, for this particular canyon, the threshold speed for establishment of the rotor by a cross-canyon mesa-level wind is around 2 m s^{-1} , even in this very stable case.

Techniques of flow visualization are among the least expensive and most informative ways to identify physical processes in airflows which are not well understood. The stable downslope wind in the canyons of Pajarito Plateau are weak but extremely steady. To determine certain fundamental features such as vertical velocity profile, nonaxial velocity components, and turbulent diffusivity and to relate them to temperature lapse rate, a series of visual tracer experiments were performed. In the first hour of daylight, there is sufficient light for tracer photography, while solar heating is insufficient to break up the nocturnal flow in Los Alamos Canyon. Orange smoke flares were fired in several configurations and photographed from several vantage points. The most useful configuration proved to be a vertical traverse as shown in Fig. 2 which shows the first wedges of upslope in the transition from nocturnal to daytime regimes. Figure 3 is the velocity profile derived from a number of traverses. The velocity maximum which has been reported in larger canyons does not appear in these runs. There are qualitative indications that, although the airflow is weak (1 m s^{-1}) and the lapse rate extremely stable, the diffusivity is significant.

Fig. 2. Smoke plume from vertically traversing source in Los Alamos Canyon.

Fig. 3. Velocity profiles in Los Alamos Canyon during downslope [(+) 0725 MST] and transition [(•) 0755 MST] flow regimes on November 12, 1975. Negative speeds indicate upslope motion.

Fig. 4. Comparison of results from tracer experiment in Los Alamos Canyon (—O—) to the dispersion predicted by Cramer's model² (—).

This fact has been substantiated further in initial results of fluorescent particle tracer experiments performed during nocturnal downslope conditions. Figure 4 shows the lateral diffusion parameters, σ_y , at 1 and 2 km downwind for one tracer experiment compared with model projections.² This comparison indicates mixing equivalent to near-neutral conditions even though temperature profiles in the canyon were very stable. If there is a conclusion to be drawn from these fragmentary results, it would be that, in complex terrain environments, some direct measure of turbulence such as σ_A must be used to estimate diffusion rates. Traditional indicators such as temperature lapse rate, wind speed, and time of day would perform poorly in these few cases, while the σ_A estimated from wind vane records is quite consistent with observed dilution rates. Of course, many more trials are necessary to verify these indications.

In addition to the Los Alamos Canyon studies, we conducted more generic efforts designed to further the understanding of the role of terrain influence on meteorological transport. A workshop on the subject was set up as a joint effort by LASL and Sandia laboratories and held in Albuquerque, New Mexico, on September 27-30, 1976.

An acoustic sounder began producing data on shear layers in the Jemez Mountains environment in

July 1976. Preliminary experiments in which pibal wind soundings were correlated with acoustic sounder records during periods of significant echoes suggest that as many as three scales of airflow regimes may be identified in a layered structure which changes with time. The three flows are a shallow Jemez Mountains-Pajarito Plateau slope flow beneath a deeper valley drainage in the Rio Grande Depression. Above this, the large-scale gradient wind dominates.

A two-dimensional hydrodynamic code, SOLA, has been adapted to study a variety of forced topographic flow problems. Applications to the cross-canyon eddy in Los Alamos Canyon and a cross-ridge flow in the vicinity of the Four Corners power plant complex have given a basis for design and interpretation of experiments.

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Radiation Exposures Measured in Rodents Inhabiting a Liquid Radioactive Effluent Receiving Area at Los Alamos

[F. R. Miera, Jr.]

A preliminary study was completed to determine the external gamma radiation exposures to rodents inhabiting a low-level radioactive effluent disposal area at LASL. A 50-x 50-m site near the effluent outfall was chosen for this study due to the relatively high but variable levels of ¹³⁷Cs in the

soils. Rodents were trapped in the study area, implanted with thermoluminescence dosimeters (TLD), and released for subsequent recapture and TLD retrieval. A detailed description of the experimental design and sampling methodology has been described elsewhere.¹

The data in Table I demonstrate that the contaminated area has a pronounced effect on the radiation doses to the rodents living in the area. Radiation exposures averaging 26 mrad/day were measured in harvest mice (Reithrodontomys megalotis), while lower averages were obtained for deer mice (Peromyscus maniculatus) (8.3 mrad/day), piñon mice (Peromyscus trueii) (1.9 mrad/day), and chipmunks (Eutamias minimus) (1.4 mrad/day). In all cases, the exposures averaged higher than the 0.5 mrad/day which can be attributed to background sources in the Los Alamos area.²

Species which exhibited high radiation doses were caught most often (i.e., 50 to 70% of the time) along the effluent stream channel where radiation intensities at the ground surface measured 20 to 87 mrad/day. Conversely, low doses in rodents were related to a low frequency of capture in this zone (i.e., less than 20% of the time).

Species differences in radiation dose are at least partially attributable to differences in habitat requirements and mobility. The smaller, less mobile harvest mice and deer mice were associated with the dense vegetation cover and high radiation intensities along the effluent stream channel, while the larger, more mobile piñon mice and chipmunks were associated with the open forest adjacent to the study plot.

This study demonstrated that liquid effluent release has increased the radiation dose received by

TABLE I

RADIATION DOSES RECEIVED BY NATIVE RODENTS IN A LIQUID RADIOACTIVE EFFLUENT DISPOSAL AREA

Species	n	Dose (mrad/day) ^a	
		Mean	+ S. D.
Harvest mouse (<u>Reithrodontomys megalotis</u>)	8	26	10
Deer mouse (<u>Peromyscus maniculatus</u>)	4	8.3	9.6
Piñon mouse (<u>Peromyscus trueii</u>)	5	1.9	0.9
Chipmunk (<u>Eutamias minimus</u>)	13	1.4	1.2

^a Primarily due to ¹³⁷Cs gamma rays.

small ground-dwelling rodents in the area. Maximum average doses of 26 mrad/day were observed in harvest mice, although individuals of this species were exposed to as much as 50 mrad/day. The total dose over the lifetime of this species (assuming a 1-yr life span) averages 9.5 rad for the species or a maximum of 18 rad for the individual. The exposure to three other species was factors of 3 to 18 times less than those for harvest mice. Habitat preferences and mobility are at least partially responsible for these differences.

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Measurement of Radon-222 Flux from Inactive Uranium Mill Tailings Piles

[M. L. Marple, W. E. Clements, and B. A. Perkins]

Uranium mill tailings piles are an anomalous local source of ^{222}Rn to the atmosphere due to their high ^{226}Ra content (see Table I). Radon diffuses through the tailings and into the atmosphere at various rates depending on the porosity, permeability, and radium content of the tailings and meteorological conditions. The amount of radon transported to the atmosphere across a unit area of the surface per unit time is referred to as the radon flux (or exhalation) and is usually expressed in

units of $\text{atoms cm}^{-2} \text{ s}^{-1}$ or $\text{Ci cm}^{-2} \text{ s}^{-1}$ ($1.76 \times 10^{16} \text{ }^{222}\text{Rn}$ atoms have an activity of 1 Ci). The objectives of this study are to (1) measure the ^{222}Rn flux (using the accumulation method¹) from three inactive uranium mill tailings piles (sites A, B, and C) near Grants, New Mexico, and (2) evaluate its attenuation by overburdens established for revegetation purposes as part of an ecological investigation of the rehabilitation of inactive piles (see reports on rehabilitation and vegetation stabilization elsewhere in this document).

Radon flux is measured primarily at site C, a carbonate leach tailings pile. Other measurements have been made on both acid (site A) and carbonate tailings (site B) at another site. Measurements are made on revegetation plots within close proximity of one another at all sites. These plots are areas (approximately 3-x 3-m) of bare tailings or tailings covered with an overburden which have been raked and seeded (primarily with native grass species). The three types of plots are bare tailings (control), a covering of boulders and cobbles of Tres Hermanos sandstone, and a soil covering. The soil overburdens are clay to an approximate depth of 30 cm on tailings A and B and shale to an approximate depth of 40 cm on pile C. The measured background flux in the area is approximately $1 \text{ atom cm}^{-2} \text{ s}^{-1}$.^{1,2} Table II gives the averages of flux measurements made from May to November 1976 at the three locations.

The large temporal and spatial variations in the measured ^{222}Rn flux are not unexpected and could be due to many factors such as depth of the tailings

TABLE I
RELATIONSHIP BETWEEN MEAN RADIUM CONTENT AND AVERAGE RADON FLUX

Location	^{226}Ra (pCi/g)	^{222}Rn (atoms $\text{cm}^{-2} \text{ s}^{-1}$)	Ratio ^a
Site A acid tailings ^b	530 ^c (1) ^d	322 ± 254 (4)	0.61
Site B carbonate tailings ^b	930 ± 184 (2)	560 ± 351 (3)	0.60
Site C tailings near revegetation plots	1142 ± 62 (3)	1042 ± 274 (14)	0.91
Site C tailings at other points	768 ± 329 (5)	589 ± 269 (6)	0.77

^aRatio of radon flux to radium content.

^bNear revegetation plots only.

^cStandard deviation of individual measurements.

^dNumber of measurements.

TABLE II
MEAN ^{222}Rn FLUX (atoms $\text{cm}^{-2} \text{ s}^{-1}$)

Location	None ^a	Overburden	Rock
		Soil	
Site A acid tailings	322 $\pm$ 254 ^b (4) ^c	203 $\pm$ 21 (5)	136 $\pm$ 35 (2)
Site B carbonate tailings	560 $\pm$ 351 (3)	338 $\pm$ 140 (6)	775 $\pm$ 58 (3)
Site C carbonate tailings	1042 $\pm$ 274 (14)	711 $\pm$ 343 (6)	714 $\pm$ 199 (6)

^aMeasurements made on soil and rock plots before covered and on control plots.

^bStandard deviation of individual measurements.

^cNumber of measurements.

(especially at sites A and B) and seasonal and diurnal changes in temperature, winds, pressure, and soil moisture. Flux variations due to meteorological and soil conditions are discussed by Tanner,³ Wilkening *et al.*,¹ and Clements and Wilkening.⁴ The flux levels for these three tailings piles are well within the range of measurements reported for other piles.⁵ The mean values obtained on the soil overburden are approximately 30% less than that from the bare tailings surface. This attenuation is also reflected in the individual measurements and is consistent with that expected from a simple diffusion model.⁶ Measurements on the rock plots are difficult to interpret due to their inhomogeneous composition and the accumulation of wind-blown material among the rocks. On the rock plot at site C, the attenuation is probably due to sand which has accumulated to a depth of 5 cm; however, this cannot account completely for the reduction. On the rock plots at sites A and B, the results cannot be attributed to particulate accumulation since it is negligible, but it is probably related to moisture and depth of tailings differences from the other plots.

Flux values were also obtained on an established revegetated area at site B, in addition to the above measurements on the experimental plots. A large area of tailings was covered several years ago with local topsoil to a depth of 5 to 50 cm, and a good cover of vegetation (primarily *Atriplex canescens*, *Kochia scoparia*, *Aristida* sp., *Sitanion hystrix*, *Sporobolus airoides*, and *Sporobolus cryptandrus*) now grows on the area. Four measurements of radon flux over this area ranged from 415 to 571 atoms $\text{cm}^{-2} \text{ s}^{-1}$. The mean of these measurements

is 43% higher than the mean for the site B carbonate soil plot, possibly due to a 50% higher radium content (~ 1245 pCi/g as compared to ~ 831 pCi/g) of the tailings underlying the revegetated area.

Radium-226 analyses were made of the tailings from all three piles as an aid in comparing flux values. Approximately 75 g of air-dry tailings were milled and sealed in a tin can for a minimum of 6 wk to ensure secular equilibrium of the radium daughters; the gamma emissions were counted with a Ge (Li) detector coupled to a multichannel analyzer. The background radium content of the soil in the Grants mineral belt is 1 to 14 pCi/g.⁷ Table II shows the relationship between mean radium content and average radon flux at various locations without overburdens on all three tailings piles.

The overall average radium content of site C tailings is 896 ± 445 pCi/g ($\pm$ S. D., $n = 21$). The results in Table II indicate that the radium content and radon flux near site C revegetation plots may be higher than elsewhere on the pile. The average radium concentrations in site B carbonate and in site A acid tailings are 1047 ± 322 pCi/g ($n = 10$) and 481 ± 221 pCi/g ($n = 5$), respectively. The ratios of radon flux to radium content indicate a somewhat smaller dependence of flux on the radium for piles A and B than for pile C. Many other factors can affect this ratio, and it cannot be used alone quantitatively to predict relative flux values. Another consideration is that the reported ^{226}Ra concentrations represent a measure of total radium, whereas the important quantity for comparison with flux measurements is the "effective radium" (i.e., that radium which supplies radon to the soil gas); this quantity can vary considerably with soil type.⁸

The average radium concentrations of the soil overburdens were 149 ± 119 pCi/g ($n = 3$) for the shale soil and 62 ± 46 pCi/g ($n = 9$) for the clay soil. These levels are much higher than background due to probable contamination from deposition and in place-ment of the overburdens on the plots.

In summary, the radon flux from three inactive uranium mill tailings piles ranged from 65 to 1414 atoms $\text{cm}^{-2} \text{s}^{-1}$ under a variety of atmospheric conditions. Results indicate that soil overburdens of depths of 30 and 40 cm may attenuate the flux by as much as 30%. Thus, unless overburdens are substantially deeper than those used in this study, the tailings piles still present a significant local source of ^{222}Rn to the atmosphere due to their large exhalation compared to background levels. Additional measurements will be made under varying atmospheric conditions to obtain annual averages for radon flux. The data will be used to estimate a mean source strength in order to evaluate the effects of tailings piles on surrounding air quality under various meteorological conditions.

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Ecological Investigation of Rehabilitation of Uranium Mill Tailings of the Southwestern United States

[C. L. Wienke and D. R. Dreesen]

This study was initiated to develop a stabilization methodology for abandoned uranium mill tailings piles in the semi-arid Southwest. The impetus for this research program stems from the concern that tailings piles may serve as a source of radioactive and toxic stable chemicals to the surrounding environs. An effective tailings stabilization program will require a clear definition of the processes that govern transport of tailings and associated contaminants away from the pile. Information is needed on the importance of wind and water as vectors of tailings components and on the contribution of the pile to ambient radiation background. Specific research objectives, which have been tailored around these needs, are to determine (1) the extent of tailings transport away from the pile as indicated by gross alpha concentrations in surface soils; (2) chemical quality of surface and groundwater in the vicinity of the pile; (3) radon flux from the surface of the pile as influenced by various treatments; and (4) ability of native vegetation species to grow on the pile under various experimental regimes.

The inactive uranium tailings pile at Ambrosia Lake, New Mexico, was selected for detailed investigation because of its proximity to LASL and the cooperation of the corporation presently controlling the pile. This tailings pile was generated from 1958 to 1963 using an alkaline mill process.

An engineering survey¹ was made of the pile and the surrounding area to provide an accurate

physical description of the pile and to establish a sampling grid. The pile is roughly a square about 620 m on a side with a surface area of about $3.7 \times 10^5 \text{ m}^2$. The thickness of the tailings varies from < 1 m to about 10 m.

Gross alpha determinations on surface soil and tailings were made as a function of direction and distance from the pile to delineate tailings migration. Gross alpha concentrations were determined in samples from 296 locations using a ZnS scintillator technique described elsewhere.

The alpha concentrations in soils from the sampling transects demonstrate that tailings material has migrated off the pile (Fig. 1). The mean gross alpha concentrations in surface tailing was 4100 pCi/g ($n = 56$; $s = \pm 1100$), while background soils in the area ranged from 20 to 50 pCi/g.

The presence of other sources of alpha-emitting contaminants such as uranium ore storage piles, ore haulage roads, mining operations, and exploration activity has complicated the interpretation of alpha contamination patterns away from the tailings pile. Evidence of wind-blown tailings transport was observed on the east side of the pile where eolian sand dunes had alpha activities from 1000 to 2000 pCi/g. Tailings have also been transported by water from the pile through natural and manmade breaches in the southern and western dikes. Tailings which have migrated through these breaches have alpha

Fig. 1. Alpha activity with distance from the pile.

activities of 3000 and 5000 pCi/g, respectively.

Thirty samples of tailings and soils were first counted for gross alpha and then analyzed for ^{226}Ra to determine if gross alpha concentration could be used as a rapid method to survey ^{226}Ra concentrations. A constrained exponential function gave the best fit of ^{226}Ra as a function of gross alpha. The equation is in the form of $y = a(e^{bx} - 1)$, where the dependent variable y is ^{226}Ra in pCi/g, the constant a equals 417, and the regression coefficient b equals 2.5×10^{-4} (Fig. 2). The degree of fit shows that gross alpha estimates ^{226}Ra content with good reliability. At the most extreme gross alpha activities of 18 and 7086 pCi/g, the estimated ^{226}Ra concentrations and confidence intervals (± 2 standard deviations) are 1.9 ± 1.0 pCi/g and 1963 ± 192 pCi/g, respectively.

Monitoring wells were drilled on and around the tailings pile to obtain water-level data, water samples for trace element analysis, and depth of tailings and to determine the type of basement rock. The water-level data indicate that water is flowing under the tailings pile from the northeast to the southwest (Fig. 3). Sources of recharge were identified.¹ A preliminary set of water samples was collected from the monitoring wells, filtered, and acidified for trace element analysis. A comparison of samples taken up-gradient (NE) of the pile with those taken down-gradient (SW) shows elevated concentrations down-gradient for arsenic,

Fig. 2. Radium-226 as a function of alpha activity.

Fig. 3. Altitude of water (average of water-level data, July/August/September 1976).

nickel, lead, vanadium (significant 98%); chromium (significant 95%); and copper, molybdenum, and uranium (significant 90%). Selenium showed no significant difference up-gradient vs down-gradient. These preliminary data indicate the probable leaching of contaminants out of the tailings into near-surface aquifers. Although the shallow aquifer sampled at this tailings pile site is not used for potable supply, the desirability of designing a stabilization effort to reduce this transport mechanism is clear. Confirmation sampling is planned. Of numerous possible stabilization techniques considered, the one given most thought is recontouring the pile from a shallow, diked basin to a gently sloping mound flanked with diversion channels and covered with rock. There is archaeological evidence provided by ruins in Turkey that this type of structure is stable for thousands of years and that vegetation will naturally propagate between the rocks.

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Long-Term Ecological Effects of Exposure to Uranium

[W. C. Hanson and F. R. Miera, Jr.]

The Los Alamos Scientific Laboratory's dynamic testing program has expended about 100 000 kg of natural and depleted uranium during the 33 yr of operation. Nearly three-quarters of that amount was dispersed at one location (E-F Site) within the western central portion of the LASL environs. This represents a unique opportunity to study the

long-term ecological consequences of a natural heavy metal that is of concern because of its radioactivity and chemical toxicity. Few other locations provide such a gradient of uranium concentrations available in soils, plants, and animals whereby an integrated picture of possible chemical toxicity responses and food chain transmission potential may be evaluated.

These studies began in October 1974 by selection of three LASL firing sites and a control area that provided areas of high, moderate, and low uranium concentrations in soil that originated mostly from different uranium particle sizes associated with the particular type of tests conducted at the sites.¹ Two sites were studied most intensively: E-F Site, where soil samples averaged 2400 ppm of uranium in the upper 5 cm and 1600 ppm at 5- to 10-cm depths; and Lower Slobovia Site, where two subplots averaged about 2.5 and 0.6% of the E-F Site concentrations. Important uranium concentration differences with depth and distance from detonation points were ascribed to the different explosive tests conducted in each area. During the second year of study, emphasis was placed on studies at E-F Site to describe the uranium concentrations and distributions in soil relative to their depth in the soil profile and distance from the detonation point, the redistribution of uranium by storm runoff, and the possible responses of soil invertebrate communities to uranium chemical toxicity.²

Uranium Concentrations in Soil Samples.

Uranium in E-F soils averaged 2400 and 1600 $\mu\text{g/g}$ (= ppm) at 0- to 5- and 5- to 10-cm sampling depths. Although these differences were not statistically significant ($P \geq 0.05$), they reflect vertical movement of uranium into the soil profile. The mechanisms for this movement could be erosion processes, mechanical disturbances, and/or penetration of uranium fragments into the soil during the explosive tests.

Uranium concentrations in the top 5 cm of Lower Slobovia soils were significantly higher ($P \leq 0.05$) than those at the 5- to 10-cm depths. This contrasts with observations at E-F Site, although both areas were used for explosive tests for about the same length of time.

Important differences between E-F Site tests and those at the other sites may partly explain the observed uranium distribution patterns. Explosive tests at E-F Site deposited relatively large fragments; particles were observed in the range of ≈ 2 mm to several centimeters in diameter. Tests at Lower Slobovia produced consistently smaller diameter uranium particles. The greater particle size range at E-F Site is apparent in the variability of uranium concentrations in soil samples from that location; the coefficient of variation (standard deviation $\div$ mean) in E-F Site soils ranged from 1.54 in the surface 5 cm to 2.85 in the 5- to 10-cm profiles, reflecting considerable inhomogeneity of the uranium in the soil. At least some of this extreme variation came from samples that contained relatively large pieces of uranium. Although variability in soil uranium at other areas was also appreciable (coefficients of variation of 0.93 to 1.7), it averaged somewhat less than at E-F Site.

Uranium Concentrations in LASL Biota.--Vegetation samples at E-F Site contained about 320 ppm in November 1974 and about 125 ppm in June 1975. These differences were probably due to (1) variable external deposition over considerable time; (2) different species of plants available for sampling at different times; and (3) greater amounts of fresh growth in the June samples which tended to dilute the uranium concentrations. Ratios of plant-to-soil uranium concentrations varied from 0.08 in November to 0.05 in June -- values that are within the range reported from other studies of plants in high uranium areas.³

Small mammals trapped in November contained a maximum of 210 ppm of uranium in gastrointestinal tract contents, 24 ppm in the pelt, and 4 ppm in the remaining carcass. In June, maximum concentrations were 110, 50, and 2 ppm in similar samples and 6 ppm in lungs. These data emphasize the importance of resuspension of respirable particles in the upper few millimeters of soil as a contamination mechanism in several components of the ecosystem.

Uranium Inventory and Transport Studies.--A polar coordinate sampling design was devised for the soil uranium inventory at E-F Site during 1976. Sampling loci were located at the intersection of radii that extended 45° angle increments from the

detonation point and concentric circles at intervals of 10, 20, 30, 40, 50, 75, 100, 150, and 200 m from the detonation point. A 20-x 50-cm frame was placed at each sampling locus, and two soil cores were collected to a depth of 30 cm on the outer perimeter of the frame, 50 cm apart. Six core segments were obtained from each core, yielding 864 samples; results for this report are based upon about 200 of those, representing the 0- to 2.5-cm segment from all sampling loci, to determine the horizontal distribution of uranium from the detonation point. In addition, uranium concentrations in the top 15-cm segments of all samples collected within 50 m of the detonation point and the entire 30-cm core of selected samples are presented to describe the vertical distribution of uranium in the soil profile.

We estimate that about 70 000 kg of uranium is available for surface transport, mainly by thunderstorm runoff, from the E-F Site environs and that most of the transported material will move into the Potrillo Canyon drainage. A permanent sampling network accordingly was established along the drainage pathway from the detonation point to Potrillo Canyon and then down the canyon for 9 km.

Results of the surface (0 to 2.5 cm) inventory study showed that the highest uranium concentrations occurred within 10 m of the detonation point where mean values were about 4500 ppm. Concentrations decreased rapidly at sampling loci beyond 50 m of the grid center and were usually < 15% of the values

Fig. 1. Three-dimensional plot of uranium concentrations ($\mu\text{g/g}$) in surface soil (0- to 2.5-cm depth) samples obtained at E-F Site.

found within 10 m of the detonation point (Fig. 1). The figure demonstrates that the highest uranium concentrations were found in the immediate vicinity of the center and that clusters of elevated values occurred to the west, south, and northeast of the detonation area. Additional analytical results from remaining soil samples obtained in this study will more precisely define the spatial distribution at this site.

Incomplete analyses of soil samples collected to depths of 30 cm within a radius of 50 m of the detonation area indicated that significant migration and/or penetration of uranium into the soil profile to the maximum sampling depth has occurred (Fig. 2). Values in the upper sampling increments (0 to 2.5 and 2.5 to 5.0 cm) were somewhat variable compared to results obtained in deeper sample increments. The greatest departure from this general statement occurred at the 10-m sampling radius where a single value of 22 000 $\mu\text{g U/g}$ soil in a 20- to 30-cm-depth sample introduced strong variance in the sample population. It should be noted that the mean radial value of 100 $\mu\text{g U/g}$ measured in the 50-m samples from 20- to 30-cm depths is about 50 times greater than the background values for this area, emphasizing the mobility of uranium in the soil profile.

Characteristics of storm runoff transport of uranium were defined during heavy rainfall events of 3.5 cm precipitation over several hours on September 5, 1975, and 1.0 cm in 0.5 h on

Fig. 2. Distribution of uranium as a function of depth and distance in soil at E-F Site. Uranium concentrations at each depth represent mean radial values obtained using a polar coordinate sampling system.

September 17, 1976. Estimated flow rates of 6 to 7 ℓ/sec on the mesa top and 30 to 35 ℓ/sec in Potrillo Canyon resulted from the first event; no estimate was made during the second event. Highest total uranium concentration for both events were observed in the standing water samples taken within the detonation crater, 87 mg/ℓ in 1975 and 282 mg/ℓ in 1976, with nearly all (99 and 83%, respectively) in solution (Table I). This is much greater solubility than inferred from the available literature.⁴ The uranium concentration in the suspended sediments for the first rainfall event was 3900 $\mu\text{g/g}$, a value comparable to average surface soil uranium concentrations in that area. Standing water sampled 20 m southwest of the detonation point contained much lower concentrations in the water fraction during both events; the presence of large corroding fragments of uranium in the detonation crater presumably accounts for that difference. These results implicate storm runoff as an important vector in the transport of uranium from E-F Site.

Surface (0-to 2.5-cm) alluvium samples from the Potrillo Canyon intermittent stream bed that were collected at a point 250 m from the detonation area contained 300 ppm, or about 10% of the values measured at the detonation point; at 2800 m from the detonation point, the values were about twice background. Within 200 m of E-F Site, the concentrations were highly variable to depths of 15 cm, but at distances between 350 and 5000 m from the site, the uranium was homogeneously distributed to depths of at least 20 cm. An estimated 58 kg of uranium was located in the upper 15 cm of alluvium in the canyon below E-F Site, with 76% of it within 300 m of the source and the remainder distributed down the canyon to 9000 m. The 58-kg inventory represented < 0.1% of the estimated total uranium expended at E-F Site during the 1943-1973 period, indicating that only minor amounts of the material have moved any appreciable distance from the point of origin.

Soil Invertebrate Populations in High Uranium Areas.--Studies of the litter- and soil-inhabiting invertebrates were performed by extracting the contents of 1 dm^2 soil cores by the Tullgren funnel method.⁵ Over 9800 specimens representing 100 to 110 species were isolated from 217 samples. Species

TABLE I
URANIUM CONCENTRATIONS IN STANDING WATER AND RUNOFF SAMPLES
COLLECTED FROM E-F SITE ON SEPTEMBER 5, 1975, AND SEPTEMBER 17, 1976

Sampling Location	Sample Date	Water ($\mu\text{g U/L}$)	Suspended Sediments ($\mu\text{g U/L}$)	Total ($\mu\text{g U/L}$)	Uranium in Solution (%)
Standing water at detonation point	1975	$86 \times 10^3 \pm 2 \times 10^3$	590 ± 30	86.6×10^3	99
	1976	$235 \times 10^3 \pm 5 \times 10^3$	$47 \times 10^3 \pm 4 \times 10^3$	282×10^3	83
Standing water 20 m southwest of detonation area	1975	63 ± 6	$1.3 \times 10^3 \pm 0.2 \times 10^3$	1.3×10^3	5
	1976	240 ± 20	890 ± 30	1.1×10^3	21
Runoff 100 m southwest of detonation point (mesa top stream channel)	1975	52 ± 5	100 ± 8	152	34
	1976	-	-	-	--
Runoff 250 southwest of detonation point (canyon stream channel)	1975	37 ± 2	54 ± 5	91	41
	1976	125 ± 9	410 ± 20	535	23

of the Order Acarina (ticks and mites) were most abundant, representing 78% of total animals and occurring in 93% of the samples. There were ≥ 40 species of ticks and mites, of which 10 were considered common. All but one of the 15 families of Acarina identified thus far have been defined as predators -- an observation that may warrant further investigation.

In general, the soil and litter macrofauna populations and species diversities were apparently reduced at the high uranium study areas compared to their control area counterparts. Collembola populations were significantly lower at both E-F and Lower Slobovia experimental sites than at their respective controls.

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Ecological Fate and Effects of Trace Elements from Coal Utilization in the Semi-Arid West

[L. E. Wangen, D. R. Dreesen, and C. L. Wienke]

This study was initiated to investigate the environmental transport and distribution of trace elements released as a result of coal combustion in the Four Corners area of New Mexico. The physical and chemical properties of trace elements originally present in the coal matrix are changed by extraction and combustion. Some of these processes are known to convert various toxic materials from a relatively inert, immobile state to physical and/or chemical forms that are more available for uptake or movement in the environment. For example, the high-temperature combustion process volatilizes many species, thereby enabling their escape to the atmosphere and subsequent dispersal over large geographic regions. A less studied phenomenon is the potential mobilization of trace contaminants from coal ash after disposal such as that resulting from wind and water erosion. The possibility of water contamination by leaching and surface runoff of trace contaminants from strip-mined areas and ash disposal sites is particularly noteworthy in an area where the limiting natural resource is the quality and quantity of water.

Air Particulate Sampling and Analysis.--The particle size distribution of various trace elements in airborne particulate matter is fundamental to an assessment of the magnitude of particulate deposition on terrain surrounding a major coal-fired power

plant. Techniques were developed and verified for sampling and analysis of airborne particulate matter as a function of particle size using six-stage cascade impactors and two-stage filters. The necessity of using greased impactor slides to collect the relatively dry air particulate matter in this region is shown by the data from a representative 1-wk sample (Table I).

We have settled on the use of a solution containing 5% Apiezon L grease in cyclohexane. The above data were obtained using two single-orifice cascade impactors of the Battelle design operated side-by-side in the Four Corners area. It is evident that these differences are due mainly to re-entrainment of larger particles and subsequent deposition on the interior walls of the impactor.

Sufficient data have been obtained to indicate the size distribution of the Four Corners area aerosol. The cumulative mass distribution by particle size averaged over 8 weekly samples is shown in Fig. 1. The data indicate that less than 15% of total particulate mass is below 5 μm in equivalent diameter (unit density spheres). The mean total particulate concentration, as determined using a cascade impactor for 8 weekly samples collected between July 22 and October 8, was $87.6 \mu\text{g}/\text{m}^3$ at STP. The comparable value for 4.0- μm and below fraction was $11.3 \mu\text{g}/\text{m}^3$. The submicron fraction accounted for about 5% of the total particulate concentration. These data are significant because power plant stack particulate emissions are nearly all under 2 to 4 μm in size. Thus, to have a reasonable chance of distinguishing trace elements in particulates from the Four Corners power plant plume from other sources, it will be necessary to separate the small particle fraction from the total particulate mass.

Results of trace element analyses on airborne particulate samples using instrumental neutron activation analyses suggest that the dominant

Fig. 1. Cumulative mass distribution by particle size averaged over 8 weekly samples.

material consists of windblown soil. However, the submicron fraction shows evidence of elevated levels of certain trace elements such as sulfur, gallium, copper, and vanadium which are of interest in coal combustion studies.

Deposition Calculations.--Calculations using a source depletion Gaussian plume model have been made to estimate an upper limit to the historic deposition of trace elements from the power plant plume. The results of these calculations for 10 yr of plant operation, during a period when particulate control consisted of mechanical cyclones, are listed in Table II. Note that these calculations were for the

TABLE I
AIR PARTICULATE MATTER IN THE FOUR CORNERS REGION

	Impactor Stage Concentration ($\mu\text{g}/\text{m}^3$) STP						BF	Total
	1	2	3	4	5	6		
Stage 50% cutoff diameter (μm)	16	8	4	2	1	0.5	-	---
Ungreased	0.04	0.08	2.1	4.0	3.2	2.1	4.9	16.42
Greased	33.7	15.3	11.2	10.7	3.4	1.2	3.0	78.50

TABLE II

CALCULATION OF TRACE ELEMENT ENRICHMENT IN SOIL DUE TO 10-YR DEPOSITION
FROM A POWER PLANT PLUME IN DIRECTION OF MAXIMUM CALCULATED IMPACT

Downwind Distance (km)	Deposition (g/cm ² /10 yr)	Percent Ash in Top 1 cm of Soil	Cf/Ci ^a (assuming indicated trace element enrichment in deposited ash compared to soil)			
			2	10	50	100
1	0.0055	0.37	1.01	1.04	1.18	1.37
2	0.0198	1.32	1.03	1.13	1.66	2.32
4	0.0235	1.57	1.03	1.16	1.78	2.57
6	0.0175	1.17	1.02	1.12	1.58	2.17
8	0.0137	0.92	1.02	1.09	1.46	1.92
10	0.0112	0.75	1.02	1.08	1.38	1.75
14	0.0082	0.55	1.01	1.06	1.28	1.55
20	0.0060	0.40	1.01	1.04	1.20	1.40
30	0.0037	0.25	1.01	1.02	1.12	1.25
40	0.0028	0.18	1.00	1.02	1.09	1.18
50	0.0020	0.13	1.00	1.01	1.06	1.13
70	0.0012	0.08	1.00	1.01	1.04	1.08
100	0.0008	0.05	1.00	1.01	1.02	1.05

^aCf = final concentration in top 1 cm; and Ci = concentration of native soil prior to deposition of ash.

ASSUMPTIONS: (1) 10 yr of accumulation mixed with top 1 cm of soil; (2) fly ash emission rate of 2500 g/sec; (3) deposition velocity of 3 cm/sec used to estimate deposition based on ground-level concentrations calculated using a source depletion Gaussian plume model; and (4) soil has a density of 1.5 g/cm³.

direction (SE) of maximum ground-level plume impact and were made under restrictive assumptions. Thus, they should be considered as a maximum credible upper limit. The numbers in the right four columns are the hypothetical factor by which natural concentrations would be increased over those concentrations that would exist with no deposition. The numbers at the top of these four columns refer to a hypothetical mass enrichment factor. For example, the numbers in the body of column 6 show the increase in soil concentration for any elements which happen to have a mass concentration in deposited ash that was 50 times its concentration in natural soils of the Four Corners area. These data are significant because they suggest that, for the proposed worst case, one would not expect to measure a buildup of trace elements in soils due to deposition from a plume except for those elements that are highly enriched in ash compared to soil. If a relative standard deviation of about 50% is assumed for the natural variability of trace element concentrations in soils, this enrichment would have to be of order 50. In general, the elements for which this magnitude of enrichment might occur would be those with

maximum volatility and, thus, most concentrated on small particles. These calculations suggest that, in general, one should not expect to measure an elemental increase in native soils due to plume deposition downwind of this modern coal-fired power plant.

Elements in Soils and Vegetation Southeast of the Four Corners Plant.—A cooperative sampling effort, including personnel from UCLA, ASU, USDA, and LASL, was undertaken in September to investigate whether elements are building up in soils and vegetation southeast of the Four Corners power plant. The model calculations suggest that such a buildup should not be expected for soils; however, recent work by the Denver Branch of the U. S. Geological Survey strongly indicates that vegetation may show such an accumulation.

Surface soil, subsurface soil, and four species of vegetation were collected at each of 7 distances southeast of the power plant at geometrically increasing intervals. Two 3-sample composites of surface and subsurface soil were collected at each distance. Subsurface soil samples were collected below the caliche layer which ranged from 25 to

TABLE III

LICHEN AND MOSS ABUNDANCE AND DIVERSITY THROUGHOUT SOUTHERN UTAH AND NORTHERN ARIZONA

Location	Elevation (m)	Substrate	Number of Species		Percent Cover	
			Lichens	Mosses	Lichens	Mosses
San Francisco peaks	3750	Rhyolite	27	2	36.8	0.3
Navajo National Monument	2200	Navajo sandstone	34	1	20.4	0.4
Navajo National Monument	2200	Dakota sandstone	30	1	16.9	0.4
Kanab-Page area	1520	Navajo sandstone	31	1	6.6	0.1
Capital Reef National Park	1520	Navajo sandstone	35	2	35.9	0.7
Henry Mountains	2500	Porphyritic intrusive	32	2	31.7	0.3
Cedar Breaks National Monument	2750	Basalt	35	2	35.9	0.7
Brian Head (Cedar Breaks area)	3440	Rhyolite	35	2	30.3	0.2
Monticello area	2130	Dakota sandstone	39	1	23.2	0.2
Moab area	1830	Navajo sandstone	21	0	13.7	0

150 cm below the surface. The subsurface soils were sampled to provide information on natural variations in area soils and to serve as controls. These samples are presently being analyzed at LASL and UCLA.

Lichen Study.--Studies were undertaken by the University of Arizona, under contract to LASL, to determine whether lichens could be used as indicators of trace element pollution on the Colorado Plateau. Field studies were performed to determine the distribution and abundance of lichens on the Colorado Plateau.

A summary of lichen diversity and abundance for the Colorado Plateau is given in Table III. A majority of the sampling is completed, and the balance will be completed during the next summer season. Although the values for lichen cover may be regarded as fairly precise, the data on the number of species are regarded as tentative until definitive identification of species is made.

In addition, lichens and lichen substrate samples were collected from 9 locations around the Four Corners power plant and were analyzed for various trace elements. Preliminary results suggest that copper may be elevated in lichens collected at impact as compared to control sites. Although iron was not subjected to statistical treatment because of data heterogeneity, it appears that iron concentrations are elevated near the power plant.

Elements Mobilized in Coal Ash Disposal Ponds.--Identification of those elements solubilized from the ash is necessary to assess the potential for water contamination by disposal of coal ash in ponds.

Water samples were collected from influent and effluent streams at the Four Corners power plant and were analyzed for the trace elements arsenic, boron, beryllium, cadmium, chromium, copper, fluorine, molybdenum, selenium, and vanadium. In addition, laboratory experiments on the extractability of trace elements in ash collected by the electrostatic precipitators were completed. Results show good agreement between those elements highly extractable in alkaline solution and those elements elevated in effluent waters. They suggest that boron, fluorine, molybdenum, and selenium should be carefully evaluated with respect to environmental contamination in alkaline waters of this region.

Accumulation of Elements by Native Vegetation in Ash Pond Decant Channels.--The occurrence of elevated levels of some trace elements in effluent waters from the power plant compelled the investigation of trace element content of vegetation growing in the effluent channels. The dominant riparian species is saltcedar (*Tamarix pentandra*). Leaf and branch material was collected at 3 locations in each of 5 different channels. Channels selected included blowdown channel from the cooling reservoir, decant channel from scrubber ash slurry disposal ponds, seepage channel from the ash ponds, downstream from the 3 effluent releases, and upstream as a control. These vegetation samples were divided; half was washed with distilled water and the other was not washed. The samples were dried and ground. The elements for analysis were boron, sulfur, molybdenum, selenium, fluorine, nickel, and

copper. Boron analyses have been completed, and preliminary indications show higher concentrations in the decant channel and the downstream samples than in the upstream control for both washed and unwashed plant material.

Soil Microbe Study.--This study was undertaken to investigate the adaptability of bacteria to various concentrations of selected trace elements. Soil bacteria constitute an essential part of an ecosystem in that they provide for the breakdown of organic matter and, therefore, the return of nutrients to bioavailable form.

Bacterial populations in surface soil samples taken from 2 sites near the Four Corners power plant have been tested for their tolerance to copper. The hypothesis of this technique is that, if soil bacteria in an "impact" area have been influenced by a metal(s) burden, they will have adapted so as to be more tolerant of the metal(s) present than the indigenous populations in a "control" area. Initial testing showed that bacteria from the "impact" site (near ash disposal ponds) were less tolerant to copper than bacteria from the "control" site. There is some question as to whether the control site was properly chosen, and experiments are planned to determine this, as well as the significance of reduced tolerance to copper at the "impact" site.

Temporal Changes in Soil Plutonium Distribution in the Trinity Site Environs

[T. E. Hakonson and J. W. Nyhan]

A major objective of LASL's environmental plutonium study at Trinity Site is to compare recent findings with those from similar studies conducted from 1947 to 1956. Sufficient data are available at this time to provide a preliminary assessment of the changes in soil plutonium distribution that have occurred over the last 29 yr. Comparative information of this type will be useful in evaluating the long-term adequacy of safety standards for soil plutonium.

Several sources of data were used for comparison, including several documents pertaining to the early studies¹⁻⁶ and several from the recent LASL studies.^{7,8} The validity of making comparisons of plutonium data from studies differing in design, methodology, and analytical sophistication was

evaluated insofar as possible. It is our opinion, based upon this evaluation, that the following comparisons are valid for the general interpretations that have been made. Library soil samples from the early studies are being reanalyzed for plutonium using modern analytical procedures to provide a quantitative basis for assessing the comparability of the data.

The concentrations of plutonium in the surface 2.5 cm of soil at a site (Chupadera Mesa) about 44 km from Trinity Ground Zero decreased from an average of about 600 mCi/m² to 14 nCi/m² over a 24-yr period (Table I). The average soil plutonium concentration in 1973 was significantly different ($P \leq 0.01$) from the data for previous years. The variability associated with the concentration data in Table I was similar between years, with the coefficient of variation ranging from about 0.5 to 1.0 for sample sizes of 3 to 15.

Migration of plutonium into the soil profile serves as one possible explanation of the depletion of plutonium from the surface 2.5 cm of soil. The data in Table II support this hypothesis by

TABLE I

COMPARISON OF AVERAGE PLUTONIUM CONCENTRATIONS IN SURFACE (0-to 2.5-cm) SOILS FROM CHUPADERA MESA^a

1949	1950	1951	1956	1973
570	670	520	550	14

^aIn nCi/m².

TABLE II

PLUTONIUM IN SOIL PROFILES FROM CHUPADERA MESA^a

Depth (cm)	1951 (n = 3)	1973 (n = 8)
0-2.5	6.8 (0.82) ^b	0.44 (0.48)
2.5-5.0	0.7 (1.7)	0.34 (1.0)
5.0-7.5	0	0.51 (1.5)
7.5-10	0	-- --
10-15	-	0.22 (1.7)
15-20	-	0.22 (1.8)
20-25	-	0.20 (1.8)

^aIn pCi/g.

^bCoefficient of variation (standard deviation/mean).

demonstrating that elevated plutonium concentrations were detectable at all depths to 25 cm in 1973. Additionally, nearly 75% of the plutonium inventory in the soil profiles from 1973 was associated with depths below 2.5 cm. In 1951, nearly all plutonium was associated with the surface 2.5-cm soil. World-wide fallout levels of plutonium in soils from the Trinity Site area averaged about 0.05 pCi/g soil.⁸

The total inventory of plutonium in the soil profiles in 1973 accounted for about 35% of the profile inventories in 1951. The differences are probably not significant, given the large variability associated with the plutonium concentration data. However, in addition to plutonium migration into the soil, other factors could contribute to depletion of plutonium from the soil surface. Studies are under way to evaluate the relative importance of wind transport of plutonium by surface creep and saltation processes.

This preliminary assessment indicates that plutonium from the Trinity Site test in 1945 has migrated into the soil profile over a 29-yr period. The significance of this observation is that the vertical migration of plutonium into the soil effectively removes a portion of the total plutonium inventory from transport processes which interact with surface soils (e.g., wind and water). The implication is that plutonium resuspension processes and concomitant inhalation problems may be of reduced significance with increasing time from an initial contaminating event. However, increased penetration of plutonium into the soil profile could alter the availability of the element to vegetation. Relatively high plant/soil plutonium concentration ratios have been observed on the Chupadera Mesa study area.

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Elemental Analysis of Complex Materials by Prompt Gamma-Ray Spectrometry

[E. S. Gladney, D. B. Curtis, and E. T. Journey]

Environmental studies often require large numbers of chemical analyses on many types of materials. These requirements challenge the analytical chemist to develop cost-effective techniques that provide the necessary accuracy and precision on many types of matrices. Development of methods using prompt gamma-ray spectrometry which meets the previous criterion for analysis of several elements is summarized here.

Neutron capture processes produce a host of excited nuclei which de-excite almost instantaneously ($t_{1/2} \approx 10^{-13}$ to 10^{-14} sec) by the emission of prompt gamma rays. The energies of these gamma rays are characteristic of the emitting nuclei, and their intensities are proportional to the elemental abundances. Hence, the detection of prompt gamma rays has utility for nondestructive, quantitative analysis of trace elements with large neutron capture

cross sections and for major and minor elements with smaller cross sections. The technique requires a facility which will allow the sample to be neutron-irradiated and simultaneously observe the prompt gamma rays with a Ge (Li) detector. Such a setup exists at LASL's Omega West reactor and has been utilized for analytical purposes. A diagram of the experimental configuration is shown in Fig. 1.

The sample is inserted into a lead-lined channel which resides in the thermal column of the Omega West reactor. At the position of sample residence, the neutron flux is about 2×10^{11} n/cm² s and is highly thermalized. A collimated beam of gamma rays is extracted from the sample at right angles to the axis between the sample and the reactor core. Collimation is such that the detector sees only gamma rays produced in the sample. A Ge (Li) detector in anticoincidence with a sodium iodide annulus is used to detect the characteristic photons. The anticoincidence system reduces the background pulses due to Compton scattering. A boron curtain between the sample position and the reactor core may be lowered to reduce the neutron flux during sample changes. Lowering the curtain allows this operation to be carried out safely without shutting down the reactor.

Boron is an element which is difficult and time consuming to measure chemically. The effects of this element on the environment are not well known; however, for many vegetation types, the tolerance range between deficiency and toxicity is rather narrow.^{1,2} A recent paper discusses the phytotoxicity of boron as an air pollutant.³ A thorough investigation of prompt gamma-ray

spectrometry for analysis of boron and cadmium has been completed, and the work has been accepted for publication in a future issue of *Analytical Chemistry*.⁴ The utility of the technique has been demonstrated using National Bureau of Standards (NBS) standard reference materials (SRM). Results of analyses of the SRMs fall within $\pm 5\%$ of the accepted values, and the precision (one standard deviation of the mean for replicate measurements of the same sample) is also about $\pm 5\%$ for a variety of matrix types. Boron can typically be detected in quantities as small as 0.05 μ g in a total sample weight between 0.5 and 1.0 g. Long irradiation periods also allow the analysis of approximately 0.2 μ g of cadmium with good accuracy and precision. However, the detection limit for cadmium is too high to be useful for many environmental problems. Prompt gamma rays are advantageous for only high levels of cadmium or for rare or unusual sample matrices where the nondestructive aspect of the method is attractive.

Oxides of sulfur are well-known pollutants associated with the combustion of certain types of coal. Environmental studies of the effects of sulfur again require analyses for this element in a variety of complex matrices. Existing analytical techniques for sulfur are generally either expensive, destructive, or sufficiently insensitive to detect the element in a workable quantity of material. Again analysis by prompt gamma-ray spectrometry offers a rapid, nondestructive, accurate, and precise method for measuring quantities of sulfur down to 1 mg in up to 1 g of most materials. The characteristics of the analysis have been investigated using NBS SRMs. Results of the work are being prepared for publication.

The prompt gamma-ray spectrum has a multitude of lines and offers many applications for multi-element analyses for major elements. Therefore, a complete analysis for major elements often requires the use of many techniques and is both time-consuming and expensive. In many cases, prompt gamma-ray spectrometry may do this type of analysis with a single irradiation, requiring relatively little expenditure of time or money. An example of this application is the analysis of geological materials. We have successfully measured aluminum, silicon, and iron in U. S. Geological Survey standard

Fig. 1. Capture gamma facility at the Los Alamos Omega West reactor.

rocks and are currently analyzing most of the major elements (sodium, calcium, magnesium, potassium, phosphorus, titanium, and manganese) in these types of material. Another possibility is the complete chemical characterization of coal.

In summary, prompt gamma-ray spectrometry promises to be an extremely useful analytical technique. It can do reliable, fast, nondestructive analyses for trace levels of boron and cadmium. In addition, the method promises to provide rapid multi-element analyses for many major elements that cannot be measured by the traditional neutron activation technique and that are difficult to measure by other methods.

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Ecological Investigation of Mercury in Mortandad Canyon

[T. E. Hakonson and E. S. Gladney]

The objective of this program is to define the environmental behavior of mercury with respect to its distribution, transport, and potential significance in food webs at Los Alamos. The study has relevance to potential environmental mercury problems resulting from uranium mill tailings, geothermal energy utilization, coal-fired power plants,¹ and nuclear waste management at facilities such as LASL.

The mercury studies were incorporated into the ongoing canyon radioecology program for the following reasons: (1) several hundred grams of mercury are released to the canyons each year; (2) there is an existing data base on canyon ecosystems structure and function; (3) radionuclide concentration data are available for comparative purposes; and (4) considerable efficiency is gained by combining the mercury studies with the existing sampling program.

The group Analytical Section has recently developed a flameless atomic absorption procedure for mercury which can be used with reliability in a variety of sample matrices (Table I).² An outstanding advantage of this technique is the ability to analyze for mercury in almost any nonexplosive matrix which is small enough to fit into the combustion apparatus. Such diverse matrices as single mouse organs, skulls, pelts, gastrointestinal tract contents, as well as more conventional vegetation and soil samples, can be analyzed successfully by this technique. The sensitivity of the technique is about 10 ng mercury per sample.

Initial results on soil samples from the canyon indicate that concentrations of mercury are present at levels well above the 12 ± 9 ppb that were measured in background samples (Table II). Concentrations in stream channel soils averaged from 74 to 99 ppb mercury in three depth profiles, while stream bank soils generally averaged higher at 120

TABLE I
MERCURY CONCENTRATIONS IN NATIONAL BUREAU OF
STANDARDS STANDARD REFERENCE MATERIALS

<u>SRM</u>	<u>Found</u>	<u>Mercury Concentration (ppb)</u>	
		<u>$\bar{x} \pm SD$</u>	<u>NBS Certified Value</u>
<u>Orchard leaves</u>			
1571	147		
	167	158 $\pm$ 10	155 $\pm$ 15 ^a
	160		
<u>Bovine liver</u>			
1577	16		
	15	16 $\pm$ 2	16 $\pm$ 2 ^a
	18		
<u>Coal</u>			
1632	120		
	113	111 $\pm$ 10	120 $\pm$ 20
	100		
<u>Coal fly ash</u>			
1633	140		
	153	141 $\pm$ 12	140 $\pm$ 10
	130		

^aProvisional certificate.

TABLE II
MERCURY CONCENTRATIONS IN MORTANDAD
CANYON SITE 1 SOILS^a

Depth (cm)	Channel (10) ^b Mercury (ppb)	Bank (40) Mercury (ppb)
0-2.5	99 (1.4) ^c	150 (0.92)
2.5-7.5	74 (0.62)	170 (1.3)
7.5-30	75 (0.6)	120 (1.9)

^a Background mercury levels in canyon soils average 12 ± 9 ppb ($n = 10$).

^b Number of samples.

^c Coefficient of variation (standard deviation/mean).

to 170 ppb mercury. Higher concentrations of mercury in bank soils likely reflect the affinity of this element for organic matter.¹ Channel soils contain less than 0.6% by weight organic carbon, while bank soils average about 5%.

The distribution of mercury in channel and bank soils was similar in several ways to that observed for ¹³⁷Cs. Mercury was distributed rather uniformly throughout the top 30 cm of channel and bank soils and was similar to previous observations on ¹³⁷Cs. In addition, the high variability in soil mercury concentrations was analogous to that observed for ¹³⁷Cs in canyon soils. The coefficient of variation for mercury concentrations ranged from 0.6 to 2.3, which indicates potential problems in designing field studies for this element.³

There are also major differences in the distribution of mercury and ¹³⁷Cs in bank and channel soils. For example, mercury is depleted from channel soils and enriched in bank soils relative to ¹³⁷Cs (Table III). Mean concentration ratios (ppb Hg/pCi

TABLE III
MERCURY/¹³⁷Cs CONCENTRATION RATIOS
IN MORTANDAD CANYON SITE SOILS

Depth (cm)	Channel ($n = 10$)	Bank ($n = 40$)
0-2.5	0.17 ^a (0.79) ^b	1.9 (1.5)
2.5-7.5	0.24 (0.53)	3.2 (1.1)
7.5-30	0.19 (0.51)	6.6 (1.2)

^a Concentration ratio = ppb Hg/pCi ¹³⁷Cs/g.

^b Coefficient of variation.

¹³⁷Cs/g) ranged from 0.17 to 0.24 in channel soils and from 1.9 to 6.6 in bank soils. In addition, it appears that the ratio in bank soils increases with depth, indicating a greater mobility of the mercury.

In summary, initial results of the mercury studies indicate that this element is well mixed to 30-cm soil depths and is concentrated preferentially in stream bank soils. In addition, there are indications that mercury is relatively more mobile than ¹³⁷Cs in stream bank soils.

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Chemical Quality of Effluents and Their Influence on Water Quality in a Shallow Aquifer

[W. D. Purtymun, J. R. Buchholz, and T. E. Hakonson]

Industrial liquid effluents generated by Los Alamos Scientific Laboratory facilities are collected and processed at a treatment plant to remove most of the radioactive constituents. The plant, which became operational in June 1963, releases the liquid effluent into Mortandad Canyon. Hydrologic studies in the canyon were initiated in 1960 and have continued as part of the overall evaluation of the impact of the effluents on the environment.¹⁻³ The objective of this study was to investigate the change in water quality in a shallow aquifer downstream from the liquid waste stream.

Measurements were made on the volume of effluent and runoff entering the canyon, as well as the amount of water in the shallow aquifer. The chemical quality of effluent, runoff, and aquifer water as a function of time was assessed from 10 different chemical analyses on several hundred individual water samples. Chemical analysis techniques and methods of chemical mass inventory have been described in a previous paper.⁴

TABLE I
QUALITY OF WATER IN A SHALLOW AQUIFER DOWNSTREAM FROM A LIQUID WASTE TREATMENT PLANT

Year	Calcium	Magnesium	Sodium	Carbonate	Chemical Constituents (mg/L)				Dissolved Solids	Total Hardness	Specific Conductance at 25°C	
					Bicarbonate	Chloride	Fluoride	Nitrate			(µmhos/cm)	pH
1962 ^a	11	4	32	0	91	7	0.6	3	320	45	360	6.8
1963	14	5	53	0	90	9	0.5	16	310	67	360	7.2
1964	19	7	100	0	123	21	0.7	68	500	76	510	7.5
1965	16	5	99	0	143	13	0.7	40	400	59	440	7.6
1966	18	4	97	0	172	25	0.4	28	400	62	470	7.7
1967	21	7	140	0	147	16	0.7	15	340	82	400	7.7
1968	17	6	110	0	138	12	0.7	8	300	68	360	7.9
1969	23	5	140	0	129	11	0.5	25	370	81	410	8.2
1970	26	7	132	0	143	15	0.5	137	530	84	560	7.6
1971	44	11	228	0	176	54	0.9	366	990	153	1130	7.7
1972	40	11	215	0	223	54	0.7	248	910	144	1000	7.5
1973	33	7	164	0	221	34	1.0	204	786	112	910	7.7
1974	27	6	188	0	254	32	1.0	207	785	93	970	7.7

^aAnalyses for 1962 prior to release of effluents.

Liquid effluents from the treatment plant are the major source of chemicals entering Mortandad Canyon. Most of the measured chemical concentrations generally average 2 to 20 times higher in effluent than in other surface water sources in the canyon. One exception was magnesium which averaged higher in storm runoff water. Many of the chemicals in the effluent from the treatment plant have increased since 1963, although not in a consistent pattern. Parameters exhibiting increases include magnesium, sodium, bicarbonate, chloride, nitrate, dissolved solids, and conductivity. Relatively stable or decreasing values were observed for calcium, carbonate, fluoride, total hardness, and pH.

The release of industrial effluents into the canyon has changed the quality of the water in the aquifer (Table I). The pH of the water has changed from 6.0 to 8.0 due to the alkaline nature of the liquid effluents. Most of the remaining chemical parameters have increased since 1962, although not consistently with time. The levels of fluoride and bicarbonate have approximately doubled, while values for calcium, magnesium, sodium, chloride, nitrate, dissolved solids, and specific conductance have increased by factors of 3 to 7. Carbonates remained nondetectable in aquifer water during the entire

observation period due to their conversion to HCO_3^- and CO_2 under the conditions in the aquifer.

Annual average concentrations of sodium, chloride, nitrate, and total dissolved solids in the effluent were compared with corresponding concentrations in solution in the aquifer to determine the relationship between chemical release and levels appearing in the aquifer (Fig. 1). The linear least-squares regressions were all highly significant ($P < 0.01$) with correlation coefficients (r) ranging from 0.75 to 0.80.

The slopes of the four regression lines indicate that concentrations of the chemicals in solution in the aquifer averaged from 30 to 50% of those measured in the effluent when prerelease concentrations (i.e., intercept values) were taken into consideration. The chemical effluents from the treatment plant are diluted with about equal volumes of storm runoff and cooling water from upstream sources. Thus, reduction in chemical concentrations in the aquifer could be the result of dilution alone. The intercepts of the regression lines for sodium, chloride, and nitrate were not significantly different from pre-effluent release levels of chemicals in the aquifer, indicating a rapid "turnover" of the water in storage. This is supported by results of tracer

Fig. 1. The relationship of concentrations of sodium, chloride, nitrate, and total dissolved solids in effluents released from the treatment plant and corresponding concentrations in the shallow downstream aquifer.

studies indicating water transit times of about 1 yr.³

The mass of chemicals added to Mortandad Canyon and in storage in the aquifer was estimated from the respective concentration and volume data. About 625 000 kg of the 8 chemicals listed in Table II were released into Mortandad Canyon from 1963 to 1974. Nearly 90% of this mass consisted of nitrates, bicarbonates, carbonates, and sodium, with calcium, magnesium, chloride, and fluoride comprising the remainder.

Mass inventories of most of the chemicals in solution in the aquifer during 1974 were higher by factors of 2 to 65 over pre-effluent release inventories (Table II) and averaged 1 to 65% of the total chemical releases. The largest increases over

TABLE II

MASS INVENTORY OF CHEMICALS RELEASED IN EFFLUENT AND IN STORAGE IN THE AQUIFER AS A FUNCTION OF TIME

Constituents	Effluents (kg x 10 ³)		
	1963-1974	1962 ^a	1974
<u>TA-50</u>			
Calcium			35
Magnesium			1.8
Sodium			170
Carbonate			84
Bicarbonate			150
Chloride			31
Fluoride			1.3
Nitrate			150
Total dissolved solids			640
<u>In storage</u>			
Calcium		0.2	0.5
Magnesium		0.08	0.1
Sodium		0.6	3.6
Carbonate		0	0
Bicarbonate		1.8	4.8
Chloride		0.1	0.6
Fluoride		0.01	0.02
Nitrate		0.06	3.9
Total dissolved solids		6.4	15

^aPrior to effluent release to the canyon.

prerelease inventories were noted for sodium and nitrate, reflecting relatively larger concentrations of these chemicals in the treatment plant effluent.

Chemical concentrations in solution in the aquifer have increased over prerelease levels; however, there has not been a steady accumulation of these materials in the water with time. The rapid loss of water and its associated chemicals from the aquifer prevents chemical accumulation and indicates that cessation of effluent release to the canyon would rapidly improve the quality of water in the aquifer.

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Los Alamos National Environmental Research Park

[L. J. Johnson, Coordinator]

This program provides an outdoor laboratory area where environmental research can be carried out as a part of the energy research and development activities at the Los Alamos Scientific Laboratory (LASL). Developing an understanding of the relationship and possible effects of pollutants on other potential energy development impacts on natural systems and

environmental resources is the general aim of this program.

Formal designation by ERDA of the Los Alamos National Environmental Research Park (LA/NERP) was announced on November 15, 1976. An oblique westerly looking aerial view of the LA/NERP is shown in Fig. 1. The LA/NERP comprises nearly 11 000 ha (27 500 acres) surrounding LASL's energy technology research facilities. Features which make this a unique locale for environmental research include a relatively sharp elevational gradient, within which fall four distinct continental life zones: Lower Sonoran, Upper Sonoran, Transition, and Canadian.

Primary research emphasis during 1976 was on tasks providing fundamental characterization of the LA/NERP environs. A survey and draft report¹ on the archaeological resources of the LA/NERP were completed. Approximately 300 separate ruins were identified, primarily on the mesa tops. The locations of

Fig. 1. Aerial view of the Los Alamos National Environmental Research Park (LA/NERP).

these sites are shown on a map which is now a part of the land use planning at LASL. About a dozen of these ruins are considered especially outstanding; three have been proposed as national historic places.

A soil survey of the Los Alamos area was completed, and a report² is in preparation. Five of the 10 nationally recognized soil orders were identified: Alfisols, Aridisols, Entisols, Inceptisols, and Mollisols. A detailed map has been prepared containing 61 mapping units. The report provides information on soil color, texture, structure, consistency, clay films, coarse and fine fragment

distributions, permeability, pH, and soil horizon boundaries for each mapping unit. This fundamental information is of use for environmental research activities and LASL engineering studies.

Some work on the geology and seismicity of the Los Alamos area was completed and reported^{3,4} to provide information for environmental and engineering studies at LASL. The LA/NERP is located on the Pajarito Plateau which forms a topographic high on the western edge of the Rio Grande rift. The Rio Grande rift is formed by a number of north-south trending faults that extend from southern Colorado

TABLE I
FLORA DISTRIBUTION BY FAMILY AND SPECIES NUMBER ALONG AN ELEVATIONAL GRADIENT
AND IN DISTURBED HABITATS OF THE LASL ENVIRONS

<u>Overstory Vegetation Type</u>	<u>Elevation (m)</u>	<u>Number of Families</u>	<u>Number of Species^a</u>
Fir-aspen-spruce	2865	18	28
Subalpine meadow	2865	10	24
Ponderosa pine-fir	2560	9	22
Ponderosa pine/piñon-juniper	2255	12	25
Piñon-juniper	1950	8	17
Juniper	1645	8	11
<u>Disturbed Habitat</u>			
Ponderosa pine/piñon-juniper	2000-2190	12	22

^aNumber of grass species have not been included.

TABLE II
RELATIVE ABUNDANCE AND DISTRIBUTION OF SMALL MAMMALS IN
OVERSTORY VEGETATION TYPES OF THE LASL ENVIRONS

<u>Overstory Vegetation Type</u>	<u>Elevation (m)</u>	<u>Number of Mammalian Species</u>	<u>Relative Abundance^a</u>
Fir-aspen-spruce	2865	6	21.8
Subalpine meadow	2865	2	37.2
Ponderosa pine-fir	2560	5	13.3
Ponderosa pine	2255	3	2.0
Piñon-juniper	1950	3	3.8
Juniper	1645	3	13.1
<u>Canyon Study Areas</u>			
Ponderosa pine-fir	2135-2190	7	7.4
Ponderosa pine/piñon-juniper	2010-2090	6	5.8
Juniper	1755	4	8.6
<u>Disturbed Habitats</u>			
Ponderosa pine/piñon-juniper	2000-2190	2	14.2

^aNumber of animals captured per 100 trap-nights.

through New Mexico into northern Mexico. Seismic frequency for the Los Alamos area was estimated using geologic data, historical records, and instrument data. This analysis indicated that the strongest earthquake projected to occur within about 100 km of Los Alamos during a 100-yr period would range up to magnitude 5.5.

Preliminary studies were completed to characterize the understory flora and small-mammal fauna according to composition and distribution within the LA/NERP.⁵ The results represent the ecological distribution of the biota along a 1500-m east-west elevational gradient and encompass 310 km². Results of the vegetation analysis (summarized in Table I) depict a general decrease in number of families and species occurring within plots with decreasing elevation.

Small-mammal trapping studies yielded 1269 captures of individual animals through 13 460 trap-nights. Nine species were identified, representing 3 families: Sciuridae, Cricetidae, and Soricidae. Eight species of nontrap-susceptible small mammals, or those which did not occur within the study plots, were also identified, representing an additional 3 families. These results are presented in Table II, expressed as number of species and number of animals captured per 100 trap-nights. Table III identifies the species captured (with common name in parentheses).

An intensive study of mule deer (Odocoileus hemionus) in the LA/NERP was begun in January 1975

TABLE III

SPECIES OF SMALL MAMMALS CAPTURED

Cricetidae

Reithrodontomys megalotis (western harvest mouse)

Peromyscus maniculatus (deer mouse)

Microtus montanus (montane vole)

Peromyscus truei (piñon mouse)

Microtus pennsylvanicus (meadow vole)

Clethrionomys gapperi (Gapper's red-backed mouse [vole])

Neotoma mexicana (Mexican woodrat)

Sciuridae

Eutamias minimus (least chipmunk)

Soricidae

Sorex nanus (dwarf shrew)

because they represent an important potential receptor and vector in pollutant transfer to humans. Objectives of the study are to determine the population distribution, movements, density, and health of the deer.

Thus far, 28 deer (20 adult females, 4 adult males, 1 juvenile female, and 3 juvenile males) have been live-trapped and marked with neck bands and ear tags prior to release. Information provided by approximately 200 resightings of marked individuals indicates that Laboratory deer move to lower elevations as snow depths increase during the winter and then return to higher elevations during the spring and summer. Individual deer usually restrict their movements to one or two mesa tops; however, it is apparent that male deer move considerably greater distances than females. It appears that juvenile males move over a larger area than adult males, which may be a reflection of dispersal in the juveniles.

Deer pellet group plots are used as an index to deer densities, as well as indicators of distribution. A stratified-random sampling technique based on habitat type (piñon-juniper, ponderosa pine, fir-aspen) was employed to estimate relative deer populations. A total of 100 rectangular plots (88 m²/plot) were read in the spring and fall. Population density estimates, based on pellet group counts, indicate that there are 1084 ± 416 deer (95% confidence interval) residing on 103 km² of LA/NERP and nearby U. S. Forest Service land. Pellet group densities demonstrate that the fir-aspen habitat is the most heavily utilized vegetation type during the summer with approximately 18 deer/km². Piñon-juniper habitat showed the lowest deer use with approximately 6 deer/km² during the summer.

Characterization of litter- and soil-inhabiting invertebrates at selected LA/NERP locations was initiated. Invertebrates were extracted from 1 cm² soil cores by use of the Tullgren funnel technique⁶ into a 70% alcohol solution. This was followed by sorting, identification, and counting. Analyses from 5 sampling periods were completed: July-August 1975, November 1975, January 1976, March 1976, and May 1976. Over 9800 specimens representing 100 to 110 species were isolated from 217 samples. Species of the Order Acarina (ticks and mites) were most abundant with a relative density (percent of total

animals) of 78% and a frequency (percent of occurrence in samples) of 93%. The mean number of animals per sample ranged from 10 to 90. On a seasonal basis, the mean number of animals per sample was highest during the November, March, and May collection periods, while the July-August and January means were about half as large.

Efforts to identify threatened or endangered fauna or flora in the Los Alamos area continued during 1976. Species or subspecies of plants and animals which have been determined by the U. S. Fish and Wildlife Service to be potentially in New Mexico have been sought for. Thus far, one endangered species, the peregrine falcon (*Falco peregrinus*), has been identified and studied. Continuous records of peregrine falcon eyries in the vicinity of Los Alamos since 1964 have been made available. All eyries occupied during the 1964-1973 period were entered in early January to obtain eggshell fragments. No samples were obtained for 1974 or 1975 because the female falcon did not lay eggs. A possible cause of the reproductive decline was noted in a 1973 eggshell fragment analyzed to be 20% thinner than normal. This sample contained DDE residues of 960 ppm -- as high as any other values reported in North America. It was presumed that the DDE was obtained by the falcon during its winter habitation in Mexico and Central America because of the discontinuance of DDT use in the United States.

Future major work objectives under the LA/NERP program include continuation of the basic environmental characterization tasks, preparation of a LA/NERP brochure and overall map to provide environmental setting and resources information to interested parties, and establishment of liaison with other regional agencies and groups interested in information exchange and pursuit of research activities at the LA/NERP.

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Radiation Ecology Studies of Northern Alaska Ecosystems

[W. C. Hanson]

Radiological studies of arctic ecosystems have been conducted for many years in circumpolar countries because of the human radiation exposure aspects of the lichen-reindeer/caribou-man food chains that are characteristic of the northern regions. Extensive investigations have been made by measurements of ⁹⁰Sr and ¹³⁷Cs, the two world-wide fallout radionuclides that have contributed most of the long-term radiation exposure to the northern peoples. By contrast, transuranic elements in natural systems have been studied sparingly because of their relatively low solubility and transference in biological systems, their more modest rates and amounts of deposition upon the landscapes, and the difficulty of their analyses.

This report summarizes measurements of ¹³⁷Cs and plutonium isotopes in samples of soil, lichens, and caribou and ¹³⁷Cs whole-body burdens of Eskimos at Anaktuvuk Pass, Alaska, that were obtained as part of continuous investigations of worldwide fallout in arctic ecosystems that began in 1959 and, since 1962, have centered at Anaktuvuk Pass in the Central Brooks Range.^{1,2} Information is included for ¹³⁷Cs and plutonium isotopes in soils and lichen communities of the Thule, Greenland, region where studies were conducted during participation in Danish expeditions of 1968 and 1974 which investigated the ecological consequences of plutonium released to the Thule environs by the nonnuclear explosion of four unarmed nuclear weapons during the crash of a U. S. Air Force B-52 aircraft on January 21, 1968.^{3,4}

Cesium-137. -- Another year of measurements of ¹³⁷Cs concentrations in the 3 trophic levels of the Anaktuvuk Pass lichen-caribou-Eskimo food web are summarized in Fig. 1. Several important

Fig. 1. Cesium-137 concentration in Eskimos, caribou flesh, and lichens at Anaktuvuk Pass, Alaska, during the period 1962-1976.

observations emerge from these data: ^{137}Cs concentrations in the *Cladonia-Cetraria* lichen carpets that have been continuously sampled during the study have apparently stabilized near 10 to 11 pCi/g dry wt; (2) ^{137}Cs concentrations in caribou flesh have been consistently lower during the summers of 1965, 1968, and 1975 -- years in which they spent considerable time on winter ranges in close proximity to Anaktuvuk Pass; (3) the years of depressed ^{137}Cs concentrations in caribou flesh also coincide with changes in seasonal use of caribou for food by the Eskimos; and (4) the substantial decline in the western arctic caribou herd during the period 1970-1976 has suddenly instituted a great change in the availability of caribou to the Anaktuvuk Pass residents and significantly lowered their ^{137}Cs body burdens.

Measurements of ^{137}Cs whole-body burdens were made during the normal winter (January) low and summer (July) high periods of the annual cycle (see Table I). The combined effects of low availability of caribou during 1975 and 1976, ample employment of family heads on new school construction within the village, and the sudden collapse of the school construction jobs in March 1976, followed by the caribou shortage that has prevailed throughout the year, are readily apparent in the results.

The inventory of cesium-137 in contiguous *Cladonia-Cetraria* carpets at Anaktuvuk Pass (see Table II) increased steadily from 6.2 nCi/m² in

TABLE I

CESIUM-137 WHOLE-BODY BURDENS OF ANAKTUVUK PASS RESIDENTS DURING WINTER AND SUMMER PERIODS (1976)

Population	Number	Body Burden (nCi)
<u>January 22-26</u>		
Adults (> 21 yr)	41	90 ± 6.9
Minors (15-21 yr)	5	36 ± 8.1
Children (< 15 yr)	10	14 ± 1.0
<u>July 7-10</u>		
Adults (> 1 yr)	33	100 ± 11.0
Minors (15-21 yr)	14	40 ± 8.0
Children (< 15 yr)	16	15 ± 1.4

the initial sampling in 1962 to maximum values of about 50 nCi/m² in 1965 and has subsequently shown a downward trend, with occasional increase due to worldwide fallout pulses about 1 yr following high-yield atmospheric nuclear tests by China and France. As noted in Table II, the estimated ^{137}Cs deposition and amount in the lichen carpet were in close agreement, although the landscapes had been exposed to an undetermined amount of fallout during the period prior to HASL measurements at Fairbanks that began in 1960. Comparison of fallout deposition rates at New York during periods prior to 1960 and after 1960 suggests that the above deposition estimates for the Fairbanks-Anaktuvuk Pass areas should be about doubled. Fallout deposition at Anaktuvuk Pass was estimated by extrapolating the deposition at Fairbanks, some 400 km southeast of Anaktuvuk Pass, to the study area by multiplying by 0.67, the ratio of annual precipitation rates at those two locations. During 1975, soil samples from Anaktuvuk Pass contained 36 nCi $^{137}\text{Cs}/\text{m}^2$, compared to 34 nCi $^{137}\text{Cs}/\text{m}^2$ in lichen mats -- an exceptional agreement; the 1976 lichen samples taken in July contained 35 nCi/m² and average biomass. Additional soil and lichen samples were obtained in September to verify these values. A similar circumstance occurred in July 1964 when values in lichens attained 41 nCi/m², compared to a cumulative ^{137}Cs deposition of 26 nCi/m². This mobilization of ^{137}Cs from an unidentified source suggested the experiments on half-times of radionuclides in lichens and modeling of arctic food chains that have been reported previously.⁵⁻⁷

TABLE II

WORLDWIDE FALLOUT CESIUM-137 INVENTORY IN CLADONIA-CETRARIA CARPETS
AT LONG-TERM SAMPLING SITES NEAR ANAKTUVUK PASS DURING THE PERIOD 1962-1976^a

Sampling Date	Number	Biomass (kg/m ²)	Cesium-137 Inventory (nCi/m ²) ^b	Deposition (nCi/m ²) ^c
July 3, 1962	6	0.52	6.2 ± 2.5	5.14
July 7, 1963	3	0.61	14 ± 1.4	9.82
July 12, 1964	4	1.53	41 ± 2.6	11.01
July 26, 1965	10	1.45	48 ± 4.3	10.97
August 2, 1966	7	1.26	34 ± 1.2	2.13
August 2, 1967	10	1.20	30 ± 1.2	0.63
June 30, 1968	10	1.16	30 ± 1.3	0.60
July 29, 1969	5	2.95	44 ± 1.6	0.66
August 2, 1970	6	1.66	24 ± 5.3	0.91
August 9, 1971	1	3.96	24	0.67
July 25, 1972	2	2.82	44 ± 1.0	0.00
July 2, 1973	3	2.42	23 ± 0.8	0.06
September 26, 1974	2	1.74	44 ± 1.3	0.32
July 21, 1975	2	3.33	34 ± 4.2	0.12
July 8, 1976	2	2.76	35 ± 3.0	0.00
				43.04

^aValues are based upon samples of 0.25- to 0.50-m² replicates from contiguous sampling areas.

^bMean ± standard deviation.

^cCumulative fallout deposition at Anaktuvuk Pass between successive sampling periods based on 0.67 of monthly measurements at Fairbanks.⁸

TABLE III

COMPARISON OF CESIUM-137 DISTRIBUTION IN LICHEN COMMUNITY COMPARTMENTS
IN PURE STANDS OF VARIOUS SPECIES AT ALASKA AND GREENLAND STUDY SITES

Taxon	Number	Percent of Cesium-137 Inventory			Cesium-137 (total
		Principal Lichen	Other Lichens	Vascular Plants	nCi/m ²)
<u>Alaska</u>					
<u>Cladonia alpestris</u>	4	84	7	9	40.0
<u>Cetraria cucullata</u>	2	53	21	26	14.5
<u>Cetraria delisei</u>	5	81	3	16	39.2
<u>Alectoria ochroleuca</u>	1	83	14	3	24.3
<u>Stereocaulon paschale</u>	5	66	19	15	40.0
<u>Greenland</u>					
<u>Cetraria nivalis</u>	8	69	8	24	28.0
<u>Cetraria delisei</u>	4	60	12	28	27.2
<u>Alectoria ochroleuca</u>	6	86	5	9	12.7
<u>Stereocaulon paschale</u>	1	86	3	11	22.6

Distribution of the ^{137}Cs inventory among various community components in nearly pure stands of various lichen species from Alaska and Greenland is summarized in Table III. Inventories in Alaska were about twice as great as those of Greenland, with 70% located in the principal lichen species, and substantially lesser amounts in subordinate species and in vascular plants and parts. The relatively high value for vascular plants was attributed to dead materials that had collected fallout materials deposited in earlier years when fallout deposition was greater, the slow breakdown of such litter, and the more dynamic translocation of elements by the lichens. Cesium-137 inventories in Greenland lichen communities were about 60% as great as those in Alaska, or about proportional to their differences in annual precipitation. However, the estimated ^{137}Cs deposition measured at Thule was 37% of that estimated for Anaktuvuk Pass, suggesting that the Thule lichen communities were more efficient at accumulating ^{137}Cs and perhaps other radionuclides from the environment. The close agreement between most of the ^{137}Cs inventories in soils and the lichen communities of Thule indicated that the two components were balanced by some physical transport mechanism, such as the wind, in the region.

Plutonium-238, Plutonium-239, and Plutonium-240.--Soil samples of the Thule region during 1974 contained an average of $15.2 \pm 1.6 \text{ fCi}$ ($= 10^{-15} \text{ Ci}$) per g dry wt of $^{239,240}\text{Pu}$ in those areas considered to be uncontaminated by the January 1968 accident debris. The $^{239,240}\text{Pu}$ inventory was estimated to be $0.85 \pm 0.10 \text{ nCi/m}^2$ from those samples. Three sampling locations within an area of about 16 km^2 near the small habitation (2 families) of Narssarssuk contained 4 to 80 times that amount. Alluvium from intermittent streams that drained the landscapes across which the soil samples were taken corroborated the soil measurements and indicated that the plutonium is slowly being transported toward the lower beach terraces bordering Bylot Sound. The nature of variability in replicate samples from the Narssarssuk area indicated that, in most samples, the radioactivity was associated with small particles of indeterminate size. However, a few samples contained sufficient activity to suggest the presence of $^{239,240}\text{PuO}_2$ particles up to $3.2 \mu\text{m}$ in diameter.

Lichens from several Thule locations during 1974 contained a mean concentration of $0.26 \pm 0.04 \text{ pCi } ^{239,240}\text{Pu/g}$ and a total inventory of 0.168 nCi/m^2 . This was about 20% of the plutonium inventory in soils of the surrounding area. During 1968, comparable values were $0.25 \pm 0.03 \text{ pCi/g}$ and 0.124 nCi/m^2 ; however, it was estimated that $0.016 \text{ nCi } ^{239,240}\text{Pu/m}^2$ had been deposited by worldwide fallout upon the Thule landscapes during the sampling interval.

Estimated inventories of fallout radionuclides in various lichen species during the period 1971-1973 at Anaktuvuk Pass (Table IV) were generally greatest in Cladonia alpestris and lowest in Cetraria delisei, although the data are not sufficient at this time for conclusive interpretation. Considerable variation between population samples from the various lichen communities remains to be analyzed by longer term studies and additional radiochemical analyses. The major lichen population in both Alaska and Greenland samples contained 72 to 74% of the total inventories of plutonium and cesium radionuclides; about 8% was in the subordinate lichens and about 20% in vascular plants and debris.

Estimates of fallout radionuclide inventories in the arctic landscapes of interest in this report began during 1959 and 1960 and have been estimated recently by soil samples at Thule, Greenland, to be $0.33 \text{ mCi } ^{239,240}\text{Pu/km}^2$ ($= \text{nCi/m}^2$) and 0.40 to 0.60 mCi/km^2 in northern Alaska. Assuming an average $^{239,240}\text{Pu}/^{137}\text{Cs}$ ratio of 0.016, these values translate to $20.6 \text{ mCi } ^{137}\text{Cs/km}^2$ at Thule, Greenland, and $35.6 \text{ mCi } ^{137}\text{Cs/km}^2$ at Anaktuvuk Pass. These are reasonably close to the amounts calculated from HASL fallout deposition data for the period after 1959-1960, when the measurements began. Therefore, a large increment of the early fallout period is unaccounted for in both soils and lichen communities of the study sites, although the lichen communities of northern Alaska during 1964 contained 41 nCi/m^2 compared to an estimated deposition of 26 nCi/m^2 . Concentrations in several lichen communities at Thule agreed closely with the estimated $^{239,240}\text{Pu}$ inventory of 0.85 nCi/m^2 in soils collected during 1974 and, thus, were significantly greater than the estimate of 0.33 nCi/m^2 in 1970-1971 from HASL fallout measurements at Thule. The $^{239,240}\text{Pu}/^{137}\text{Cs}$ ratio of 0.038 ± 0.006 in the 1974 soil samples

TABLE IV
ESTIMATED INVENTORIES OF ^{238}Pu , $^{239,240}\text{Pu}$, AND ^{137}Cs IN AREAL SAMPLES^a
OF ALASKAN LICHEN COMMUNITIES DURING THE PERIODS 1968 AND 1971-1973

Year	Taxon	Number	^{238}Pu	$^{239,240}\text{Pu}$	^{137}Cs
1968	<u>Cladonia-Cetraria</u>	1	0.019	0.28	30.0
1971	<u>Cladonia alpestris</u>	1	0.030	0.47	42.1
	<u>Cetraria delisei</u>	1	0.013	0.18	40.4
	<u>Alectoria ochroleuca</u>	1	0.002	0.04	9.2
1972	<u>Cladonia-Cetraria</u>	2	0.024	0.28	44.0
	<u>Cladonia alpestris</u>	1	0.029	0.30	27.9
	<u>Cetraria delisei</u>	2	0.028	0.30	40.2
1973	<u>Cladonia-Cetraria</u>	3	0.024	0.33	23.4
	<u>Cladonia alpestris</u>	1	0.038	0.30	26.8
	<u>Cetraria delisei</u>	2	0.007	0.10	27.7
	<u>Stereocaulon paschale</u>	2	0.069	0.32	57.4

^aIn nCi/m².

represents a significant deviation from the value of 0.016 reported to represent the normal worldwide fallout situation. A second source contribution of plutonium from the 1968 accident debris accordingly is distributed widely over the Thule environs.

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INTRODUCTION

The following reports of the Cellular and Molecular Biology Group represent aspects of our ongoing programs selected by the principal investigators as being at a sufficient stage of completion to warrant presentation at this time. No attempt has been made to provide a comprehensive description of the programs.

The overall intent of the studies is an understanding of the biochemical events which ensure the continuity of cellular behavior from one generation to another and to detect and quantitate energy-related toxicants which interfere with normal cellular activities. The major interest of the group focuses on the mammalian cell in culture which provides a convenient and efficient means for examining perturbants which affect a large fraction of the cellular population, as well as those which produce rare genetic alterations at specific loci. A considerable effort examines the effects which result as a consequence of chemical alteration of DNA. Thus, an interrelated program is studying the chemical reactivity of enzymatically or light-activated environmental agents with DNA and chromatin, the extent of repair of alterations, and the biological consequences of damage and repair assayed by analysis

of DNA replication and RNA transcription, cell survival and cell-cycle accumulation, karyologic alterations, and mutation induction. At the same time, our experience as biologists, that cells with a constant genetic constitution can nonetheless exhibit a wide range of morphological and biochemical individuality, cautions us against the premature acceptance of mutagenesis as the exclusive mechanism for variant cell derivation. These concerns have led us to develop nucleic acid hybridization techniques which should allow us to detect alterations in the expression and utilization of messenger sequences.

A continuing objective of the program involves efforts to determine the extent to which cellular behavior in culture reflects the *in vivo* situation and to extend the utility of cell culture techniques as a tool for toxicological studies. An area which is particularly confused concerns the validity of *in vitro* criteria for tumorigenic potential. We believe that a major impediment stems from inadequacies in current procedures for establishing the tumorigenicity of cultured cells in animals, and we are involved in a systematic study of cell implantation which will ideally allow all cells, normal as well as tumorigenic, to survive.

CELLULAR METABOLISM

Chromatin Structure near the DNA Replication Fork

[C. E. Hildebrand and R. A. Walters]

Recent advances in studying the fine structure of eukaryotic chromatin have led to a generalized subunit structure of chromatin. However, the mechanisms by which this repeating subunit or nucleosome structure is replicated remain to be elucidated. It is clear that chromatin replication in eukaryotic cells requires not only duplication of the DNA but also coordinate synthesis of chromatin proteins and association of these proteins with the nascent DNA duplexes. We summarize herein studies of the incorporation of replicating DNA into the subunit structure of chromatin. Sedimentation analysis of nuclease-treated chromatin provides a direct comparison of the assembly of newly replicated DNA into nucleosomes with the subunit organization of non-replicating chromatin. Since reports from several laboratories have indicated that the "native" structure of chromatin may be altered during isolation and shearing procedures, the studies described here have been performed using isolated nuclei as substrate for micrococcal nuclease digestion.

To examine the incorporation of newly replicated DNA into chromatin subunits, nuclei from cells pulse-labeled with ^3H -thymidine for 0.5 min were digested with micrococcal nuclease, and the chromatin was analyzed in isokinetic sucrose gradients (see Fig. 1A). Several major features should be noted regarding the data in Fig. 1A. First, the peaks sedimenting at fractions 9, 12, and 14 correspond, respectively, to the monomer, dimer, and trimer of the chromatin subunit organization characterized for several cell types in other laboratories and by Strniste, Rall, and Okinaka of this Laboratory. Second, the pulse-labeled chromatin is rapidly degraded to monomer and dimer nucleosomes, compared with the uniformly labeled chromatin. In fact, Fig. 1A shows that 46% of the ^3H label in the gradient is in the mononucleosome peak, compared with 6% of the ^{14}C uniform label. In comparison, 52% of ^3H -labeled DNA and 13% of the ^{14}C -labeled DNA were rendered acid-soluble during nuclease treatment. Finally, the small peak of ^3H -radioactivity appearing between fractions 1 and 6 in Fig. 1A may be nuclease-resistant submonomer assembly intermediates involved in the replication

Fig. 1. (A) Isokinetic neutral sucrose gradient analysis of chromatin following micrococcal nuclease digestion of the nuclei from pulse-labeled CHO cells. Cells were uniformly prelabeled with ^{14}C -thymidine ($-●-$) and pulse-labeled with ^3H -thymidine ($-○-$) for 5 min. Micrococcal nuclease digestion of the isolated nuclei was performed for 2.5 min. Sedimentation was from left to right. (B) Alkaline sucrose gradient analysis of DNA from CHO cells pulse-labeled for 0.5 min with ^3H -thymidine. Gradients were calibrated with λ -phage and T2 phage DNAs. Sedimentation was from left to right.

process or may be small DNA degradation products resulting from incomplete nuclease digestion, although the discreteness of the distribution argues against this possibility.

Examination of the size of the DNA synthesized during the 0.5-min pulse revealed that 52% of the DNA sedimented at $\sim 4\text{S}$, similar to the size of the eukaryotic "Okazaki fragment" (Fig. 1B). It is

evident that very little pulse-labeled DNA has matured to higher molecular weight intermediates during the 0.5-min pulse. Hence, the results in Fig. 1B, in combination with those of Fig. 1A, suggest that newly replicated DNA is rapidly assembled into the subunit structure of chromatin at times preceding the joining of the newly synthesized DNA strands into high molecular weight DNA.

To estimate the time required for newly replicated DNA (chromatin) to display a nuclease digestion profile similar to that of nonreplicating chromatin, uniformly prelabeled cells were pulse-labeled for 1 min, harvested, washed, and chased in fresh medium for times up to 30 min. Results from sedimentation analysis of nuclease-treated nuclei obtained at various times during the chase period indicate that most of the DNA synthesized in the 1-min pulse matured during a 10-min chase to a configuration displaying nuclease sensitivity similar to nonreplicating chromatin.

While the maturation of newly replicated chromatin to a configuration having the same nuclease sensitivity as nonreplicating chromatin requires times of the order of minutes, the incorporation of newly replicated DNA into nucleosomes requires only seconds. Our results also indicate that incorporation of newly replicated DNA into nucleosomes occurs when newly synthesized DNA is predominantly the size of the lowest molecular weight replication intermediate ($\sim 4S$ by alkaline sucrose gradient analysis, see Fig. 1B). Hence, the increased nuclease sensitivity of newly replicated chromatin may be attributed to (1) preferential nuclease attack at the single-stranded gaps between unjoined nascent 4S DNA molecules; (2) enhanced nuclease degradation at the unjoined 4S ends due to the presence of RNA primers, since micrococcal nuclease has a specificity for both RNA and DNA; (3) an absence of protection of replicating DNA by chromatin proteins; or (4) a combination of the above.

The folding or "kinking" of DNA suggested for its arrangement in the nucleosome may require twisting (or supercoiling). Hence, it is possible that the packaging of newly synthesized DNA into nucleosomes is facilitated by the absence of rotational constraints on the newly replicated DNA duplex (due to the presence of gaps at the ends of nascent 4S DNA strands).

Fig. 2. Model summarizing events near the DNA replication fork.

Preliminary experiments have indicated a correlation between the maturation of newly synthesized DNA strands into successively larger replication intermediates and the maturation of newly replicated chromatin into a structure similar to that of nonreplicating chromatin. Further investigations of this relationship are in progress. The possible involvement of histone modifications and nonhistone proteins in maturation of newly replicated chromatin must also be considered.

These findings and their implications are summarized in a simple model shown in Fig. 2. Chromatin subunits in nonreplicating regions (shown just to the left of the replication fork) are packed in a highly ordered structure (a helical arrangement of nucleosomes with a repeat every 6 to 7 units as described by J. T. Finch and A. Klug). At the replication fork, the structure of chromatin subunits must be altered to allow access of the replication machinery to the parental DNA. DNA is synthesized in short segments (approximately of nucleosome size) indicated by the saw-tooth line (nascent DNA). The segments of newly replicated DNA undergo rapid packing into nucleosomes possibly requiring supercoiling or kinking. The short, newly synthesized chains are then joined (or ligated), and further packing of the nucleosomes proceeds to the prereplication configuration. This model is highly speculative and has ignored the problem of doubling the histone content of replicated chromatin (i.e., deposition of newly

synthesized histones onto the duplicated DNA strands).

These studies provide a rudimentary indication of differences in the structure (packing) of replicating and nonreplicating DNA. It will be possible with this information to ask questions concerning the differential effects on replicating and nonreplicating chromatin of chemical agents associated with fossil fuel energy technology and, hence, the mutagenic potential of such agents in dividing or nondividing systems.

The Use of Circular Synthetic Polydeoxynucleotides as Substrates for the Study of DNA Repair Enzymes

[R. L. Ratliff and J. Bingham]

Circular DNAs with no free 3'- or 5'-ends have an advantage over linear DNAs. They cannot be hydrolyzed by exonucleases and provide substrates with high sensitivity for the detection of endonucleases, especially endonucleases that are specific for damaged or altered bases and are involved in repair of damaged DNA. The first enzymatic step

in the repair process is introduction of a single-strand break in the DNA adjacent to a damaged or altered nucleotide residue by an endonuclease, removal of the damaged nucleotides by an exonuclease, replacement of damaged nucleotides by DNA polymerase, and joining the DNA strands by ligase.

We have prepared circular forms of synthetic polydeoxynucleotides using two different procedures. The first procedure is essentially the same as that used by recombinant DNA researchers. Polydeoxyadenylate or polydeoxythymidylate tails are added to the 3'-hydroxyl ends of a DNA molecule, the dA- and dT-DNAs are mixed and allowed to anneal, and a mixture of the enzymes, substrates, and co-factors needed for closure are added to the DNA solutions. In the second procedure, polydeoxyadenylate extensions are added to the 3'-termini of a synthetic DNA with terminal transferase. After removal of the unreacted dATP, polydeoxythymidylate extensions are added to the 3'-hydroxyl end of the polydeoxyadenylate. This gives a loop of dA and dT nucleotides and a double-stranded region of dA-dT base

TABLE I
LINEAR POLYDEOXYNUCLEOTIDES^a

Polymer	T _m (°C) (0.05 M Na ⁺)	% Resistant to Exonuclease III
1. d[(AC) _n -A _n]·d[(GT) _n -A _n]	79.5	99
2. d[(AC) _n -T _n]·d[(GT) _n -T _n]	82.5	100
3. d[(AG) _n -A _n]·d[(CT) _n -A _n]	73.5	100
4. d[(AG) _n -T _n]·d[(CT) _n -T _n]	75.0	97
5. d[(AC) _n -A _n -T _n]·d[(GT) _n -A _n -T _n]	57 + 70	1.5 ^b
6. d[(AC) _n -T _n -A _n]·d[(GT) _n -T _n -A _n]	57 + 75	1.7 ^b
7. d[(AG) _n -A _n -T _n]·d[(CT) _n -A _n -T _n]	58 + 71.5	3.1 ^b
8. d[(AG) _n -T _n -A _n]·d[(CT) _n -T _n -A _n]	60.5 + 71.0	5.4 ^b
9. d[(AC) _n -A _n ·d(GT)-A _n]·d[(AC) _n -T _n ·d(GT)-T _n]	60 + 84.5	3.6
10. d[(AG) _n -A _n ·d(CT)-A _n]·d[(AG) _n -T _n ·d(CT)-T _n]	58.5 + 74.0	3.2

^a Each reaction contained 200 mM potassium cacodylate (pH 7.2), 1 mM 2-mercaptoethanol, 1 mM polydeoxynucleotide (as monomer units), 0.5 mM ¹⁴C-dATP or ³H-dTTP, 4 mM MgCl₂ for the addition of deoxyadenosine triphosphate and 1 mM CoCl₂ for the addition of deoxythymidine triphosphate, and 500 units of terminal transferase. All incubations were at 37°C for 3 h.

^b % of 3'-terminal nucleotide resistant to exonuclease III: d(T_n) in Nos. 5 and 7; d(A_n) in Nos. 6 and 8; d(A_n) label with ¹⁴C; d(T_n) label with ³H.

TABLE II
CIRCULAR POLYDEOXYNUCLEOTIDES^a

Polymer	T _m (°C) (0.05 M Na ⁺)	% Resistant to Exonuclease III	Methyl Methane- sulfonate Treated % Resistant to Exonuclease III
d[(AC) _n -A _n -T _n -d(GT) _n -A _n -T _n -]	58 + 78	62	6.7
d[(AC) _n -T _n -A _n -d(GT) _n -T _n -A _n -]	58 + 78	85	11
d[(AG) _n -A _n -T _n -d(CT) _n -A _n -T _n -]	59.5 + 74.0	73	28
d[(AG) _n -T _n -A _n -d(CT) _n -T _n -A _n -]	60 + 75	60	27
d[(AC) _n -A _n -(GT) _n -T _n -]·d[(AC) _n -T _n -(GT) _n -A _n -]	61 + 80	25	4.3
d[(AG) _n -A _n -(CT) _n -T _n -]·d[(AG) _n -T _n -(CT) _n -A _n -]	59.5 + 75.5	34	5.7

^aThe reaction mixture for formation of closed circular DNA molecules contained 1 mM of each polydeoxynucleotide, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 6 mM MgCl₂, 50 µg/ml bovine serum albumin, 50 mM NaCl, dATP, dCTP, dGTP, and dTTP (0.1 mM of each), *Micrococcus luteus* DNA polymerase (14 units), and *Escherichia coli* ligase (4 units). The final volume of each reaction was 10 ml. Incubation was at 20°C for 20 h. The polymers were deproteinized and dialyzed to remove triphosphates. For alkylation of each polymer, 0.1 mM of each polymer was incubated with 0.5 mM methyl methanesulfonate at 37°C for 2 h, then dialyzed against 0.2 M KCl and 0.05 M Na⁺.

pairs. Unpaired bases are completed with DNA polymerase and the four deoxyribonucleoside triphosphates. Closure of the dog-bone circular structure is completed with DNA ligase. The second procedure has an advantage over the first in that multiple linear or branch molecules cannot be formed; therefore, the dog-bone circular structure is almost 100% sealed by ligase.

The circular synthetic polydeoxynucleotides are valuable tools for the assay of enzymes involved in repair of damaged DNA. Results using exonuclease III (Tables I and II), which is specific for double-stranded DNAs, have shown that both circular forms, as well as polymers with dA or dT tails, are resistant to exonuclease III. However, prior treatment of the different circular polydeoxynucleotides with the alkylating agent methyl methanesulfonate increases the hydrolysis of the circular polymers by exonuclease III.

Validation of the LASL Cultured Cell System as a Predictive Guide to Human Cellular Kinetic Response following Exposure to DNA-Interactive Compounds
[R. A. Tobey, H. A. Crissman, L. L. Deaven, and R. J. Kissane]

The advent of new energy technologies raises the likelihood of exposure of workers to a multitude

of poorly characterized but potentially hazardous substances, including DNA-interactive agents such as mutagens, carcinogens, and teratogens. Significant attention accordingly must be directed toward identifying and characterizing such compounds at the earliest possible stage of development. In particular, what is needed is a low-cost model screening system which simply and rapidly identifies hazardous agents and allows their classification into categories based on biochemical mode of action. Above all, the model system must be capable of yielding results which mimic responses observed in man.

We have developed a cultured cell system at LASL which permits the analysis of effects of DNA-interactive compounds on kinetic rearrangement of cells about the cell cycle. Specifically, line CHO Chinese hamster cells are exposed to test agents, and post-treatment perturbations in distribution of cells around the cell cycle are assayed by a technique which combines ³H-thymidine autoradiography, cell number determination, and flow cytometric analysis of population DNA contents.¹

We have circumvented the impracticability of calibrating the LASL screening system with compounds of interest to ERDA (i.e., agents associated with fossil fuel processing may not be administered to humans) by utilizing a series of DNA-interactive

compounds for which there are available corresponding whole-animal and human data. Because the agents that we have employed are cancer chemotherapeutic drugs which can act as mutagens, carcinogens, and teratogens under appropriate conditions, their utilization as representative compounds of the general types expected to be encountered in fossil fuel processing is justified.

To obtain an idea of the comparative data obtainable with the LASL cultured cell system and in whole-animal and human studies, data are presented in Table I which illustrate the cell kinetic responses obtained in the various systems following treatment with three major classes of DNA-interactive agents: (1) antimetabolites; (2) alkylating agents; and (3) intercalating agents plus a fourth agent which induces single-strand DNA breaks. It is readily apparent that the results generated in the LASL screening system correspond remarkably well with results obtained in whole animals and humans. The results validate the general usefulness of the LASL cultured cell system as a predictive indicator of kinetic response in man.

Note also that each category of agents produces a distinctive kinetic response, allowing separation of alkylating agents from intercalating agents, etc. Although not shown in Table I, studies with additional agents suggest that a variety of alkylating agents produce the kinetic response pattern shown for BCNU and Melphalan^{1,11} and that intercalating agents respond in the manner shown for adriamycin,¹² etc. If these trends continue as progressively larger numbers of agents are analyzed with the LASL screen, it would appear that kinetic response measurements of substances associated with fossil fuel processing may be utilized to provide an early stage classification of agents. Once an agent has been assigned to a general biochemical category, subsequent detailed biochemical studies could concentrate on a few representative agents from each category rather than the far more costly and time-consuming analyses on a compound-by-compound basis. It must be emphasized that we are advocating that cultured cell system measurements be employed as an adjunct to, rather than a substitute for, animal studies. As a long-range goal, we would like to

TABLE I
COMPARISON OF KINETIC RESPONSES INDUCED BY VARIOUS DNA-INTERACTIVE AGENTS

Type of Agent	LASL Cultured Cell Response	Whole-Animal Response	Human Response
Antimetabolite (Cytosine arabinoside)	Accumulation of cells at the G ₁ /S boundary with slippage of cells into early S phase ²	Accumulation of cells on the early S side of the G ₁ /S boundary in mice bearing B16 melanoma or Ehrlich ascites tumors ³	Accumulation of cells on the early S side of the G ₁ /S boundary in patients with acute lymphocytic leukemia ⁴
Alkylating agent (Melphalan, BCNU)	Transitory accumulation of cells in S resulting from prolongation of the DNA synthetic phase ¹	Transitory accumulation of cells in S resulting from prolongation of the DNA synthetic phase in mice bearing L1210 leukemia ⁵	Transitory accumulation of cells in S resulting from prolongation of the DNA synthetic phase in patients with multiple myeloma ⁴
Intercalating agent (Adriamycin)	Transitory enrichment of the G ₂ population, primarily at the expense of the G ₁ subpopulation ⁶	-----	Transitory enrichment of the G ₂ population, primarily at the expense of the S subpopulation of patients with neuroblastoma or acute myelocytic leukemia ⁴
Miscellaneous agent (Bleomycin)	Accumulation of cells in G ₂ at the expense of both G ₁ and S subpopulations ⁷	Accumulation of cells in G ₂ at the expense of both G ₁ and S subpopulations in mice bearing Ehrlich ascites tumors ⁸ or in rabbits with VX-2 carcinoma ⁹	Accumulation of cells in G ₂ at the expense of both G ₁ and S subpopulations in patients with malignant melanoma ¹⁰

examine the feasibility of utilizing in vivo cell kinetic rearrangements (and induction of associated chromosomal lesions) to assess sublethal damage accruing in workers exposed to technology-related agents.

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Histone Phosphorylation and Chromatin Structure in Cell Proliferation

[L. R. Gurley, J. A. D'Anna, L. L. Deaven, S. S. Barham, R. A. Walters, R. A. Tobey, and J. G. Valdez]

It is anticipated that increased energy production from fossil fuels within the next few years will result in an increased release of biologically active toxicants into the environment. There is growing concern that these substances will have an impact on cell growth, embryonic development, and carcinogenesis. Fundamental to all three of these biological processes is cell proliferation. Thus, if a rational basis is to be established for evaluating the biological impact of these environmental toxicants, a clear understanding of the mechanisms which control cell proliferation is absolutely necessary. Experiments in this Laboratory have shown that histone phosphorylation is involved in the cell proliferation process. Thus, further investigations are under way to determine if this biochemical process might be a target for environmental toxicants produced by energy-based activities.

Using synchronized cell cultures, we have determined the cell-cycle schedule of histone phosphorylation. A comparison of this schedule with cell-cycle dependent chromatin structural changes revealed that two different correlations existed between histone phosphorylation and chromatin condensation: one associated with the presumptive interphase chromatin condensations of the "chromosome cycle" and one associated with chromosome condensation at mitosis. These correlations formed the basis for a new hypothesis suggesting that histone phosphorylations may promote cell proliferation by assisting in the chromatin condensations necessary for cell division.

The potential importance of this hypothesis to an environmental impact evaluation program provided the impetus to search for other examples of an involvement of histone phosphorylation in chromatin condensation. Such a positive correlation was found when we compared histone phosphorylation in the cell lines of two related species of deer mice (Peromyscus) which differed greatly in their contents of condensed heterochromatin. Flow microfluorometric measurements revealed that P. eremicus cells contained 36% more DNA than did P. crinitus

Fig. 1. Histone phosphorylation in two *Peromyscus* cell lines having different quantities of constitutive heterochromatin: (A) transmission electron microscopy of an interphase *P. crinitus* cell demonstrating its low heterochromatin content; (B) and (C) preparative electrophoresis of histones from *P. crinitus* cells labeled 2.3 generations with ^3H -lysine and then pulse-labeled with ^{32}P for 2 h during exponential growth; (B) $^{32}\text{P}/^3\text{H}$ ratio of H1 was 1.203; (C) $^{32}\text{P}/^3\text{H}$ ratio of H2a was 0.494; (D) transmission electron microscopy of an interphase *P. eremicus* cell demonstrating its excessive heterochromatin content; (E) and (F) preparative electrophoresis of histones from *P. eremicus* cells labeled 2.3 generations with ^3H -lysine and then pulse-labeled with ^{32}P for 2 h during exponential growth; (E) $^{32}\text{P}/^3\text{H}$ ratio of H1 was 1.200; and (F) $^{32}\text{P}/^3\text{H}$ ratio of H2a was 0.779.

cells. Chromosomal C-band analysis demonstrated that this excess of DNA in *P. eremicus* cells existed as constitutive heterochromatin which was not present in *P. crinitus* cells. Furthermore, the excessive constitutive heterochromatin of *P. eremicus* cells remained condensed throughout interphase as revealed by electron microscopy (Figs. 1A and 1D). To determine if histone phosphorylation was associated with the degree of interphase chromatin condensation, the extent of ^{32}P incorporation into histones was measured while growing these cell lines under identical culture conditions. It was observed that the histone H2a phosphorylation rate was 58% greater in *P. eremicus* cells (Fig. 1F) than in *P. crinitus* cells (Fig. 1C), while the histone H1 phosphorylation rate was identical in both cell lines (Figs. 1B and 1E). These experiments suggest that H2a phosphorylation may be involved with the processes which form or maintain condensed heterochromatin aggregates during interphase.

By combining these experiments with our previous experience with synchronized Chinese hamster CHO cultures, we have proposed a unified model for the involvement of growth-related histone phosphorylations in the condensation of chromatin structures. In this model, interphase histone H1 phosphorylation would be involved with a presumptive interphase chromatin condensation cycle detected at the submicroscopic level by the heparin method of probing chromatin structure. At the microscopic level of chromatin structural organization, histone H2 would be involved with the condensation of chromatin into heterochromatin. At mitosis, the phosphorylation of histone H3 and the superphosphorylation of histone H1 would be involved with the final condensation of chromatin into the chromosome structures necessary for cell division.

It has been proposed that these chromatin structural changes are involved in the control of cell proliferation, cell differentiation, and thus carcinogenesis. The possible involvement of histone phosphorylation in these processes makes histone phosphorylation a good target for the deleterious effects of environmental toxicants. Our experimental results strongly justify further examination of histone phosphorylation-related chromatin structural changes for a possible role in programs

designed to evaluate the impact of energy-based environmental emissions.

Photoaddition Products Induced in Chromatin by Ultraviolet Light

[G. F. Strniste and S. C. Rall]

Various chemical compounds (monofunctional and polyfunctional alkylating agents) and physical agents (uv light, x rays) are known to mediate addition reactions between protein and nucleic acids.¹ The genetic material (DNA of eukaryotic cells is complexed with a variety of basic (histone) and acidic (nonhistone) proteins whose functional roles, although not well understood, are at least involved in the maintenance of DNA conformation in chromatin and in the regulation of information stored in the DNA. Addition products induced in chromatin (i.e., protein-DNA adducts, protein-protein adducts) could result in subtle alterations in the structure of chromatin. These local distortions could initiate the expression and/or repression of genetic information, triggering biochemical events in the cell which could result ultimately in cellular changes including mutagenesis, carcinogenesis, and teratogenesis. The capacity of cells to repair addition products between protein and DNA is not known.

The brief summary of work presented herein consists of recent results we have obtained from a study initiated last year concerning the photoaddition of protein to DNA in chromatin by light of primarily 254-nm wavelength.^{2,3} In addition to using chromatin isolated from hypotonically shocked nuclei and solubilized by shearing (to be referred to as sheared, soluble chromatin), we have also studied photoaddition reactions in purified chromatin subunits (nucleosomes) isolated from micrococcal DNase-treated nuclei. Chromatin prepared by nuclease digestion probably reflects more accurately the *in vivo* substructure of chromatin compared to chromatin samples isolated by other, more rigorous procedures. Nucleosomes are readily obtainable in useful quantities and thus provide a unique model system for studying protein-DNA interactions as they exist in chromatin *in vivo*.⁴

Chromatin proteins are crosslinked to DNA by uv light most likely via covalent linkage.^{2,3} We have previously developed a rapid assay for the detection of protein-DNA adducts induced by uv light

in sheared, soluble chromatin.² The assay utilizes molecular sieve chromatographic techniques in which protein linked to DNA is excluded with the DNA, while the bulk nonlinked protein is partially retained in the column bed. Similar chromatographic techniques can be employed in examining addition of protein to DNA in uv-irradiated nucleosomes. For either chromatin preparation used, linkage of protein to DNA is linear with light fluence to about 12 000 Jm⁻², although the quantity of protein linked in sheared, soluble chromatin exceeds the protein linkage to DNA in nucleosomes by about 60%. This latter result is most likely a consequence of the involvement of nonhistone proteins which contribute to about 50% of the protein mass in sheared, soluble chromatin but only 5 to 15% of the protein mass in nucleosomes. This latter finding has been substantiated by an independent technique.³

Fig. 1. Sodium dodecylsulfate-polyacrylamide (15%) slab gel electrophoretic patterns of protein from control and uv-irradiated nucleosomes: (slots a-d) control trimer, 60-min uv-irradiated trimer, control tetramer, and 60-min uv-irradiated tetramer, respectively. After staining and photography, the gel was sectioned at the points indicated by the arrows (left side) and monitored for radioactivity as previously described.³

Another method we have employed in determining the role of the various chromatin proteins in photo-addition reactions is polyacrylamide gel electrophoresis (PAGE). Chromatin samples, control or uv-irradiated, were dissociated in detergent containing buffer and subjected to electrophoresis on polyacrylamide slab gels. Figure 1 shows the result of PAGE analysis of control and 60-min uv-irradiated nucleosomes (trimers and tetramers). The individual protein bands are revealed after staining with Coomassie brilliant blue stain, as shown in Fig. 1. After photography, the slab was sliced according to the numbered arrows, and each slice was monitored for radioactive counts per minute (the proteins were labeled in culture with ^{14}C -lysine). The "core" histones (H2A, H2B, H3, and H4) are depleted from their normal banding positions in the irradiated samples (note the reduction in stain intensities in slots b and d). From radioactive measurements of individual histone bands, we were able to quantify

Fig. 2. Sodium dodecylsulfate-polyacrylamide (12.5%) slab gel electrophoretic patterns of protein from control and uv-irradiated, sheared, soluble chromatin: (a) control, nonirradiated sample; (b and c) 15-min uv-irradiated; (d and e) 45-min uv-irradiated; and (f) 60-min uv-irradiated. Slices 1-19 were analyzed for radioactivity as described in Fig. 1.

the loss of histones from their normal banding positions as a function of uv light fluence. The loss proceeds in the order of $\text{H2A} + \text{H2B} > \text{H3} > \text{H4}$. It is also apparent from analysis of Fig. 1 that histone H1 is substantially reduced (at its normal banding position) in the irradiated samples compared to the controls. In addition to mediating protein-DNA crosslinks, uv light can induce formation of protein-protein adducts. Formation of a protein band migrating slower than histone H1 appears in the irradiated samples. This phenomenon has been observed recently by others and was subsequently characterized as a H2A-H2B crosslink (histone-histone dimer).⁵ Stained material is also seen to accumulate in the stacking gel and gel interface. This material is primarily radiation-induced protein-DNA adducts and protein aggregates which, because of their size, cannot migrate into the gel.³

Results of PAGE analysis of uv-irradiated, sheared, soluble chromatin are shown in Fig. 2. The presence of a variety of discrete nonhistone bands is evident in the control sample (slices 2-10, slot a). Depletion of many of these stained bands in irradiated samples suggests extensive involvement of nonhistone proteins in photoproducts generated in uv-irradiated, sheared, soluble chromatin. Although it is not directly evident from examining the photographs, radioactive measurements of the "core" histones in control and uv-irradiated samples show the loss proceeding in the exact manner as in uv-irradiated nucleosomes (i.e., $\text{H2A} + \text{H2B} > \text{H3} > \text{H4}$). Radiation-induced H2A-H2B dimer formation is seen to increase with uv light fluence (see slice 11).

The various data from the column chromatographic assays and the PAGE depletion assay for both nucleosomes and sheared, soluble chromatin are summarized in Fig. 3. In nucleosomes, protein-DNA adducts account for about 70% of the protein photoproducts generated. The remaining 30% of the photoproducts induced are protein-protein adducts and aggregates, of which the H2A-H2B dimer is a major radiation-induced product. In fact, for nucleosomes, uv light-induced protein-DNA adducts and the H2A-H2B dimer can account for almost the entire loss of the "core" histones as determined by the gel depletion assay. For sheared, soluble chromatin, the analysis is complicated by the presence of nonhistone proteins. However, for irradiation times of up to about

Fig. 3. Fate of proteins in uv-irradiated chromatin: (—○—) loss of "core" histones (H2A + H2B + H3 + H4) determined by polyacrylamide gel electrophoresis (PAGE); (---○---) formation of protein-DNA adducts determined by column chromatographic procedures; (—△—) formation of H2A-H2B dimer determined by PAGE analysis; and (---□---) loss of nonhistone proteins determined by PAGE analysis. (Left panel) nucleosome data; and (right panel) sheared, soluble chromatin data.

30 min, losses of histone and nonhistone proteins (determined by gel depletion analysis) exceed the amount of protein involved in radiation-induced protein-DNA adducts by about 2-fold. That is, only 50% of the protein photoproducts generated in sheared, soluble chromatin by 254-nm light are protein-DNA adducts, the remaining 50% of involved protein existing as protein-protein adducts, including the H2A-H2B dimer.

Since addition reactions between protein and nucleic acids are known to result in cells exposed to a variety of chemical and physical agents,¹ an immediate question arises concerning the significance of such a class of lesions induced in the DNA of cells. More specifically (at the molecular level), how do protein-DNA adducts influence the precise biochemical mechanisms involved in replication and transcription of the genetic material? Furthermore, does the cell have the capacity to recognize and repair this "class" of lesion with an "excision-type" repair system, or are these lesions repaired post-replicatively via an "error-prone" repair mechanism? Current research at this Laboratory is being directed toward attempts to answer these questions.

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Pollutants and Genetic Information Transfer

[D. A. Smith, A. M. Martinez, and R. L. Ratliff]

The defined model DNAs available in this Laboratory provide a unique opportunity for study in cell-free systems of the effects of energy-related pollutants on the maintenance and expression of genetic information. Such model systems have provided much of our present understanding of the mechanisms of genetic information transfer and should be equally effective in studies on the molecular mechanisms of deleterious agents which react with components of the genetic apparatus. Simple model DNAs with repeating nucleotide sequences are proving very useful in several types of investigation on the effects of energy-related pollutants. To name a few applications, such studies include the effects of different metal ions on the fidelity of nucleic acid polymerases in copying DNA, studies on the nature of the adducts formed when mutagenic chemicals react with DNA and the effects of such adducts on DNA properties, and studies on the specificity of DNA repair enzymes.

Our work this year has concentrated on the fidelity of RNA polymerase and how this is affected by different metal ions. We have investigated the error frequency of Escherichia coli RNA polymerase in vitro using synthetic polydeoxyribonucleotides as templates. Error frequency was measured by (1) incorporation of complementary and noncomplementary ribonucleotides into acid-insoluble material; (2) by gel exclusion chromatography on the product; and (3) nearest-neighbor studies on the RNA product. Although all three methods of analysis are satisfactory under normal reaction conditions, when the effect of different metal ions on the fidelity of the reaction is measured, only nearest-neighbor analysis is always

TABLE I
EFFECT OF La^{3+} ON FIDELITY

LaCl_3 (nmols)	Complementary Nucleotide Incorporation [pmols r(A·U)]	Noncomplementary Nucleotide Incorporation (pmols CMP)	CMP (100 000 AMP + UMP)
0	4296	3.72	87
2	4224	9.43	223
5	2364	12.10	512
10	1464	10.61	725
30	566	10.47	1850

a satisfactory procedure. In the presence of some metal ions, precipitates or complexes are formed which give false-positive results with the acid-insoluble and gel exclusion chromatography assays. We found that the background error frequency of RNA polymerase using $\text{d(A-T)}_n \cdot \text{d(A-T)}_n$ as template was much lower than previous reports in the literature indicated it to be. Also in contrast to reports in the literature, we found that the error frequency does vary with the template used, being much higher with $\text{dA} \cdot \text{dT}$. These experiments were done with labeled CTP as the noncomplementary nucleotide. Considerable effort was expended this year improving the analytical procedures and investigating the criteria of template and substrate purity necessary to measure the incorporation of very low levels of misreading (on the order of 1 wrong base per 100 000 correct bases).

The effect of several different metal ions on the error frequency of RNA polymerase has been investigated. Although Be^{2+} increases the error frequency of DNA polymerase and although it gives positive results with the acid-insoluble and gel exclusion chromatography assays, when nearest-neighbor studies were done, it was concluded that BeCl_2 did not decrease RNA polymerase fidelity. Other metals which gave negative results included CoCl_2 , ZnCl_2 , CaCl_2 , NiSO_4 , CuCl_2 , FeCl_2 , and SrCl_2 ; however, some of these strongly inhibit the reaction. A few other metals have given positive results with the acid-insoluble assay and the gel exclusion chromatography assay and must be checked by nearest-neighbor analysis. The only metal ion which has definitely been shown to increase the error frequency of RNA polymerase is lanthanum. Table I shows that 30 nmols of lanthanum chloride increase the error frequency by about 20-fold over background and that

as little as 2 nmols increase misreading significantly.

Altered RNA Metabolism as a Function of Cadmium Accumulation and Intracellular Distribution in Cultured Chinese Hamster Cells

[M. D. Enger, C. E. Hildebrand, H. L. Barrington, M. Jones, E. W. Campbell, and J. L. Hanners]

Cadmium is a toxic metal that accumulates in the environment and in man. The level of accumulated Cd^{++} which produces overt toxic effects in man is well documented. When the level of Cd^{++} in the kidney cortex approaches 200 ppm, proteinuria and kidney dysfunction occur. This level is less than an order of magnitude greater than that found in most adult populations of the world. The effects of this subacute burden are not known. Of special concern is the possibility that the present levels of exposure and accumulated body burdens may cause mutations, cancer, or terata. A greater knowledge of the effect of cadmium on cellular metabolism and, in particular, on the expression of genetic information in cells exposed to low levels of Cd^{++} is needed to delimit this possibility. We are accordingly investigating the effects of cadmium on cell growth, viability, morphology, and DNA, RNA, and protein metabolism in cultured mammalian cells. These effects are correlated with cadmium uptake and distribution of cadmium among various intracellular forms, with special attention given to the synthesis, induction, and cadmium binding of specific, protective proteins such as metallothionein.

Preliminary studies have indicated that growth of cultured Chinese hamster ovary cells (line CHO) is affected by levels of extracellular Cd^{++} as low

as 4×10^{-7} M. At 2×10^{-7} M, no apparent growth effects occur. However, 2 or 3 days exposure at this level results in an increased polysome mass (per cell) of about 25%. This increased net synthesis of stable RNA and protein is accompanied by stimulation of isotopic precursor incorporation into cytoplasmic messenger RNA, with an up to 2-fold increase in incorporation into informosomal (cytoplasmic messenger not bound to polysomes) RNA. We now report on analyses of the time-course of this stimulation of RNA synthesis as a function of exposure time. These data are correlated with Cd^{++} accumulation and partitioning. Stimulation of incorporation into heterogeneous nuclear RNA (hnRNA) is also measured, since this population represents, in part, precursors to cytoplasmic messenger RNAs.

The effects of exposing CHO cells to 2×10^{-7} M Cd^{++} for different amounts of time are summarized in Table I. Data are expressed as precursor incorporation (into cytoplasmic species, informosomal or polysomal, and into hnRNA) or polysome mass in cadmium-treated cells relative to control populations. Data are expressed on a per cell basis but, since growth rates of treated and control populations are usually the same, also represent relative values on a per culture aliquot basis.

Cells exposed to 2×10^{-7} M Cd^{++} increase nucleoside precursor by about 10% when pulsed 8.5 to 10 h after Cd^{++} addition. Polysome mass at 10 h is 111% of control. After 12.5 to 14 h of exposure, both precursor incorporation and polysome mass are significantly increased. Effects on precursor incorporation are maximal after 24 h of exposure, and polysome mass is (relatively) greatest after 46 h of exposure. Although not increased to the same extent relative to control as cytoplasmic messenger at later times, hnRNA incorporation is significantly increased after 12 h or more of exposure.

When these effects are considered together with data for Cd^{++} accumulation and intracellular partitioning, certain correlations appear. The rate of accumulation of Cd^{++} in cells exposed to 2×10^{-7} M Cd^{++} is linear for the first 24 h and by 48 h reaches a plateau level of accumulation at 12 to 18 $\mu\text{g}/\text{Cd}^{++}/10^9$ cells (see Table II). This range represents the variations observed in 5 different experiments. The maximum intracellular level of Cd^{++} in cells grown in 2×10^{-7} M Cd^{++} is 500- to 1000-fold greater than the extracellular concentration. Although the average intracellular Cd^{++} concentration is 0.1 to 0.2 mM, this estimate does not account for intracellular partitioning

TABLE I
STIMULATION OF NUCLEOSIDE PRECURSOR INCORPORATION INTO MESSENGER-RELATED RNAs
AND INCREASED POLYSOME CONTENT IN CHO CELLS AS A FUNCTION OF EXPOSURE TO 2×10^{-7} M Cd^{++}

Exposure Time ^a (h)	Polysomal	Incorporation as % of Control		Nuclear	Polysome Mass as % of Control	
		Informosomal				
4	119	112 Ave.	99	114	106 Ave.	110
4	104		100	97		100
8	104	113 Ave.	108	102	118 Ave.	102
8	112		119	116		126
8	123		95	136		105
12	110		141	119		112
24	173	173 Ave.	188	154	138 Ave.	115
24	177		151	134		118
24	170		165	127		125
46	138		147	134		124
46 ^b	130		140	125		124
72	146		171	-		127

^aPrior to 30-min treatment with 0.05 $\mu\text{g}/\text{ml}$ actinomycin and 90-min exposure to radiolabeled uridine or

^badenosine.

TABLE II

INTRACELLULAR CADMIUM CONCENTRATIONS AS A FUNCTION OF EXPOSURE TO 2×10^{-7} M Cd^{++109} .

Exposure Time (h)	Total Content ^a	Cytoplasmic		Nuclear (%)	Nuclear (content)
		Thionein-Bound (%)	Thionein-Bound (content)		
4	3.1	7	0.20	23	0.7
8	5.0	9	0.45	22	1.1
12	6.7	13	0.90	18	1.2
24	13.2	21	2.80	16	2.1
48 ^b	15.0	31	4.70	9	1.4

^a $\mu\text{g Cd}^{++}/10^9$ cells; this is equivalent to $\mu\text{g Cd}^{++}/\text{g cells}$ or ppm, determined using Cd^{++109} of known specific activity.

^b With dilution at 24 h with equal volume of medium containing 2×10^{-7} M Cd^{++} .

of incorporated Cd^{++} so that local subcellular concentrations could be higher or lower. Table II also gives the intracellular distribution of Cd^{++} at different times during exposure. These data show significant changes in the intracellular location of Cd^{++} as the total cellular content increases. It should be mentioned that all intracellular Cd^{++} is bound to a cellular component (i.e., no free Cd^{++} is detected by molecular sieve chromatography).

Up to 8 h after beginning the exposure, very little change is apparent in the intracellular distribution of Cd^{++} . At 12 h, a significant decrease in the nuclear Cd^{++} and an increase in the cytoplasmic Cd^{++} are observed. Corresponding to the increased percentage of Cd^{++} in cytoplasm, an elevated level of Cd^{++} is found in cytoplasmic metallothionein. At 24 and 36 h, the level of Cd^{++} in metallothionein continues to rise. Other experiments show that the increased level of Cd^{++} in metallothionein is a result of increased synthesis of thionein (the apo-protein). While the level of Cd^{++} in metallothionein is rising, the percent of cellular Cd^{++} in the nucleus is decreasing. Since the intracellular level of Cd^{++} continues to increase during the first 24 to 36 h of exposure, these results may suggest that the elevated thionein synthesis in the cytoplasm decreases the transfer of Cd^{++} through the cytoplasm to the nucleus. Further, at 48 h, the net decrease in Cd^{++} in the nucleus indicates that it was removed, most likely as a result of the increased levels of cytoplasmic

thionein. It is not yet known by what mechanism Cd^{++} stimulates RNA synthesis. From these data, it appears that the effect occurs when the cellular content is about 5 ppm; this is apportioned such that the level of cytoplasmic Cd^{++} not bound to thionein is about 3.5 ppm and the level of nuclear Cd^{++} is about 1 ppm. Whether the stimulation correlates with any of these values is not yet known, but dose-response studies using CHO cells and a resistant variant which apportions intracellular Cd^{++} differently may offer better definition. However, the presence of Cd^{++} in the nucleus, the stimulation of nuclear incorporation, and the fact that the points of maximal RNA stimulation and nuclear Cd^{++} content coincide (24 h) suggest that Cd^{++} may play a direct role in affecting transcription. The fact that nuclear RNA incorporation is increased to a lesser extent than cytoplasmic incorporation may indicate increased nucleocytoplasmic transport.

Polysome and Cd^{++} contents both plateau after about 24 h of exposure. Steady state apparently ensues at this point. The increased polysome mass is less than the increased precursor incorporation at this time. If incorporation reflects synthesis, then there is probably increased messenger turnover or accumulation of unbound messenger, since not all of the increase (~70%) can be accommodated by association with ribosomes (increased 25%). Kinetic studies are being performed to elucidate this point.

The increased synthesis of thionein has been proposed as a cellular mechanism for detoxification

of heavy metals. In this study, it is evident that increased thionein synthesis occurs in response to exposure of cells to a low level of Cd^{++} . It is difficult to determine at this stage of our studies whether the increased synthesis of thionein is a consequence of induction of gene expression or a result of changes in the level of synthesis and processing of specific RNAs. In this context, it is interesting to note the temporal correlation between the changes in RNA synthesis and processing described above and the increased synthesis of thionein.

Preliminary results for the increased synthesis of metallothionein in response to exposure of cultured cells to Cd^{++} are consistent with observations of the metabolic response of tissues in animals exposed to low levels of Cd^{++} . Thus, it is likely that cultured cell systems will provide a useful model for studying the induction of mechanisms which operate to detoxify heavy metals in humans and other animals. Furthermore, the tissue culture system allows a more detailed examination of the mechanisms underlying changes in gene expression reported here.

Expanded Mechanistic and Mathematical Treatment of Thymidylate Chromophore Destruction by Water Free-Radicals

[F. N. Hayes and D. E. Hoard]

An expanded theoretical treatment of radiation damage to nucleotides in solution has been perfected. The treatment is based on a comprehensive list of various chemical processes taking place during irradiation under a given set of experimental conditions; each such process is represented in the computer program by a differential equation expressing the rate of change in concentration of some component of the solution (reactant, intermediate, or final product) as irradiation proceeds. At any selected point (dose), the instantaneous concentration of each component is calculated by integrating the appropriate differential equations, making use of LASL computer subroutines.¹ It is no longer necessary with the new treatment to make a number of simplifying assumptions required by the methods we used earlier,^{2,3} including the steady-state approximation. In addition, the new method is very versatile; the concentration of any selected

chemical species at any desired point in an experiment can be determined at will.

Results of experiments performed earlier in which thymidine 5'-monophosphate (dTMP) solutions containing specific radical-scavenging solutes were x-irradiated have been reanalyzed by the new treatment. The specific experimental conditions were (1) M aqueous tert-butyl alcohol, neutral pH, nitrogen atmosphere; (2) 0.1 M aqueous tert-butyl alcohol adjusted to pH 3.0 with perchloric acid, argon atmosphere; and (3) neutral aqueous solution, oxygen atmosphere. Under conditions (1), (2), or (3), respectively, the principal species of water free-radical attacking dTMP is the hydrated electron, the hydrogen atom, or the hydroxyl radical. Under condition (1), some 76% of the attacks by hydrated electrons were found to lead to dTMP chromophore destruction. Under condition (2), about 56% of the hydrogen atom attacks gave destruction of the chromophores; under condition (3), about 76% of the hydroxyl radical attacks destroyed the chromophores. The model incorporates not only the initial attack by radicals of the target (the 5,6-double bond of the base moiety) but also processes leading to the final saturation of this double bond. When no oxygen is present, these processes are thought to include (1) attack by another radical and (2) electron exchange taking place between the dTMP adduct radicals after their initial formation. Process (2) is far more significant quantitatively. Depending on their identity, the dTMP adduct radicals may be reoxidized to give the original chromophore or may, through oxidation or reduction, be converted to carbonium ion or carbanion transients. Such transients then almost universally react with water to give chromophore destruction. [The one exception appears to be a carbonium ion intermediate formed by oxidation of the dTMP-hydrogen atom adduct where addition of the hydrogen atom has occurred at carbon atom 6. In this case, proton elimination takes place, giving chromophore restitution. It was estimated from the results obtained under condition (2) that some 78% of the initial attacks of the 5,6-double bond of dTMP by hydrogen atoms occur at this position.]

An example of results obtained at three different initial dTMP concentrations is summarized in Table I. For each species of radical are listed in

TABLE I

COMPUTED PATHWAYS OF dTMP CHROMOPHORE DESTRUCTION BY 25 000 RAD OF X IRRADIATION

Experiment	Free-Radical Species	Radicals Produced (mol dm ⁻³) x 10 ⁵	Radical Production Destroying Chromophores	Adducts Fixed by Radical-Radical Coupling	Percent of Adducts		Adducts Fixed by Reacting with Other Solutes	
					Adducts Fixed by Adduct-Adduct Electron Transfer	Adducts Fixed by		
1	Initial dTMP Concentration 1.0 x 10 ⁻⁴ mol dm ⁻³							
	OH	9.44	0.072	0.001	0.071		0	
	H	1.73	6.99	0.174	6.80		0	
	E	7.50	32.8	0.339	32.4		0	
	2-HMMP ^a	10.7	0.116	0.002	0.114		0	
	Initial dTMP Concentration 2.5 x 10 ⁻⁴ mol dm ⁻³							
	OH	9.45	0.189	0.002	0.187		0	
	H	1.69	15.0	0.278	14.7		0	
	E	7.50	50.7	0.399	50.3		0	
	2-HMMP ^a	10.4	0.308	0.004	0.304		0	
	Initial dTMP Concentration 1.0 x 10 ⁻³ mol dm ⁻³							
	OH	9.45	0.794	0.007	0.787		0	
H	1.66	32.0	0.428	31.6		0		
E	7.50	67.1	0.401	66.7		0		
2-HMMP ^a	9.84	1.33	0.012	1.32		0		
2	Initial dTMP Concentration 1.0 x 10 ⁻⁴ mol dm ⁻³							
	OH	7.90	0.748	0.007	0.741		0	
	H	8.58	19.0	0.155	18.8		0	
	E	7.18	0.813	0.007	0.805		0	
	2-HMMP ^a	9.43	0.129	0.001	0.128		0	
	Initial dTMP Concentration 2.5 x 10 ⁻⁴ mol dm ⁻³							
	OH	7.91	1.90	0.011	1.89		0	
	H	8.48	30.4	0.163	30.3		0	
	E	7.20	2.07	0.012	2.06		0	
	2-HMMP ^a	8.70	0.347	0.002	0.345		0	
	Initial dTMP Concentration 1.0 x 10 ⁻³ mol dm ⁻³							
	OH	7.95	7.26	0.022	7.24		0	
	H	7.96	41.4	0.140	41.2		0	
	E	7.26	8.03	0.024	8.00		0	
	2-HMMP ^a	7.47	1.54	0.005	1.54		0	
	3	Initial dTMP Concentration 1.0 x 10 ⁻⁴ mol dm ⁻³						
		OH	7.00	48.9	0.000	0.000		48.9
		H	1.50	0.119	0.000	0.000		0.119
E		6.90	0.005	0.000	0.000		0.005	
Initial dTMP Concentration 2.5 x 10 ⁻⁴ mol dm ⁻³								
OH		7.00	59.4	0.000	0.000		59.4	
H		1.51	0.334	0.000	0.000		0.334	
E		6.90	0.013	0.000	0.000		0.013	
Initial dTMP Concentration 1.0 x 10 ⁻³ mol dm ⁻³								
OH		7.06	67.3	0.000	0.000		67.3	
H		1.51	1.41	0.000	0.000		1.41	
E		6.94	0.054	0.000	0.000		0.054	

^aSignifies the 2-hydroxymethyl-2-methylpropyl radical.

columns 2 through 5 (1) the total yield of radicals of that species produced by a dose of 25 000 rad; (2) the percent of those radicals participating in chromophore destruction; (3) the percent of

dTMP-radical adducts fixed by radical-radical coupling; (4) the percent of the adducts fixed by adduct-adduct electron transfer; and (5) the percent of the adduct radicals fixed by reaction with

nonnucleotide solutes. [Products containing the 2-hydroxy-2-methylpropyl radical will be formed under conditions (1) and (2) because 2-hydroxy-methyl-2-methylpropyl free-radicals are generated upon the reaction of hydroxyl radicals with *tert*-butyl alcohol. It is assumed that these attack dTMP and combine with dTMP adduct radicals at specific rates of 10^2 to $10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, respectively.]

It is evident that, under conditions (2) and (3), where respectively hydronium ions and molecular oxygen scavenge hydrated electrons, contribution of electron attack to the total chromophore destruction is significantly reduced. Though it may be that, in all irradiated biological systems, enough electron scavengers are present to reduce that amount of electron damage to DNA to insignificant levels, the foregoing results suggest that, under some conditions, electron damage may indeed be extensive. A complete description of the foregoing work is being prepared for publication.

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THE CELL SURFACE

Properties of the Cell Surface

[P. M. Kraemer and B. J. Barnhart]

The advent of widespread use of new alternate energy technologies means that man and the surrounding ecosystem will be exposed to additional chemical and biological agents which may be mutagenic, carcinogenic, or teratogenic. Assessment of many of the biological effects of these agents will be performed at the cellular level utilizing tissue culture techniques. Any interaction between living cells and extracellular agents must occur via the cell surface. Thus, quantitative and qualitative alterations in the biochemistry of the cell membrane might be expected to alter the biological effectiveness of suspected mutagens and/or carcinogens. It is not unlikely that these agents could themselves act as

modifiers of the cellular matrix leading to phenotypic variations in cell morphology and cell-to-cell and cell-to-substratum interactions which are generally accepted parameters of importance in cyto-differentiation, morphogenesis, and malignant transformation.

We have shown using detachment-variant sublines of Chinese hamster cells (line CHO) a direct relationship between the rate of cell detachment from the substratum and the hyaluronic acid (HA) content of the trypsin-removable cell surface material. However, when transferred from suspension to monolayer culture, the same variant sublines showed no difference in attachment kinetics. These results suggest that either altered levels of HA do not influence attachment as they do detachment with trypsin or EGTA or that the variant sublines do not have altered HA when grown in suspension culture. To answer this question, we examined the cell surface glycosaminoglycans of cells growing attached or unattached to substrate.

One set of experiments compared cells growing as subconfluent monolayers in tissue culture (T) plates with cells growing unattached in bacteriological (B) plates. After 18 h of incubation in medium containing ^3H -glucosamine and $\text{Na}_2^{35}\text{SO}_4$, the cultures were harvested and processed into fractions representing trypsin-removable material (TRM), residual cell fractions following trypsin treatment, and medium fractions. Figure 1 illustrates the radioactivity elution profiles for the TRM of parental CHO cells. It is apparent that the attached cells from T-dishes have a greatly elevated level of radioactivity ascribable to HA. This 13-fold difference in HA content was much less evident in the residual cell fractions and did not occur in the medium fractions.

We also compared monolayer and suspension cultures of a detachment variant which we have shown has reduced HA relative to parental CHO cells when grown in monolayer. Our data showed that the slowly detaching variant, DV2, with less than the wild-type level of HA in monolayer exhibits a further reduction in HA when grown in suspension. Thus, the enhanced level of this complex carbohydrate seen in attached parental CHO cells is also evident for a variant which may have altered regulatory capabilities with respect to HA synthesis. We conclude

Fig. 1. DEAE-Cellulose separation of trypsin-removable material from attached and unattached CHO cells. Attached cells were grown on tissue culture plates (T), and unattached cells were grown on bacteriological plates (B). Each profile represents 10^7 cells that had doubled in cell number during incubation in labeling medium containing ^3H -glucosamine and $\text{Na}_2^{35}\text{SO}_4$. GP = glycopeptides, HA = hyaluronic acid, HS = heparin sulfate, and CS = chondroitin sulfate. Open circles represent counts per minute (cpm) of ^3H label and open triangles cpm of ^{35}S label.

that cell spreading and/or anchorage to substratum induces increased hyaluronic acid synthesis.

GENETIC STUDIES

Somatic Cell Genetics

[B. J. Barnhart, S. H. Cox, P. M. Kraemer, and R. T. Okinaka]

It is generally recognized that greater biological risks are and will continue to result from the environmental impact of new energy technologies. One area of primary concern is the genetic danger from use of fossil fuels and uranium- and plutonium-fueled nuclear reactors. Genetic alterations are best determined by measuring the increase in frequency of

gene mutations. It is most advantageous to assess mutagenicity in model *in vitro* systems as opposed to time-consuming and expensive whole-animal studies. It is recognized that assessment of genetic toxicology must be performed in the latter systems but that preliminary assessment can and should be obtained using mammalian cell tissue-culture methods.

We have developed such a tissue-culture model system for quantitating gene mutations. Using the Chinese hamster cell line CHO, we are measuring forward mutation frequencies at the HGPRTase locus and reversion frequencies for temperature-sensitive and glycine-requiring mutants. These *in vitro* short-term assay systems have been optimized for a known chemical mutagen (e.g., ethyl methanesulfonate) and a known physical mutagen (e.g., near-uv radiation of 265 μm).

High-LET radiation from radionuclides is suspected of being mutagenic, carcinogenic, and teratogenic; however, there is a paucity of data addressing these allegations. Using our model *in vitro* mammalian cell assay systems, we are beginning to quantitate the relative mutagenicity of high-LET alpha radiation from ^{238}Pu and of low-LET 250-kVp x rays.

In addition to the high-LET radiation mutagenesis studies, we have initiated a new project to determine whether environmental pollutants derived from fossil fuel energy production are activated by near-uv radiation (300- to 400- μm) to an active mutagen state. Many of these chemicals are known to be converted from a promutagen to a mutagen by metabolic activation (i.e., via the liver monooxygenase system). It is equally important to know if these promutagens can be activated by the uv radiation from sunlight to produce somatic mutations and thereby cancers on the exposed skin of man and animals. There is some evidence in the literature in support of light activation of the promutagens which otherwise require metabolic activation to react with DNA.

The ongoing project to decipher the biochemical genetics of cellular anchorage to the growth substratum has shown that cell-surface hyaluronic acid modulates the accessibility of cell-to-substratum attachment sites to detachment mediators such as trypsin and EGTA. Our data also indicate that cells in the anchorage state synthesize and deposit more hyaluronic acid into the cell surface matrix than do

Fig. 1. Kinetics of trypsin-mediated detachment from a plastic substratum of 4 detachment variants and the parental CHO cells.

Fig. 2. DEAE-Cellulose separation of trypsin-removable material from detachment variants and the parental CHO cells. Cells were labeled with ^3H -glucosamine and $\text{Na}_2^{35}\text{SO}_4$. GP = glycopeptides; HA = hyaluronic acid; HS = heparan sulfate; and CS = chondroitin sulfate.

cells grown in suspension. Both parental and detachment-variant sublines of CHO grow at the same rate in suspension and monolayer cultures. However, while all sublines had similarly low levels of cell surface hyaluronic acid when grown in suspension culture, cell anchorage resulted in an increased level that was directly proportional to the detachment kinetics of the respective cultures (Figs. 1 and 2). Cell hybridization experiments have shown that the substratum-detachment characteristics of the variant sublines are recessive traits, as are their respective rounded or flattened cell morphologies. The relative capabilities of these hybrids and variant sublines to synthesize and deposit hyaluronic acid on the cell surface remains to be determined.

CYTOGENETICS

Factors Affecting the Induction of Chromosomal Aberrations by Cadmium

[L. L. Deaven, E. W. Campbell, and C. Sanchez]

The development of alternative energy technologies in the United States will be accompanied by a certain amount of occupational risk to individuals working in conversion plants, and the general environment will be exposed to increased levels of toxic by-products. Occupational health hazards include acute toxicity or accumulation of toxic materials as a result of chronic low-level exposures, while environmental contamination usually involves exposure of a variety of organisms to nontoxic levels of potentially mutagenic or carcinogenic compounds. One method which has proven useful in the detection and assessment of either type of exposure is chromosome analysis. We accordingly have been studying the cellular and chromosomal responses to toxic materials associated with proposed alternative energy technologies.

One class of compounds, heavy metals, is found in varying concentrations in nearly all known fossil fuel sources. We selected cadmium as the element for initial studies for a number of reasons: (1) additional quantities of cadmium will be released into the general environment as a result of increased coal utilization and oil shale extraction; (2) cadmium is known to be toxic to man (acute toxicity results from ingestion of 3 g or inhalation of 40 mg, while chronic exposures can result in

pulmonary disorders, renal damage, or osteoporosis; (3) other projects at this Laboratory are concerned with the cellular localization of cadmium and its effects on informational macromolecules; and (4) the existing data on cytogenetic effects of cadmium are equivocal, with some investigators demonstrating chromosome damage while others do not.

Cadmium can be sequestered by a specific protein, metallothionein, and by this mechanism stored in the liver or kidney. Cadmium exposure, then, may include constant or intermittent low levels of the metal in the circulatory system, with higher levels accumulating in the kidney and/or liver.

Experiments were designed to simulate these conditions with cells grown in vitro.

Chinese hamster cells (lines V79, CHO, and 3 lines derived from lung, skin, and kidney tissue) were utilized for these studies. The cells were grown in spinner or monolayer culture in Ham's F-10 medium, supplemented with 15% bovine cadet serum or 20% fetal calf serum. Cadmium chloride stock solutions were prepared in 0.1 M HCl in polyethylene containers and diluted with sterile H₂O for experiments. Cadmium was added to exponentially growing cell cultures at concentrations ranging from 1×10^{-4} M to 1×10^{-7} M. Examinations for cytogenetic

Fig. 1. Chromosomes of Chinese hamster ovary cells after exposure to 1×10^{-6} M Cd⁺⁺ for 24 h (A), for 48 h (B and C), and for 72 h (D).

effects were made at varying intervals, depending on the cadmium concentration and cell line under study.

Chromosome damage varied with serum concentration and cell line, but the results in each case are relative to the results found in CHO cells growing in 15% serum and treated with 1×10^{-6} M CdCl_2 . Under these conditions, CHO cells show no damage up to 24 h after addition of the metal (Fig. 1A). At 48 h after treatment, approximately 40% of the cells have chromatid damage in one or more chromosomes (Fig. 1B); a few of the cells at this time have multiple chromatid aberrations affecting many chromosomes (Fig. 1C). At 72 h, 80% of the cells in metaphase have shattered chromosomes (Fig. 1D). While a picture of increasing effect over time emerges from these studies, it should be pointed out that the effects are not uniformly distributed among all cells (i.e., some normal metaphase cells can be found at each of the collection points illustrated).

When the cadmium concentration is lower than 1×10^{-6} M, cells growing in medium supplemented with

15% serum show no chromosome damage and little or no effect on growth rate even after exposure for 12 wk. When cells growing under these conditions are then challenged with a dose of cadmium (2×10^{-6} M) which stops growth in normal cells (Fig. 2) and induces chromosome aberrations as illustrated in Fig. 1, they are tolerant to the cadmium with respect to growth rate and induced chromosome aberrations (Fig. 3). Other studies by Hildebrand *et al.* reported herein have shown that these tolerant cell populations contain abnormally high levels of metallothionein.

When serum levels were increased from 15 to 20%, CHO cells were also more tolerant to cadmium. In order to induce chromosome aberrations in these cultures, cadmium concentrations had to be doubled (2×10^{-6} M). Chinese hamster cells grown from biopsy specimens of lung, kidney, and skin from the same animal were all more resistant to cadmium than CHO cells. In order to inhibit the growth rate in these cells, the cadmium concentration had to be increased to 2×10^{-5} M. They were also more resistant with respect to chromosome damage. Preliminary

Fig. 2. Effects of 2×10^{-6} M CdCl_2 on Chinese hamster (line CHO) cells; (-●-) control and (-□-) 2×10^{-6} M CdCl_2 .

Fig. 3. Effects of 2×10^{-6} M CdCl_2 on Chinese hamster (line CHO) cells previously grown in the presence of 2×10^{-7} M CdCl_2 ; (-●-) control and (-□-) 2×10^{-6} M CdCl_2 .

results with V79 cells indicate that they react to cadmium like cells derived from the lung, kidney, and skin.

These studies demonstrate that (1) cadmium can induce chromosomal aberrations in mammalian cells in vitro; (2) cells grown in vitro differ considerably in the extent of their resistance to cadmium; (3) serum concentration can alter the resistance to cadmium in a given cell line; (4) cells may become tolerant to cadmium when grown in the presence of nontoxic levels of metal; and (5) cells grown from several different organs of the same animal are equally susceptible to cadmium effects. They also illustrate the need for detailed analysis of the effects of pollutants at the cellular level before meaningful interpretations of in vivo data can be made. For example, cadmium has been implicated as the source of chromosome damage in peripheral lymphocytes of industrial workers with elevated blood levels of lead and cadmium.¹ However, our studies suggest that lymphocytes should be protected from even very high cadmium levels by the serum content of blood. The positive^{2,3} and negative⁴ reports in the literature of cadmium-induced chromosome damage similarly may be the result of culture methodology. Finally, these results

suggest that cells derived from different tissues of the same animal are equally capable of synthesizing metallothionein and, thus, acquiring tolerance to cadmium. The presence of relatively high levels of metallothionein and cadmium in the kidney is probably due to the gradual exposure of kidney cells to cadmium as it is sequestered from the blood rather than an innate difference in cell type, as has been suggested previously.⁵

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INTRODUCTION

Flow-systems technology in the United States was born with the developments at the Los Alamos Scientific Laboratory in the mid to late 1960s, and the Biophysics and Instrumentation Group remains in the forefront of present-day developments. In contrast with other laboratories where one specific biological area has been the motivation for activity, LASL has concentrated on technological developments designed to suit a variety of biological needs. For that reason, the LASL effort is multidisciplinary in approach, responding to the needs of several federal agencies. The goal of the LASL program is to provide to ERDA programs at LASL or elsewhere developments which can be used in screening programs.

There have been several personnel added to the group during the past year. Two postdoctoral appointees have joined the group: Robert A. Hoffman,

Ph.D., is studying the problems associated with making cell impedance measurements in flow systems, and James B. Robertson, Ph.D., is performing radiobiological studies with pions and using flow systems in those studies as appropriate. The federally funded STEP program continues to provide support people. During 1976, Robert A. Cox, B.S., and Susan Favaloro, B.S., joined the group in support of the radiobiological and NCI programs. Two "alumni" of the STEP program (Melvin Oka and Joseph Frank) have returned to graduate school to pursue their studies.

Mark E. Wilder, M.S., a biologist, is applying flow systems to the study of immunological questions related to oil shale technologies. An additional technician, Anita P. Stevenson, also joined the group during 1976.

The activities of the group fall into several categories: (1) development of flow-systems

instrumentation for rapid cell analysis and sorting subject to a specific need; (2) development of cell staining and preparational techniques suitable for use with flow-systems instruments; (3) biological and medical applications of these instruments to ERDA needs; (4) radiobiological studies at the biomedical channel, at LAMPF and other high-LET facilities; and (5) electronic and computer support for many of the activities in the LASL biomedical program. Specific information on two of the areas is given below.

The multiangle light-scatter system development has continued during the past year, with emphasis on validation of the methodology with biological models. The problems investigated include determination of abnormal red blood cell morphology, discrimination of microalgal types and application to water quality measurements, and classification of exfoliated lung cells. A flow cytometer with high light-collection efficiency was used to measure DNA content of several types of sperm, in collaboration with the Lawrence Livermore Laboratory, and to measure the DNA content of chromosome samples with improved resolution.

The program which is examining the effects of physical and chemical agents associated with oil shale technology on the immune system has developed

techniques for discriminating between T and B lymphocytes. Techniques for assessing damage to lung epithelium by toxic agents associated with production of synthetic fuel from oil shale are being investigated with flow systems. The development of cell preparation and staining methods has continued to evolve as new problems are presented.

The flow-system approach is a very powerful tool for use in basic biology, as well as in clinical diagnosis. A book, Flow Cytophotometry and Cell Sorting, by Melamed (Memorial Hospital, New York), Mullaney (LASL), and Mendelsohn (LLL), is in preparation with publication scheduled during 1977.

Our pioneering work in cell analysis continues to draw national and international attention. P. F. Mullaney, J. M. Crowell, H. A. Crissman, G. C. Salzman, and J. A. Steinkamp were invited by the German government to participate in the Workshop on Technologies for Automation of Cervical Cancer Screening. L. S. Cram was on the organizing committee for the Second International Symposium on Rapid Methods and Automation in Microbiology, held in Cambridge, England. Our personnel have also been invited to speak on the state-of-the-art in the flow-systems field at symposia sponsored by the National Cancer Institute and other agencies.

DEVELOPMENT OF FLOW-SYSTEMS INSTRUMENTATION FOR
RAPID CELL ANALYSIS AND SORTING

Flow Analysis and Separation of Respiratory Cells:
Preliminary Characterization Studies in Hamsters

[J. A. Steinkamp, K. M. Hansen, J. S. Wilson, and
G. C. Salzman]

The application of advanced flow analysis methods to measure physical and biochemical properties in respiratory cells provides a new approach for analytically assessing damage to lung epithelium of humans exposed by inhalation to toxic agents associated with production of synthetic fuels from oil shale. This includes the development of automated cytological methods for determining early atypical changes in lung cells from model animal systems, the end objective being examination of sputum samples from exposed humans. Analytical flow methods developed at the LASL are presently being applied to characterize initially exfoliated respiratory cells from the Syrian hamster which was selected as the model experimental animal.

Respiratory cell samples composed of macrophages, leukocytes, ciliated columnar, and epithelial (basal) cells were obtained by lavaging the lungs with normal saline.¹ To demonstrate non-specific esterase activity and subsequent cell viability, unfixed samples were stained with fluorescein diacetate (FDA)² and analyzed for total fluorescence. Unfixed samples were also stained with acridine orange (AO)³ and analyzed for green nuclear and red cytoplasmic fluorescence. Fixed samples were stained for DNA content with mithramycin⁴ and analyzed for DNA and cell size by measuring nuclear fluorescence and small-angle light scatter (whole-cell size), respectively. Two-color fluorescence analysis of DNA content and protein was accomplished by staining fixed samples with propidium iodide (PI) and fluorescein isothiocyanate (FITC),⁴ respectively. Lung cell samples stained in liquid suspension were analyzed for fluorescence and light scatter as they flowed through a chamber intersecting a laser beam.⁵ Optical signals proportional to cellular parameters were measured (cell-by-cell basis) and displayed as frequency distribution histograms using a multichannel pulse-height analyzer. Cells were also separated and identified microscopically.

Typical fluorescence distributions recorded on hamster lung cells after treatment with FDA show two distinct regions made up primarily of macrophages and leukocytes.⁶ A third (smaller) region has not been positively identified. Preliminary results have also shown that lung cells can be characterized using AO,⁶ similar to that previously described for differentiating human leukocytes.³ The green fluorescence distributions are nearly unimodal, illustrating uniform nuclear staining, whereas the red fluorescence distributions (cytoplasmic granulation) show three distinct regions similar to FDA-stained lung cells.

DNA content distributions recorded on respiratory cells using mithramycin show a major unimodal peak representing cells having 2C diploid DNA content and a much smaller peak having 4C DNA content (binucleated and cell doublets).⁶ The cell size distributions (small-angle light scatter) are broad, indicating heterogeneous mixtures composed of small debris, erythrocytes, and nucleated cells. Gated single-parameter methods have been used recently to display size distributions of nucleated and non-nucleated cells.⁶ Two-color fluorescence analysis of DNA content and total protein in respiratory cells stained with PI-FITC yielded similar results to those obtained with mithramycin.

Flow analysis methods can also be used to determine nuclear and cytoplasmic diameter based on fluorescence and light-scatter signal time duration measurements.⁷ For example, diameter measurements recorded on mithramycin-stained lung cells show unimodal nuclear diameter distributions, illustrating similar-sized nuclei where the cytoplasmic diameter distributions are broader to indicate two or three regions. Data recorded from two-color fluorescence signal time duration measurements⁶ on PI-FITC stained lung cells yielded similar results.

Flow multiangle light-scatter techniques⁸ have also been used to characterize initially hamster respiratory cells based on differences in light-scatter patterns. Preliminary experiments indicate that at least three and possibly four cell groups are identifiable.⁶ Future experiments will involve separating cells differing in light-scatter patterns and determining what types correspond to the regions of the cluster diagrams.

These results demonstrate the potential capability of applying multiparameter flow-systems technologies to analyzing and separating exfoliated respiratory cells, the goal being development of cytological indicators for estimating damage to lung epithelium of humans exposed by inhalation of toxic agents associated with production of synthetic fuels from oil shale. As this technology becomes adapted to the analysis of these cell types, measurement of physical and biochemical properties as a function of exposure will be performed.

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Analytical Support for Flow Systems and Other Research Activities

[J. H. Jett, D. E. Jackson, and J. M. Crowell]

Progress in analytical and computing support of

flow-systems technology and other research activities conducted within the Health Research Laboratory which continue at a low level is summarized herein. Emphasis in computer support related to flow systems has been in two major areas. First, the PDP-11 operating system RSX-11M, which is a multi-user system, is being tailored for use in a real-time data acquisition environment for multiple experiments in the flow-systems laboratory. The other area of effort is in conversion of programs developed on the larger computers of the LASL Central Computer Facility (CCF) to operate on the in-house PDP-11 systems. This has been done for the life-cycle analysis code and the histogram comparison code. In addition, programs which synthesize DNA histograms from cells or chromosomes have been written and will be used to develop new analytical techniques. Two techniques for analysis of DNA histograms from synchronized cell populations are being refined. A matrix inversion technique is being formulated, as well as refinements to the technique of fitting the DNA histogram data to the sum of a small number of Gaussian functions.

The MUMPS system operates on our PDP-11/40 computer system in a multi-user time-shared mode. At the present time, there are two primary uses for the MUMPS system. Building a data base of pathology and related information on animals involved in the "hot particle" program is the primary task for which MUMPS is being used. At the present time, there are approximately 2 000 000 characters of information in that data base which covers some 8000 animals. Results from isotope retention studies are the second largest data base in the system. It contains 1 000 000 characters. Programs to manipulate and analyze these data bases have been written and continue to evolve.

Other uses of the MUMPS system involve smaller projects where manipulation of character string information is required. Examples of these applications include keeping a catalog of synthesized stable isotope compounds and processing and storage of DNA molecule length measurements from electron microscope pictures.

Analytical support has been supplied to several programs. Two different techniques (with C. E. Hildebrand of Group H-9) for calculating the

distribution of v-bodies resulting from enzyme digestion of DNA molecules have been developed. Results of these calculations are being compared with experimental results to attempt to understand the kinetics of digestion and later to understand the changes in those kinetics due to modifying agents.

Statistical analyses (with L. S. Gomez of Group H-4) of the response of rats (testes weight loss and spermatogenic stem-cell renewal) to negative pions have been performed. The stability of karyotypes of transformed cells (with J. M. Lehman of the University of Colorado and R. Beckman of Group T-13) is being investigated with statistical methods. The objective of the study is to detect the chromosomal change which is responsible for tumorigenicity, if there is one. This study is in progress. However, it is clear that, in spite of the apparent variability in karyotype for each clone, each clone has distinguishing characteristics which separate it from other clones. The implications of this type of data for flow-systems analysis of chromosomes are being studied.

BIOLOGICAL APPLICATIONS OF THE MULTIANGLE LIGHT-SCATTERING FLOW SYSTEM

Classification of Microalgal Species

[B. J. Price, V. H. Kollman, and G. C. Salzman]

The objective of this study is to compile information concerning the condition of aquatic ecosystems using algae as bio-indicators of freshwater and marine environments. It is thought that light scattering can be a useful tool for discriminating among algal species due to the wide morphological diversity in these organisms. Light-scatter patterns for three species were obtained initially. The samples were taken from asynchronous populations grown on agar slants. The species looked at were *Anacystis nidulans* (ICG No. 625), *Chlamydomonas reinhardtii* (Harvard strain minus mating type), and *Chlorella pyrenoidosa* (strain 71105). Both *Chlorella* and *Chlamydomonas* are implicated in water pollution studies as being present in significant amounts in polluted water. *Anacystis* is a rod-shaped unicellular blue-green alga, 1 to 3 μ m in length. Its cluster patterns are shown in Fig. 1. The abscissa in this and succeeding figures is

Fig. 1. Cluster patterns for *Anacystis nidulans*. The percent of cells in each cluster is indicated.

TABLE I

MEAN SCATTERING ANGLE AT THE CENTER OF EACH RING AND THE TOTAL POLAR ANGLE SUBTENDED BY A CELL AT EACH RING FOR A CELL-TO-DETECTOR DISTANCE OF 30 mm^a

Ring Number	Mean Angle (°)	Total Angle Subtended (°)
1	0.00	0.14
2	0.28	0.11
3	0.43	0.11
4	0.58	0.11
5	0.74	0.12
6	0.89	0.12
7	1.1	0.13
8	1.2	0.14
9	1.4	0.16
10	1.6	0.18
11	1.9	0.20
12	2.1	0.23
13	2.4	0.26
14	2.7	0.30
15	3.1	0.34
16	3.5	0.39
17	4.0	0.44
18	4.5	0.51
19	5.1	0.58
20	5.7	0.66
21	6.5	0.75
22	7.3	0.83
23	8.2	0.93
24	9.2	1.0
25	10.3	1.1
26	11.6	1.2
27	12.9	1.4
28	14.4	1.5
29	15.9	1.6
30	17.6	1.7
31	19.3	1.8
32	21.1	1.8

^a The angles have been corrected for refraction effects of the 3-mm-thick quartz window whose inside face is 7 mm from the cell.

Fig. 2. Cluster pattern for Chlorella pyrenoidosa.

Fig. 3. Cluster patterns for a mixture of Anacystis and Chlorella.

labeled "Parameter" and refers to ring number on the light-scatter detector. Table I relates each detector ring number to a scattering angle. The percent of cells in each cluster is indicated. The presence of the second cluster (7%) at higher scattering intensity is probably due to the fact that a small percent (< 10% as verified by microscopic count) of the asynchronous population is in

Fig. 4. Cluster patterns for Chlamydomonas reinhardtii. The percent of cells in each cluster is indicated.

Fig. 5. Cluster patterns for a mixture of Chlorella and Chlamydomonas.

the process of reproducing by fission in a longitudinal manner. Because of their greater size, these cells will scatter more light, particularly at the lower angles where diffraction effects dominate the scattering.

Figure 2 shows the cluster pattern obtained for Chlorella, a spherically shaped green alga 3 to 5 μm in diameter. The major difference in this cluster pattern compared to Anacystis (Fig. 1) lies in the shapes of the clusters. This becomes apparent in Fig. 3 where a mixture of Anacystis and Chlorella is analyzed. It is evident that, for rings 6 through 19, the scattered light decreases for Anacystis, while an increase is seen for Chlorella. This results in excellent discrimination between the two species for rings 15 through 20. Figure 4 shows the cluster patterns obtained for Chlamydomonas, a slightly aspherical green alga 15 to 20 μm in diameter. There are two populations virtually indistinguishable in size but differing somewhat in light-scattering intensity over rings 15 through 19. Biochemical studies by the Organic and Biochemical Synthesis Group (H-11) indicate that there is a major rate-limiting step in the growth cycle of this organism which effectively divides the population into two subpopulations with a 60:40 ratio. This agrees well with the percent of cells found in each cluster of Fig. 4.

A mixture of Chlorella with Chlamydomonas is shown in Fig. 5. Even though the two species are quite similar in shape, they can be discriminated rather well on the basis of size, as comparison of the two clusters between rings 6 and 11 demonstrates.

We have shown that it is possible to distinguish among certain algal species on the basis of their individual light-scattering properties. The next step is to apply the same techniques to a larger number of species implicated in pollution studies in order to obtain scattering "signatures" for each species. Recognition of individual species in mixtures containing several species should be possible subsequently. It is hoped that this will eventually lead to a rapid and accurate method for water quality evaluation in lakes and streams.

Identification of Human Abnormal Erythrocyte Morphologies

[B. J. Price, M. Ingram, and G. C. Salzman]

We have begun a study of the light-scattering properties of human erythrocytes (red blood cells). There is a wide diversity of erythrocyte shapes and sizes that can be induced either artificially from normal erythrocytes (normocytes) or are the result

of disease such as sickle cell anemia. Although increasing attention has been given to the diagnostic importance of the many types of red blood cell shape changes that occur in various disease states, quantitation of the changes is usually not possible. For the most part, one would like to know the number of cells showing various degrees of a given abnormality.

In our initial study, a series of 11 human normocyte populations from male and female donors were studied to see what variation in light-scattering properties, if any, were detectable. A striking similarity was found among these normal samples. Figure 1 depicts the cluster pattern from a typical normal sample. The abscissa labeled "Parameter" refers to detector ring number. The first 26 rings are in the forward scattering direction, while ring 27 is the backscatter detector which is described elsewhere in this report.

Table I relates detector ring number of scattering angle. These results were then compared with 7 other erythrocyte morphologies associated with (1) macrocytic anemia (reticulocytosis during response to therapy); (2) hemolytic anemia (etiology undetermined); (3) sickle cell anemia; (4) iron deficiency; (5) cells at nearly their critical hemolytic volume; (6) stomatocytosis (damage from aortic valve prosthesis); and (7) echinocytic transformation of normocytes. Of these 7, the sickle cell anemia and echinocytic erythrocytes are the most

Fig. 1. Cluster pattern from typical normal human erythrocyte population.

Fig. 2. Cluster pattern for *Chlorella pyrenoidosa*.

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of disease such as sickle cell anemia. Although increasing attention has been given to the diagnostic importance of the many types of red blood cell shape changes that occur in various disease states, quantitation of the changes is usually not possible. For the most part, one would like to know the number of cells showing various degrees of a given abnormality.

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(PARAMETER) AND THE POLAR HALF-ANGLE SUBTENDED BY A
CELL AT EACH RING FOR A CELL-TO-DETECTOR DISTANCE
OF 30 mm^a

Parameter Number	Mean Angle (°)	Polar Half-Angle Subtended (°)
1	0.00	+ 0.07
2	0.28	+ 0.06
3	0.43	+ 0.06
4	0.58	+ 0.06
5	0.74	+ 0.06
6	0.89	+ 0.06
7	1.10	+ 0.07
8	1.20	+ 0.07
9	1.40	+ 0.08
10	1.60	+ 0.09
11	1.90	+ 0.10
12	2.40	+ 0.13
13	3.10	+ 0.17
14	3.50	+ 0.20
15	4.00	+ 0.22
16	4.50	+ 0.26
17	5.10	+ 0.29
18	5.70	+ 0.33
19	6.50	+ 0.38
20	7.30	+ 0.42
21	8.20	+ 0.47
22	9.20	+ 0.50
23	10.30	+ 0.51
24	11.60	+ 0.51
25	12.90	+ 0.70
26	14.40	+ 0.75
27	176.00	+ 1.00

^aThe angles have been corrected for refraction effects of the 3-mm-thick quartz window having an inside face 7 mm from the cell.

morphologically distinct. Figures 2 and 3, respectively, show the cluster patterns obtained from these 2 samples. In the sickle cell anemia case (Fig. 2), there are 2 clusters. While the bottom cluster (31%) differs significantly from normal cells with respect to both scattered light intensity and shape, the upper cluster (69%) does not appear significantly different in scattered intensity to warrant classification of cells in this cluster as abnormal. The same can be said about the upper (54%) cluster in Fig. 3A, as well as the major clusters for each of the other 5 abnormal morphologies (data not shown). However, there are

Fig. 2. Cluster patterns from sickle cell anemia population. The percent of cells in each cluster is indicated.

Fig. 3. Cluster patterns from echinocytic erythrocyte population. The percent of cells in each cluster is indicated.

subtle differences in the shapes of these clusters, especially beyond ring 11 ($> 1.9^\circ$). Since the normocyte samples are virtually indistinguishable from each other both in scattered intensities and shape, it can be concluded that shape differences in abnormal cell clusters are in some way related to cell morphology. Therefore, it is apparent that cluster analysis alone is unsuitable for unambiguous discrimination among these various erythrocyte morphologies. It is also possible that the light scattered at angles beyond 22° may be more useful for this purpose. Both of these possibilities are being explored at present. Given the inherent structural simplicity of mammalian red cells, as compared to nucleated cells, we anticipate that this study will not only provide a rapid and unambiguous diagnostic for certain erythrocyte disorders but will also yield useful information on how we may effectively interpret light-scattering results from more complicated cellular systems.

A High-Efficiency Flow Cytometer

[M. J. Skogen-Hagenson]

A flow chamber has been developed which collects about 60% of the total fluorescence from each cell for analysis compared to between 2.5 and 30% for conventional flow cytometers. The chamber, an ellipsoid of revolution, has semimajor and semiminor areas of 2.0 and 1.0 cm. The interior is coated with a flash of gold for increased reflectivity. Cells flow through the water-filled chamber (see Fig. 1), intersecting a focused argon-ion laser beam at one focus of the ellipsoid. Fluorescence from nearly 4π steradians is reflected toward the secondary focus for analysis. The large collection angle makes this chamber less sensitive to asymmetric particle orientation in the flow stream, as well as allowing the analysis of weakly fluorescent particles as shown in Fig. 1.

At the secondary focus, fluorescence and light scattered from each particle exit from the chamber through a 3.0-mm aperture. A bioconvex lens then collimates the light so that an interference filter can block the scattered light, allowing only fluorescence to strike the photomultiplier tube. The laser beam, delivering 700 mW (TEM_{00}) at 488 nm, is split by a glass slide so

Fig. 1. Schematic diagram of the high-efficiency flow cytometer.

that the dc power can be sampled at the time of each fluorescence event. The main beam is focused by crossed cylindrical lenses with focal lengths of 30 and 2.1 cm.

Each integrated fluorescence signal and the corresponding amplified dc laser level are stored in computer memory during data acquisition. During data analysis, each fluorescence event is normalized to the average laser power, and fluorescence distributions are generated for display. Results of several applications of the high-efficiency flow cytometer are summarized below.

When operating at low laser power (i.e., 35 mW), as do several commercially made flow cytometers, it is especially important to collect as much of the particle fluorescence as possible due to poor fluorescence yield. With its improved light collection, the high-efficiency flow cytometer has consistently obtained better coefficients of variation than conventional flow cytometers.

The amount of nucleotide material in a bacterium is on the order of 0.001 the amount found in a diploid mammalian cell. The high-efficiency flow cytometer has been used to detect nucleotide fluorescence from *Micrococcus glutamicus* stained with propidium iodide. When analyzed by Sorter II, a flow system with $f/1.6$ collection optics (i.e., fluorescence collection of about 2.5%), the fluorescence signal could not be detected above noise.

Fig. 2. Fluorescence distributions of aneuploid CHO chromosomes: (A) analysis by the high-efficiency flow cytometer; and (B) analysis of the same sample by FMF II.

Preliminary work with chromosome karyotyping has yielded encouraging results. Analyses of the same aneuploid CHO chromosome preparation by FMF II and the high-efficiency cytometer (shown in Fig. 2) demonstrated the improved resolution obtained with the high-efficiency chamber. In addition, the improved sensitivity of this chamber resulted in detection of a ninth chromosome peak, a peak undetected by FMF II.

The shape of fluorescence distributions obtained from stained cells is dependent on the shape and orientation of the cells in the flow stream. Cell asymmetry results in anisotropic fluorescence emission to which small-angle fluorescence collectors are particularly sensitive. The high-efficiency flow cytometer has obtained symmetric fluorescence distributions from bull sperm which are cylindrically asymmetric (see Fig. 3). Analysis of the same

Fig. 3. Fluorescence distributions of sperm analyzed by the high-efficiency flow cytometer: (A) a symmetric distribution produced by bull sperm which are asymmetric; and (B) a symmetric distribution produced by cylindrically symmetric abalone sperm.

samples by a conventional flow microfluorometer produces distributions with right lateral extensions. This implies that the right lateral extension produced by conventional flow microfluorometric analysis is due to directionally dependent collection optics. Cylindrically symmetric abalone sperm result in normal (symmetric) fluorescence distributions (see Fig. 3) when analyzed by each type of flow system, as one would expect.

Evaluation of the Dose-Effect Relationship for the Immune Response to Biologically Active Agents Associated with Oil Shale Technology

[M. E. Wilder and L. S. Cram]

The objective of this project is the application of automated flow-systems technology to the evaluation of damage to the immune system resulting

from exposure to physical and chemical agents associated with the developing oil shale technology. The program utilizes sophisticated multiparameter flow-system cell analysis and cell sorting instrumentation developed at LASL, thus permitting early and accurate assessment of cellular damage to the immune response system. Two projects are described: (1) the extension of nonspecific *in vitro* blastogenesis stimulation techniques to hamster lymphocytes, and (2) attempts to differentiate and isolate T and B lymphocytes in a viable state by exploiting differences in their respective metabolism.

Hamster Lymphocyte Stimulation.--The animal being exposed to physical and chemical agents associated with oil shale is the hamster. It was of primary interest, therefore, to extend our ability to stimulate lymphocyte blastogenesis to this animal. We have confirmed the finding of Ron *et al.*¹ that hamster splenic lymphocytes require approximately 10 times more photohemagglutinin (PHA) for maximal stimulation than most other species. Figure 1 is a superposition of four fluorescence distributions from control and PHA-stimulated lymphocytes normalized with respect to the area of the major peak (approximately channel 60). The distributions were obtained from cultured hamster lymphocytes exposed to from 0 to 120 μ g of PHA/ml and quantitatively stained for DNA by the low salt-propidium iodide

Fig. 1. Response of hamster lymphocytes to PHA: (—) control (no PHA); (---) 10 μ g PHA/ml; (....) 60 μ g PHA/ml; and (-.-.-) 120 μ g PHA/ml.

technique.² Note that the ordinate is a log scale. It can be seen in Fig. 1 that the cultures exposed to PHA have many more cells which were stimulated to enter S and $G_2 + M$ than the culture which was not exposed to PHA. Whereas other rodents, cattle, and man require only 6 μ g of purified PHA for maximal stimulation, hamsters require 60 μ g of the same material. Also found to be of critical importance in the stimulation of hamster lymphocytes but not mentioned by Ron *et al.* is that the serum used to supplement the incubation medium must be heated to 56°C for 30 min to inactivate complement.

Soulillou *et al.*³ have reported the augmentation of blastogenic response in rats with 2-mercaptoethanol (2-ME). We have found that 2-ME at 5×10^{-5} M has the same effect on hamster lymphocytes. Photohemagglutinin (10 μ g) in the presence of 2-ME induced nearly the same amount of blastogenesis as 60 μ g of PHA alone. Besides a 6-fold reduction in amount of PHA needed to stimulate the lymphocytes, a further advantage of the use of 2-ME is the fact that serum is not necessary. Therefore, mitogenic response can be measured in completely chemically defined medium.

T and B Lymphocyte Cell Separation.--Current methods of identifying and separating T and B cells depends on immunologic tagging of surface markers or on differential physiologic or physical parameters. All these methods have technical problems which make them difficult to use. It has been suggested that T and B cells might differ in their esterase enzyme systems. Therefore, we have begun to investigate this aspect of lymphocyte metabolism in the hope of finding a new, less difficult method of separating viable T and B cells.

The nonfluorescent compound fluorescein diacetate (FDA) can pass through the outer membranes of viable cells. When it does, the esterase enzyme system of the cell removes the acetate molecules from the compound, leaving free fluorescein present inside the cell.⁴ Since fluorescein cannot easily pass through cell membranes, viable cells (i.e., those cells with an intact outer membrane) concentrate fluorescein in amounts which can be detected easily by the FMF instrumentation available at LASL.

We have analyzed pure populations of human lymphocytes incubated with FDA and have obtained distributions which clearly indicate two subpopulations

Fig. 2. Fluorescence distribution of peripheral lymphocytes isolated on a Ficoll-hypaque gradient and depleted of monocytes by incubation in tissue culture flasks. Lymphocytes were incubated with FDA (2.5 $\mu\text{g/ml}$) for 20 min at 20°C.

(Fig. 2). To check the identity of the subpopulations, we have subfractionated human lymphocytes into T- and B-enriched cell subpopulations on the basis of a combination of "E" (T cell) rosetting⁵ and Ficoll-hypaque centrifugation.⁶ The results of incubating these subpopulations in FDA are shown in Fig. 3A. Figures 3B and 3C are the results of artificial mixtures made from the isolated subpopulations. Figure 3B is a mixture of a 1:1 ratio of T and B cells and Fig. 3C a mixture of 5.5:1 T cells to B cells, which is about the ratio at which these cells are found in normal peripheral blood. Note the similarity between the distribution produced by this mixture and the one produced by unfractionated lymphocytes (Fig. 2). If the subpopulations are incubated at reduced temperature (4°C), the difference between their fluorescence is larger. This is an unexpected result and not easily explained.

Differential enzyme activity is not the only possible explanation for these results. Other possible factors are (1) membrane transit rates of FDA and fluorescein; (2) subfraction artifacts due to the rosetting technique; and (3) differential cell size. Experiments are in progress to assess the role of these possible factors.

In summary, we have been successful in stimulating hamster lymphocytes to undergo nonspecific blastogenesis *in vitro* and are now able to reduce the amount of mitogen required by a factor of 6 with the inclusion of a small amount of 2-mercaptoethanol. We have also shown the feasibility

Fig. 3. Isolated lymphocytes were allowed to form rosettes with sheep erythrocytes. The mixture containing lymphocytes, erythrocytes, and rosettes was recentrifuged on a Ficoll-hypaque gradient. Nonrosetting lymphocytes (B cells) were found at the interface, while erythrocytes and rosetted lymphocytes (T cells) were found at the bottom of the gradient. Cells from both areas were recovered, washed, and incubated with FDA (0.25 $\mu\text{g/ml}$) at 20°C either as isolated populations or artificial mixtures: (A) isolated T cells (—), enriched B cells (---), incubation time 20 min; (B) mixture of T and enriched B cells, 50% each, incubation time 55 min; and (C) mixture of T and enriched B cells, 85% and 15% B, incubation time 65 min.

of separating T and B lymphocytes on the basis of differences in their metabolism of fluorescein diacetate.

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CELL PREPARATION

Flow Microfluorometric Analysis and Characterization of Protein Contents and Proliferating Kinetics in Ascites and Solid Tumors

[H. A. Crissman, R. J. Kissane, and A. P. Stevenson]

Flow microfluorometry (FMF) has been shown to be a rapid and reliable approach for simultaneous determination of both DNA and protein content in single-cell populations, thus providing information for characterizing the dynamics of tumor cell growth and proliferation. The FMF technique is especially useful for analysis of slowly traversing or arrested cells such as encountered in many tumor systems because the methodology does not rely on cellular incorporation of ^3H -thymidine.

FMF analysis of the DNA and protein content of mouse ascites and solid tumor cell populations grown *in vivo* has provided information on protein distribution of cells in various phases of the cell cycle, as well as age-associated changes in proliferation kinetics of these tumor systems. L1210 ascites cells and solid Lewis lung carcinomas (LLCs) were dispersed, fixed, and stained for both DNA and protein, respectively, using the fluorochromes propidium iodide (red) and fluorescein isothiocyanate (green).

Analysis of the DNA distributions of L1210 cells on days 3, 4, 6, and 7 following implantation of 10^5 cells revealed changes in cycle kinetics patterns concomitant with increased cell density or tumor age. DNA distribution patterns for Lewis lung

tumors showed a 2C peak and a cell proliferation pattern extending between the 4C and 8C peaks. Tumor cells exhibited elevated protein distributions compared to normal cells; however, tumor cells were quite heterogeneous in cell size and morphology. Analysis of cellular DNA and protein in various tumor systems permits characterization of the growth kinetics at various stages of tumor development, and such information is of predictive value in chemotherapeutic regimen scheduling.

DNA and protein contour profiles of a primary tumor and lung metastases of the LLC from C57Bl/6

Fig. 1. Computer-generated contour profiles of DNA and protein content of propidium iodide-fluorescein isothiocyanate stained primary and lung metastases of the Lewis lung carcinoma. The contour lines are at 50, 100, 150, 200, 350, and 500 cells.

mice are shown in Fig. 1. Computer-generated density contours were obtained at threshold settings of 50, 100, 150, 200, 350, and 500 cells. Based on results obtained in both cell sorting experiments and DNA content analysis of diploid spleen cells, the portion of cells designated 2C in the DNA profile (x axis) shown in Fig. 1 represents normal cells and the remaining portion of the profile G_1 (4C), S, and $G_2 + M$ (8C) populations of the tumor cells, respectively. The percentages of cells in G_1 , S, and $G_2 + M$ were 38.7, 54.9, and 6.4, respectively, for the primary tumor and 58.8, 32.0, and 9.2 for the metastatic population. It is apparent that, in general, tumor cells have a greater protein mass than most but not all normal cells. Also, the protein profiles of the primary and lung metastases appear to be quite different, particularly through the S and $G_2 + M$ regions. Specifically, the primary tumor appears to contain cells having a wider range in protein content through these phases of the cell cycle.

Spectrophotofluorometric and Flow Microfluorometric Analysis of DNA Staining in Mammalian Cells Using Fluorescent Antibiotics

[H. A. Crissman, R. J. Kissane, and A. P. Stevenson]

The effects of various fixative agents, pH, ionic strength, stain concentration, and magnesium concentration on DNA staining with the antibiotics olivomycin, chromomycin A3, and mithramycin have been examined with DNA in solution and in mammalian cells. Ethanol-fixed Chinese hamster cell populations (line CHO) stained with mithramycin and analyzed by FMF provide DNA distribution patterns with a high degree of resolution. Glutaraldehyde-fixed cells exhibit about half the fluorescence intensity of ethanol-fixed cells and yield distributions with G_1 peaks having coefficients of variation (CVs) 1.5 times as large as ethanol-fixed cells. However, the percentages of cells in G_1 , S, and $G_2 + M$ were quite comparable. DNA distributions obtained for formalin-fixed cells were unacceptable for computer analysis.

Similar values were obtained by FMF for the fluorescence intensities of CHO cells stained over a pH range of 5.0 to 9.0. Below pH 5.0, there was a large decrease in fluorescence intensity and,

from pH 9.0 to 10.0, a significant increase in intensity of stained cells.

The fluorescence intensity of cells stained in mithramycin solutions containing 0 to 1.75 M NaCl increased gradually up to about 1 M NaCl. The intensity of cells stained in 1 M NaCl was 1.5 times greater than cells stained in the absence of NaCl.

Cells stained with increasing concentrations of mithramycin up to 250 μ g/ml showed a sharp increase in fluorescence intensity. DNA distributions for cells stained at all concentrations yielded virtually the same percent of cells in the various phases of the cell cycle. Cells stained with mithramycin solutions containing 0 to 70 mM $MgCl_2$ showed a gradual increase in fluorescence intensity as a function of increasing $MgCl_2$ concentration. At a concentration of 70 mM $MgCl_2$, cells exhibited a 100% increase in intensity above cells stained in 15 mM $MgCl_2$. Cell staining in the absence of $MgCl_2$ was extremely poor. Other metal ions tested included the chlorides (15 mM) of cadmium, calcium, cobalt, copper, lead, manganese, mercury, and zinc. None of these divalent metal ions proved superior to magnesium, and only cells stained in the presence of zinc, lead, and cadmium yielded bimodal DNA distributions.

Spectrophotofluorometric analysis of olivomycin, chromomycin A3, and mithramycin complexed with magnesium yielded spectral patterns quite similar but not identical in shape (Fig. 1). Addition of magnesium to the solutions induced a spectral shift in the emission peaks toward the longer wavelengths by about 23 nm and also produced a 10-fold decrease in fluorescence intensity of the dye. Addition of DNA in solution to the mithramycin-magnesium complex increased fluorescence 10 times but did not produce any major spectral shift. Increasing the ionic strength of the mithramycin solution did not change the spectral patterns of either the mithramycin-magnesium or mithramycin-magnesium-DNA complex, nor did it produce a change in fluorescence intensity of these complexes. These results and those obtained by FMF analysis indicated that the increase in fluorescence intensity of stained cells as a function of increasing ionic strength is probably due to changes in chromatin structure, providing a greater number of binding

Fig. 1. Fluorescence excitation and emission spectra for olivomycin, chromomycin, and mithramycin unbound (—) and bound (---) to calf thymus DNA. The antibiotic concentration was 3.3 $\mu\text{g/ml}$ in 0.15 M NaCl and 15 mM MgCl_2 , and the final DNA concentration was 1 mg/ml. Spectra for the unbound dye were expanded vertically 10 times for comparative purposes.

sites for the dye complex. We are continuing our studies in this area in order to elucidate the above phenomenon.

PHYSICAL DOSIMETRY AND RADIOBIOLOGY STUDIES Heavy Particles in Radiotherapy: A Comparative Study

[M. R. Raju, H. I. Amols (Group MP-3), E. Bain, S. G. Carpenter, R. A. Cox, and J. B. Robertson]

The clinical use of heavy particles in radiotherapy has made rapid progress during the past 3 yr. Therapeutic trials of fast neutrons are in progress at 5 installations in the United States and in several centers in Europe and Japan. Therapeutic application of protons is in progress at Uppsala, Sweden; in the Soviet Union; and at the Harvard cyclotron; helium and heavy ions at Berkeley; and negative pions at Los Alamos. Therapeutic application of negative pions is expected very soon

at Vancouver, Canada, and Zurich, Switzerland.

A comparative radiobiological study of heavy particles to evaluate the complementary therapeutic potential of heavy particles is in progress with a grant (CA17290) from the National Cancer Institute, and some of the preliminary results are reported herein. Fast neutrons from the Texas A & M cyclotron (50-MeV deuterons on beryllium), protons from the Harvard cyclotron, helium ions from the 184-in. cyclotron at Berkeley, heavy ions (carbon, neon, and argon) from the Berkeley BEVALAC, and negative pions from the Los Alamos Medium Energy Physics Facility (LAMPF) are used in this study. Since the biological effects of heavy charged particles at the Bragg peak position depend upon peak width, a therapeutically relevant peak width of 10 cm was chosen for all heavy charged particles in this comparative study.

The following comparative measurements are being made: (1) depth-dose distributions; (2) cell-survival measurements as a function of depth of penetration using a gel technique;¹ (3) cell-survival measurements at the beam entrance and at some positions in the peak region under oxygenated and hypoxic conditions using a metabolic depletion technique to obtain hypoxia;² and (4) skin reactions at the beam entrance (plateau) and peak center utilizing the mouse foot system used by Fowler and associates.³

The depth-dose distribution of the heavy particles used in this comparative study is shown in Fig. 1. The depth-dose distribution of all beams was normalized to the peak center. The dose

Fig. 1. Depth-dose distributions of heavy particles used for this comparative study.

distribution for protons in the peak region was made flat because it is known that there are no significant variations in relative biological effectiveness (RBE) with depth. An approximately flat distribution in the peak region was also used for negative pions because the exact correction for RBE variation was not known prior to these studies. For heavy ions, the dose in the peak region was varied to produce approximately the same cell survival in the entire peak regions of 10-cm width using the radiobiology data available prior to this investigation. The same ridge filter was used for heavy ions (carbon, neon, and argon); therefore, the slopes of the depth-dose distribution in the peak region become steeper with increasing charge of the heavy ion because of increase in nuclear interactions with increasing charge of the heavy ion. The depth-dose distributions of protons and negative pions are nearly the same except that negative pion beams produce some unwanted residual dose beyond the range because of u^- and e^- contamination and fast neutrons from stars. Also, the dose fall-off beyond the range for negative pions is not as sharp as for protons because of neutrons from negative pion stars and an increase in range straggling for negative pions compared to protons. With increasing charge, the heavy ions lose the dose localization advantage because of an increase in nuclear interactions. Ideally, this comparative study should have been done with charged particle beams of the same range. The ranges of neon and argon ions used in this study were shorter than other charged particle beams used. The beam entrance position (plateau) was very close to the proximal peak. If neon and argon beams of ranges comparable to other heavy charged particles were used, the dose localization (peak-to-plateau ratio) should have been even poorer, and the residual doses due to nuclear secondaries beyond their ranges should have been higher than for carbon ions. The residual doses for carbon ions and negative pion beams (from LAMPF) are very nearly the same.

Cell survival measurements using cultured human cells (T_1) as a function of depth of penetration for various heavy particle beams were made for a series of incident doses for each particle beam. Figure 2 shows the results of cell-survival measurements with depth of penetration for various

Fig. 2. Percent cell survival vs depth of penetration for various heavy particles.

beams with doses that cause similar cell killing. With the exception of argon ions, the data for all the other heavy charged particle beams are quite similar. In the case of argon ions, because of saturation effects at high linear energy transfer (LET), there was no increase in RBE with depth. At higher doses (not shown because of space limitations), enhancement in cell killing at the peak was observed compared to the plateau for all beams except argon ions. The data for various incident doses clearly indicate that the slope of the depth-dose curves in the peak region for helium, carbon, and neon should be shallower than that used to obtain uniform cell killing. In the case of pions, a significant increase in cell killing was observed in the last few centimeters in the peak region.

Cell-survival measurements using cultured Chinese hamster cells (V79) under oxygenated and hypoxic conditions for various heavy particle beams at the beam entrance, central position of the 10-cm wide peak, and at the distal peak (1 cm from the dose fall-off) were made. The data were fitted with the aid of a computer program using a linear quadratic equation, $S = e^{-\alpha D - \beta D^2}$, where S is a

Fig. 3. Aerated and hypoxic cell-survival curves for various heavy particles at the beam entrance (plateau), peak center, and distal peak (1 cm from the dose fall-off).

Fig. 4. RBE at the 10% cell-survival level for various heavy particles. 250-KVP X rays are used as a reference source of radiation.

Fig. 5. OER at the 10% cell-survival level for various heavy particles.

surviving fraction for a dose D and α, β are constants.⁴ The fitted curves for various beams at the plateau, peak center, and distal peak are shown in Fig. 3. The RBE and oxygen enhancement ratio (OER) values calculated at the 10% survival level are shown in Figs. 4 and 5. It can be seen that there is a slight but significant increase in RBE at the peak compared to the plateau for negative pions and carbon and neon ions. No significant differences in RBE were observed between the plateau and peak for protons. In the case of argon ions, the RBE at the distal peak was found to be significantly smaller than at the plateau and peak centers, and this is due to saturation effects at higher LETs. These results are consistent with those of Hall *et al.*,⁵ Chapman *et al.*,⁶ and Tobias *et al.*⁷ The OER for neon ions is similar to fast neutrons, and the OER for argon ions is slightly less. The OER for protons is not significantly different from that of x rays. The OER for carbon ions and negative pions is similar and slightly higher than for fast neutrons.

The acute skin reactions of various heavy particle beams were studied by exposing one of the hind feet of mice (RFJ female) to single doses. Skin reactions were scored daily for 30 days after exposure and 3 times a week from 30 to 60 days after exposure. The skin reactions were scored using an arbitrary scale.³ The average skin reaction (6 mice) plotted as a function of time after exposure

Fig. 6. Average skin reaction as a function of time after exposure.

Fig. 7. Average skin reaction (10 to 30 days) as a function of dose for various heavy particles at the plateau.

Fig. 8. Average skin reaction (10 to 30 days) as a function of dose for various heavy particles at the peak center.

Fig. 9. Late reaction (foot deformity) plotted as a function of early skin reaction in the mouse foot exposed to different heavy particles at the peak center.

is shown in Fig. 6 for argon ions and ^{60}Co gamma rays. It can be seen that, if the doses of argon ions and ^{60}Co gamma rays are matched to produce similar reactions, there are no qualitative differences between the reactions produced by ^{60}Co gamma rays and argon ions. This result is consistent with those reported by Denekamp *et al.*⁸ for neutrons and by Leith *et al.*⁹ for neon ions at the plateau. Figures 7 and 8 show the results of average skin reactions from 10 to 30 days after exposure for various heavy particle beams at the plateau and peak center, respectively. As expected, the biological effectiveness increases with increasing charge of the heavy ion. Carbon ions at the peak center and fast neutrons produce similar reactions.

The mouse foot develops a deformity in about 6 to 8 months after exposure, and the deformity can also be measured using an arbitrary scale.¹⁰ Figure 9 shows the results of deformity plotted as a function of early skin reactions when exposed at the peak center for various beams. The correlation between early and late reactions in the mouse foot system remains the same for all heavy particles used in this study. It must be stated that, although the early reactions in the mouse foot system are quite relevant to radiotherapy, the relevance of deformity to late effects observed in radiotherapy is highly questionable.

The results reported herein are helpful in relating heavy particles of interest in radiotherapy with one another. It must be stated that the real therapeutic evaluation of heavy particles can be made only from the differential effects of tumor and normal tissues in human patients. The advantages of heavy charged particle beams are evident when the peak widths are small (up to about 5 cm) but, for larger peak widths such as 10 cm or larger, the RBE at the peak is not much larger than at the entrance for single doses. This is not surprising because, in order to cover large volumes at the peak regions, we are essentially modulating the heavy charged particle beams to behave more like neutrons. The advantages of heavy charged particles can be maintained by aiming the beam whenever possible in a direction where the thickness of the treatment volume is minimal.

The OER for neon ions for the peak width used in these studies (10 cm) is comparable to fast

neutrons, and the OER for argon ions is slightly lower. Thus, the OER values for heavy ions are higher than expected values. A significant fraction of energy of heavy ions is deposited outside the core by delta rays. These results indicate that the oxygen effect depends on energy deposition over very short distances of the order of nanometers.¹¹

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Flow Cytophotometry in Radiobiology

[M. R. Raju, R. A. Cox, B. J. Price, and G. C. Salzman]

During the past 5 yr, major developments have been made in flow systems for rapid cell analysis. These developments permit quantitative and precise measurements of optical properties of individual mammalian cells. The modern flow systems are capable of simultaneously measuring various parameters such as DNA, protein, Coulter volume, light scatter, etc., and some of these instruments are also capable of sorting cells electronically based on the above parameters either singly or in combination. These flow systems have potential applications in biology and medicine. Studies to explore the potential of applying flow cytophotometry instrumentation to some of the selected problems in radiobiology are in progress, and some of the preliminary results are reported herein.

In principle, cell viability can be measured using flow cytometry instrumentation. Fluorescein diacetate (FDA) is taken up by viable cells and renders them fluorescent.¹ This principle has been used by Julius et al.² to measure viable fractions of murine thymocytes using the cell flow systems developed at Stanford. All cells produce light scatter, whereas only viable cells scatter and fluoresce simultaneously. Julius et al.² have also indicated that dead cells can be differentiated by scatter alone. We have reported results of scattering and fluorescence measurements on human lymphocytes exposed to different doses of x rays after staining the lymphocytes with FDA and have compared these results with cell viability measurements using the trypan blue dye exclusion technique. Good agreement was obtained between these two methods.³

If cell viability can be measured by scatter patterns alone from cells without fixation and staining procedures, such methods may have practical application in biological research. The

multiangle scatter spectrometer is very appropriate for this application because different angles may be optimal for different cells exposed to different agents to differentiate the viable and nonviable cells. This instrument measures on a cell-by-cell basis light scatter in 32 angles between 0° and 21° with respect to the laser beam axis, and the scatter pattern for each cell is transferred to a computer.⁴ The multiangle spectrometer is also equipped with a cell sorting capability which permits microscopic examination of cells that have a specific light-scatter pattern that is of interest in identifying viable cells from nonviable cells. In addition, the multiangle spectrometer is also equipped with a Coulter volume sensor.

Radiation damage to the mitotic apparatus of mammalian cells may not be observed by light-scatter patterns; however, membrane damage resulting from ionizing radiation on lymphocytes and hyperthermia and ultrasound on cultured mammalian cells seems to be reflected in the light-scattering patterns. Hyperthermia research is currently being pursued actively as an adjunct to radiotherapy. If a fast method can be devised using light-scatter patterns that can measure cell survival and that correlates with colony formation, this will be a significant development in hyperthermia research. Exploratory studies on viability of human lymphocytes exposed to x-rays and of cultured Chinese hamster cells (line CHO) exposed to hyperthermia and sonication are in progress.

Fig. 1. Light-scatter patterns of human lymphocytes at 2.4° .

Figure 1 shows light-scatter patterns of human lymphocytes exposed to different doses of x-rays at an angle of 2.4° where resolution between the peaks is optimal. The peaks to the right are presumably viable cells, and the peaks to the left are dead cells. It can be seen that the number of cells in the first peak, compared to the second peak, increases with increasing dose. The fraction of cells in the first peak seems to be lower than the fraction of dead cells determined by the trypan blue dye exclusion method.

Figure 2A shows the light-scatter patterns at different angles of cultured Chinese hamster cells (line CHO) treated with hyperthermia at 44°C for 90 min. The light-scatter patterns around 3.5° differentiate the two peaks with better resolution. Figure 2B shows the scattering patterns at an angle

Fig. 2. Light-scatter patterns of CHO cells treated with hyperthermia at (A) 44°C for 90 min and (B) 3.5° for different periods of hyperthermia treatment.

Fig. 3. Light-scatter patterns of CHO cells sonicated at (A) different angles and (B) 3.5° for different periods of sonication.

of 3.5° for CHO cells treated with hyperthermia for different time periods. It can be seen that the number of cells in the first peak, compared to the second peak, increases with increasing time of treatment. The fraction of cells in the first peak again was found to be lower than the fraction of dead cells measured by colony formation.

Figure 3 shows similar results for CHO cells exposed to sonication (Cole-Parmer ultrasonic cleaner, 50 W). The results also indicate that, with increasing sonication, the magnitude of the first peak (dead cells ?) relative to the second peak (viable cells ?) increases.

These preliminary results indicate that, by measuring the light-scatter patterns of cells using the multiangle light spectrometer, it may be possible to measure cell viability. However, the exact correlation with other conventional methods such as colony formation and the dye exclusion method needs further investigation. This work is in progress. A cell separator capability with our multiangle light spectrometer is very helpful in these studies, and questions such as what cells are in different peaks of the light-scatter patterns can be answered with certainty.

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INTRODUCTION

The use of isotopic tracers has proven to be an invaluable technique for elucidation of processes occurring in both the physical and life sciences. Radioactive isotopic tracers historically have been used much more extensively than stable isotopes. There are several reasons for the disproportionate use of radioactive materials: the commercial instrumentation for detection of radioactive isotopes is available at a reasonable cost and allows routine quantitative measurements; a variety of relatively inexpensive radioactively labeled compounds are commercially available; and the low background of isotopes such as ¹⁴C and ³H affords excellent sensitivity. However, there are also several limitations on the use of radioactive isotope tracers: their inherent radioactive nature precludes their use in certain circumstances such as clinical studies with children and pregnant women; location of the position of the radioactive label in a product derived from a radioactive precursor generally requires degradative chemical operations; and manipulations with radioactive isotopes always involve the possibility of radioactive contamination. Like their radioactive counterparts, stable isotopes have their own set of unique advantages and limitations. Stable isotopes can be used safely in cases such as

clinical diagnostic tests and field-condition environmental tracing experiments where the use of radioactivity is undesirable. There are also biologically interesting elements such as oxygen and nitrogen which have no suitable radioactive isotope but which have minor-abundance stable isotopes which can be used as isotopic tracers. Stable isotopes can be handled safely without special precautions. Finally, for stable isotopes possessing a nuclear spin, the position of the isotope in a molecule can be determined conveniently by nuclear magnetic resonance spectroscopy. At present there are unfortunately several circumstances which have limited the more widespread use of stable isotopes in the physical and life sciences. In spite of the dramatic price reductions that have occurred, primarily as a result of the stable isotopes program at Los Alamos, commercially available stable isotope-labeled compounds are still relatively expensive and limited in selection. The instrumentation available for stable isotope detection, while yielding more information than the presence and amount of the label, is operationally more complex than instrumentation for radioisotope detection. The background resulting from natural abundances of stable isotopes also makes their quantitative detection less sensitive than radioactive isotopes.

The naturally occurring stable isotopes of carbon, oxxygen, and nitrogen (ICONS) are of considerable importance in the life sciences since they constitute a sizable portion of the elements found in biological systems. Since the inception of the ICONS program at LASL, it has been realized that a program to promote uses of stable isotopes should concentrate on three aspects: production of the separated stable isotopes, synthesis of labeled compounds, and applications of stable isotopes in physical and life sciences research. Production of the separated stable isotopes is carried out by Group CNC-4 of the Chemistry and Nuclear Chemistry (CNC) Division. Carbon isotopes (^{13}C at 90 mol % ^{13}C and ^{12}C at 99.999 mol % ^{12}C) are obtained from the distillation of carbon monoxide. Nitrogen isotopes (^{15}N at both 40 mol % ^{15}N and 95 mol % ^{15}N and ^{14}N at 99.99 mol % ^{14}N) and oxygen isotopes (^{18}O at 95 mol % ^{18}O , ^{17}O at 20 mol % ^{17}O , and ^{16}O at 99.99 mol % ^{16}O) are produced simultaneously when nitric oxide is distilled. The synthesis of labeled compounds is done by the Organic and Biochemical Synthesis Group (H-11) of the Health Division.

The group synthesizes a variety of isotopic intermediate compounds, primarily by organic synthesis methods, and then uses both organic syntheses and biochemical syntheses to prepare other compounds, most of which have biomedical applications. The applications of stable isotopes undertaken by Groups H-11 and CNC-4 have involved clinical applications, studies of possible biological effects of stable isotopes, structural and analytical biochemistry studies and, to a lesser extent, stable isotopes as environmental tracers.

Production of stable isotopes is funded by ERDA under RPIS No. 000230 (F696). The synthesis and applications of labeled compounds program, whose annual report is given herein, is also funded by ERDA but under RPIS No. 000140 (F695) which accounts for about 35% of the total ICONS funding. The Division of Research Resources of the National Institutes of Health has established a National Stable Isotopes Resource (SIR) at LASL. The Resource, which accounts for about 15% of the total funding of the ICONS program, provides support for both Groups CNC-4 and H-11.

SYNTHESIS OF LABELED COMPOUNDS

Organic Synthesis of Labeled Compounds

[T. W. Whaley]

Owing to the fact that all compounds derive isotopic carbon and isotopic nitrogen from carbon-¹³C monoxide and ammonium-¹⁵N sulfate, respectively, a number of routine preparations were carried out during the year to produce the necessary intermediates for more complex syntheses. These materials included methane-¹³C, sodium cyanide-¹³C, methanol-¹³C, methyl-¹³C iodide, ethanol-1-¹³C, ethyl-1-¹³C iodide, ethyl bromoacetate-1-¹³C, ethyl bromoacetate-2-¹³C, and acetonitrile-2-¹³C. A few other compounds were also synthesized by existing procedures for ongoing experiments or for other groups within the Laboratory: methane-d₄ as an atmospheric tracer; ¹methyl palmitate-1-¹³C for P. Klein of the Argonne National Laboratory; carbon-¹³C disulfide for LASL's Laser Division; and a series of metal chelating agents [1-phenyl-3-thienyl-1,3-propanedione; 1,3-dithienyl-1,3-propanedione; 2-methoxy-N-(3-methylphenyl)benzohydroxamic acid] for the Analytical Chemistry Group (CMB-1).

While necessity dictates that some of the synthetic work is routine, the majority of the organic synthesis effort is devoted to developing new methods for synthesizing labeled compounds. 4-Ethylsulfonyl-1-naphthalenesulfonamide-¹⁵N (ENS-¹⁵N) was prepared for W. Reynolds of the National Center for Toxicological Research. As a result of the synthesis of ENS-¹⁵N, ²a new convenient synthesis of ENS itself was developed and published. ³ Appropriate modifications ⁴ of the ENS synthesis have been used to synthesize 4-(2-hydroxyethylsulfonyl)-1-naphthalenesulfonamide, a possible metabolite of the bladder carcinogen, and 4-vinylsulfonyl-1-naphthalenesulfonamide.

Scheme 1 shows the series of transformations which have been used to prepare valine-3,4,4-¹³C₃. The condensation of acetonitrile-2-¹³C with aminoethanethiol gave 2-methyl-¹³C-thiazoline which was

bis-alkylated with two equivalents of methyl-¹³C iodide in the presence of sodium hydride to give 2-isopropyl-¹³C₃-thiazoline. Reduction of the thiazoline with Al(Hg) gave 2-isopropyl-¹³C₃-thiazolidine which was hydrolyzed to give 2-methyl-¹³C-propanal-2,3-¹³C₂. After considerable experimentation, suitable conditions (NaCN, NH₄Cl, NH₄OH) were found for converting the labeled isobutyraldehyde into d,l-valine-3,4,4-¹³C₃. ⁵ A similar synthesis in which the 2-methyl-¹³C-thiazoline is alkylated with ethyl-1-¹³C iodide and methyl iodide ultimately gives d,l-isoleucine-2,3-¹³C₂. The isoleucine synthesis is currently being carried out.

A number of pathways to oleic-1-¹³C acid have been investigated. One sequence involved conversion of oleic acid to its dianion, followed by condensation with a carbonyl compound to give an α-carbonyl-oleic acid derivative which was readily decarboxylated; thus, the overall transformation was replacement of carbon-1 of the oleic acid with the carbonyl carbon of the carbonyl compound used in the condensation. Carbonyl compounds investigated in the condensation reaction were isopropyl formate, ethyl chloroformate, and diethyl carbonate; however, low yields (ca. 30%) were obtained, and the work was terminated.

A second pathway investigated was based on carbonation of 8-heptadecynylmagnesium bromide with carbon dioxide to prepare steric acid. To date, yields in the Grignard carbonation have been low, and the steric acid has been contaminated with an unidentified compound that appears to be a hydrocarbon. Scheme 2 shows the sequence of reactions currently being used to prepare oleic-1-¹³C acid. 1-Bromo-8-heptadecyne has been prepared from 1,7-dibromobutane and 1-decyne and converted into steric nitrile smoothly by NaCN in DMSO. The steric acid was obtained by basic hydrolysis of the nitrile. Suitable conditions for the semi-hydrogenation of the triple bond of steric acid into the cis-double bond of oleic acid are currently being investigated.

A sequence of reactions for labeling any of

the carbons of δ -aminolevulinic acid is still being developed. The synthesis of δ -aminolevulinic-¹⁵N acid has been completed using standard reactions.

Biochemical Synthesis of Labeled Compounds

[V. H. Kollman]

As in the case of the organic synthesis work, certain biosynthetic preparations have been repeated throughout the year using previously published procedures; glucose-¹³C₆ has been obtained from starch isolated from tobacco leaves following photosynthetic incubation with carbon-¹³C dioxide,⁶ and galactose-¹³C₆ has been prepared by hydrolysis of galactosylglycerol-¹³C₉ isolated from kelp.⁷

When one considers biochemical syntheses, it becomes apparent that there are two phases to the overall process: organism growth and product isolation. Although the division into these phases is more conceptual than operational, it is convenient to discuss the biosynthetic program in terms of organism growth and product isolation.

The major effort in organism growth has shifted from the previous systems (tobacco leaves, kelp, and bacterial fermentations) which produced only one or two labeled compounds to photosynthetic growth of algae in which all cellular carbon is labeled. The algae are grown in 18- $\frac{1}{2}$ shaker boxes using carbon-¹³C dioxide as a carbon source and can be produced in quantities of kilograms per month if the algae are grown and harvested continually. During the past year, Chlorella pyrenoidosa have been grown at the 90 mol % ¹³C level; Anacystis nidulans have been grown on 20 mol % ¹³C and 95 mol % ¹⁵N; and Agmenellum quadruplicatum, strain PR-6, have been grown on 20 mol % ¹³C.⁸ It is known that, under nitrogen starvation, the PR-6 algae produce high levels of carbohydrates, but the composition and quantities of the carbohydrates have not previously been identified (*vide infra*). A number of samples of various algae have been grown and made available to the Biophysics and Instrumentation Group (H-10) for light-scatter fingerprinting. Preliminary mutagenesis experiments to produce a δ -aminolevulinic acid-requiring and a glycine-requiring mutant have also been carried out. Several preliminary small-scale experiments to optimize the growth of Escherichia coli on sodium acetate-¹³C₂ have been performed. The best incorporation of

carbon to date has been about 30%, which is less than the 40 to 45% carbon yield we feel is optimum.⁸

The product isolation phase of the biosynthesis program has concentrated on isolation of amino acids from A. nidulans, C. pyrenoidosa, and A. quadruplicatum; isolation of glucose from A. quadruplicatum; and isolation of nucleotides and lipids from A. nidulans. The major emphasis has been on isolation of amino acids and glucose.

In order to isolate amino acids from algae, the lyophilized cells were first disrupted with trichloroacetic acid and extracted with ethanol-ether to remove lipids. The lipid-extracted cells were then hydrolyzed with hydrochloric acid to give a crude mixture of amino acids which were placed on a Dowex-50 cation exchange column and eluted with ammonium hydroxide. This procedure does not fractionate the amino acids but separates the amino acids from other cellular material. The purified amino acid mixture was placed on a Dowex-50 column (5-x 200-cm) and eluted with a series of buffers ranging from pyridinium formate (0.1 M, pH 3.1) to pyridinium acetate (2.0 M, pH 5.1). This chromatographic procedure provided pure fractions of aspartic acid, glutamic acid, valine, phenylalanine, tyrosine, lysine, histidine, and arginine but left the remaining amino acids as unresolved or cross-contaminated mixtures. The unresolved amino acid mixture (containing serine, threonine, proline, aspartic acid, glutamic acid, valine, leucine, isoleucine, glycine, and alanine) was then subjected to two additional chromatographic separations: first, a Dowex-1 column (elution with aqueous acetic acid) which separates glutamic acid and aspartic acid and, second, a Dowex-50 column (elution with hydrochloric acid) which separates serine, threonine, glycine, alanine, proline, valine, isoleucine, and leucine. The yield of isolated amino acids from lyophilized algae is on the order of 10% with 50- to 200-mg quantities of the individual amino acids being obtained.⁸ While the procedure outlined here is probably not suitable for preparing multigram quantities of individual amino acids, it is applicable to the preparation of a broad spectrum of amino acids in 100-mg quantities. The available literature on microbial fermentations which produce specific amino acids has been reviewed, but experimental work in this area is not planned presently.

The second major product isolation project has been the isolation of glucose- $^{13}\text{C}_6$ from A. quadruplicatum, strain PR-6. As mentioned previously, when the algae are grown under nitrogen starvation conditions, they are known to produce high levels of carbohydrates;⁹ however, the composition and quantities of these carbohydrates were previously unknown. The lipid-extracted cells contain cyanophycean starch which we have isolated and hydrolyzed with trifluoroacetic acid. The yield of crystalline glucose from the hydrolysate was 28%. We have also found that the lipid extract contains about 1% of low molecular weight carbohydrates, and we have identified these carbohydrates (spectroscopically) as sucrose, glucosylglycerol, and glucosylglycerate. The 28% yield of glucose from PR-6 algae is less than that obtained from the tobacco leaf system (ca. 50%), but the glucose is free of endogenous material which is always present in tobacco-derived glucose. This means that the algae are the only system which can be used to prepare glucose labeled at the 99+ mol % level with ^{13}C .

The isolation of nucleotides from algae has also been accomplished during the past year. DNA from lipid-extracted A. nidulans (grown on 20 mol % ^{13}C and 95 mol % ^{15}N) has been isolated using published methods.¹⁰ We have obtained 82 mg of DNA from 10 g of lipid-extracted cells, and we expect to obtain better yields on preparations now in progress. The DNA was hydrolyzed enzymatically and the nucleotide mixture separated chromatographically (Dowex-50, 0.05 M ammonium formate, pH 3.2) to give the four 5'-deoxynucleotides. When the isolations are completed, 50- to 100-mg quantities of the nucleotides will have been prepared.

APPLICATIONS OF STABLE ISOTOPES

Clinical Applications

[C. T. Gregg]

Historically, the group has engaged in research projects which could result in the use of stable isotopes in clinical diagnostic medicine. Recent work has centered around developing a simple diagnostic test for liver function. Previous results have shown that the oral administration of galactose- $^{13}\text{C}_6$ and subsequent measurement of the ^{13}C content of respired carbon dioxide as a function of time gave two curves corresponding to control patients

and patients with "cirrhotic" livers.¹¹ The error bars of the two nonoverlapping curves, however, suggested that mild liver-dysfunctional states probably would not be easily detectable by this mode of the galactose breath test. An alternative version of the galactose breath test which might prove more sensitive for intermediate liver dysfunction is currently being studied. In the alcohol-primed version of the test, a small quantity of alcohol is administered orally along with galactose- $^{13}\text{C}_6$. Figure 1 shows the suppression of galactose-derived carbon dioxide expiration by alcohol for a control subject; the upper curve shows the carbon- ^{13}C dioxide found in the breath following oral galactose- $^{13}\text{C}_6$ administration, and the lower curve shows the carbon- ^{13}C dioxide found in the breath following simultaneous oral administration of galactose- $^{13}\text{C}_6$ and ethanol. Data on subjects with cirrhotic livers have not been obtained yet, but we have found that lowering the alcohol dose from 32 ml (the amount used in similar intravenous tests) to 8 ml did not change the lower curve for the control subject. It is not clear whether the cirrhotic subjects should show greater or lesser alcohol-induced suppression of carbon- ^{13}C dioxide than control subjects, but the tests (being done in collaboration with the Occupational Medicine Group at LASL) have been started. Blood levels of γ -glutamyl transpeptidase (γ -GTP), an enzyme whose serum level is an indicator of liver dysfunction, are also being measured at the time the galactose breath test is given. Base-line

Fig. 1. Carbon-13 content of respired carbon dioxide following oral administration of galactose- $^{13}\text{C}_6$ (upper curve) and following oral administration of galactose- $^{13}\text{C}_6$ and ethanol (lower curve).

values of serum γ -GTP have been measured for 135 LASL employees. Normal ranges of serum γ -GTP for 91 males and 44 females were 9.4 to 34.4 units/l and 3.5 to 29.7 units/l, respectively. Breath tests of the metabolism of acetate- $^{13}\text{C}_2$ with and without simultaneous alcohol ingestion have also been performed. The same trend seen in the galactose test (suppression of carbon- ^{13}C dioxide in the breath) is evident, but the acetate test is much less reproducible at present.

Breath tests to study glucose metabolism in children are being done by Dr. Hans Helge of the Free University of Berlin in collaboration with Dr. Diether Neubert (Free University of Berlin) and LASL. When the respired carbon- ^{13}C dioxide from ingested glucose- $^{13}\text{C}_6$ was measured for normal and diabetic children, the diabetic children showed breath curves in which the peak of the curve was lower and later in time than normal children. This result was observed for both load and tracer doses of glucose- $^{13}\text{C}_6$. The data on blood glucose levels for these children are not available as yet. When diabetic children were given insulin, their carbon- ^{13}C dioxide curves returned to normal; this suggests that the glucose breath test may be quite useful in establishing proper insulin dosages. Another interesting observation from the Berlin group was that nondiabetic children (age 1 day) showed a breath curve having a delayed but normal height peak. A normal breath curve was established over about 9 months. Finally, a single experiment showing the transport of glucose across the placental barrier was performed by the Berlin group. A woman about to deliver a malnourished fetus (below normal weight and size) received an administration of glucose- $^{13}\text{C}_6$ directly into the amniotic fluid a few hours before labor was expected to begin. The onset of labor was unfortunately delayed, but the exhaled carbon dioxide of both the mother and infant showed ^{13}C enrichment. The inference that nutrients in the amniotic fluid are metabolized by the infant in utero suggests that nutrients added to this fluid may help to alleviate the symptoms of the malnourished fetus.

Biological Effects of Stable Isotopes

[C. T. Gregg]

The use of stable isotopes in clinical tests

quite properly raises the question of possible biological effects resulting from isotopic substitution. Previous studies in which 60% of the body carbon of mice was replaced with $^{13}\text{C}^{12}$ and in which algae were grown on 99+ mol % $^{13}\text{C}^{13}$ failed to show discernible effects. A series of in vitro experiments utilizing developing mammalian systems have not shown biological effects resulting from the presence of stable isotopes. The mouse limb bud system in organ culture, in which undifferentiated embryonic tissue develops into an elongated limb with the deposition of collagen at the sites of the bones of shoulder, leg, and paw, has shown no differences when natural-abundance glucose in the organ culture medium was replaced by glucose- $^{13}\text{C}_6$.¹⁴ The in vitro formation of blastocysts from preimplantation mouse embryos was found to be 96.3% for incubation with glucose- $^{13}\text{C}_6$ and 95.2% for incubation with natural-abundance glucose.¹⁵ In the final experiment, preimplantation mouse embryos were transplanted to pseudopregnant foster mothers following an in vitro incubation period in a glucose-containing medium. When natural-abundance glucose was used in the medium, 50% of the embryos implanted in the uterus of the foster mothers and 24% of these embryos gave rise to living fetuses. When the incubation medium contained glucose- $^{13}\text{C}_6$ (ca. 84 mol % ^{13}C), 48% of the embryos implanted and 22% gave rise to living fetuses. None of the 44 living fetuses from the isotopically enriched embryos showed any signs of abnormalities.¹⁶

Biochemical Applications

[T. W. Whaley]

The uniformly labeled 90 mol % ^{13}C amino acids obtained from C. pyrenoidosa exhibit interesting nuclear magnetic resonance spectra, with the most predominant feature being the spin-spin scalar couplings which are absent from natural-abundance carbon spectra. In collaboration with T. Walker and R. London (Group CNC-4), we have recorded cmr spectra of these amino acids and verified published values for the carbon-carbon coupling constants in these compounds. A number of three-bond couplings have been observed, and Group CNC-4 is trying to use these data to establish a Karplus-type relationship for three-bond carbon couplings.¹⁷

Also in collaboration with Group CNC-4, the

^{15}N nmr spectrum of arginine-guanidino- ^{13}C -2,3- $^{15}\text{N}_2$ has been studied as a function of pH.¹⁸ It has been observed that the resonances show pH-dependent exchange broadening and some unresolved long-range couplings. We believe that our data correct the existing literature on this subject, since other work was done with natural-abundance samples and resulted in misassignment of some of the resonance.

Several experiments producing selectively enriched DNA and nucleotides have been done with J. Zimbrick (University of Kansas) who is currently with Group H-9. An anaerobic photosynthetic bacterium, *Chlorobium thiosulfatophilum*, which can be grown with acetate and carbonate as its sole carbon sources, has been grown on a variety of ^{13}C substrates. To date, the organism has been grown on potassium carbonate- ^{13}C , sodium acetate-1- ^{13}C , sodium acetate-2- ^{13}C , and sodium acetate-1,2- $^{13}\text{C}_2$ in which the enrichments at the two carbons are not the same. The DNA from the organism grown on carbonate- ^{13}C has been isolated, and the nmr spectra do show selective enrichment. The isolation of DNA from the remaining organisms is in progress.

Fatty acids have been isolated from algae by A. Harmon, a predoctoral student from Oklahoma State University. The algae were taken from early generations of the organism growth experiment so that a distribution of fatty acids containing from zero to all ^{13}C atoms would be obtained. The methyl esters of the fatty acids have been analyzed by the gas chromatography-mass spectrometry technique, and isotopic fractionation has been observed in the peak emerging from the gas chromatograph. Interestingly enough, the isotope isomer containing all ^{13}C atoms has the shortest retention time, and correlations between ^{13}C content and retention time have been obtained.

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APPENDIX A

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APPENDIX B

LASL FOURTH LIFE SCIENCES SYMPOSIUM

"Mechanisms of Molecular and Cellular Toxicology"

September 28-30, 1976

Sponsored by the Health Division of the Los Alamos Scientific Laboratory and the Division of Biomedical and Environmental Research of the U. S. Energy Research and Development Administration

Symposium Committee

D. A. Smith, Chairman

M. D. Enger, C. T. Gregg, E. E. Campbell, R. A. Tobey, D. M. Smith, and H. A. Crissman

Tuesday, September 28, 1976

Welcome

G. L. Voelz, M.D., Health Division Leader,
Los Alamos Scientific Laboratory

SESSION I: METABOLISM

Chairman, Guido H. Daub, Department of Chemistry, University of New Mexico

"Metabolic Activation of Mycotoxins by Animals and Man"

Ronald Shank, University of California, Irvine

"Metabolism of Polycyclic Aromatic Hydrocarbons"

James K. Selkirk, Oak Ridge National Laboratory

"Biological Activity of Benzo[a]pyrene Metabolites: A Molecular Basis for polycyclic Hydrocarbon-Induced Carcinogenicity"

Roland E. Lehr, National Institute of Arthritis, Metabolism and Digestive Diseases-University of Oklahoma

"N-Nitroso Compounds"

Sidney S. Mirvish, Eppley Institute for Cancer Research

SESSION II: INTERACTIONS WITH MACROMOLECULES

Chairman, A. G. Saponara, Cellular and Molecular Biology Group, Los Alamos Scientific Laboratory

"Nucleic Acid Alkylation"

Bea Singer, University of California, Berkeley

"Infidelity of DNA Synthesis as Related to Mutagenesis and Carcinogenesis"

Lawrence A. Loeb, Fox Chase Institute

"Involvement of Free Radicals in Chemical Carcinogenesis and the Molecular Nature of Neoplastic Transformation"

Paul O. P. Ts'o, Johns Hopkins University

Wednesday, September 29, 1976

SESSION III: CELLULAR AND TISSUE STUDIES

Chairman, Ronald M. Humphrey, Department of Physics, M. D. Anderson Hospital and Tumor Institute

"Mutational Approaches to the Study of Carcinogenesis"

Ernest H. Y. Chu, University of Michigan

"Relationships between Differentiation and Carcinogenesis"

G. Barry Pierce, University of Colorado

"Chemical Mutation in Human Lymphoblasts"

William Thilly, Massachusetts Institute of Technology

"Mutagenesis and Teratogenesis in Mammalian Embryos"

Diether Neubert, Free University of Berlin

SESSION IV: DNA REPAIR

Chairman, Robert B. Painter, Laboratory of Radiobiology, University of California, San Francisco

"Repair of DNA Damage Induced by Radiation and Aryl Alkylating Agents in Mammalian Cells"

Peter A. Cerutti, University of Florida

"Repair of DNA Damaged by Physical and Chemical Carcinogens"

James E. Cleaver, University of California,
San Francisco

"Alkylation Damage and Repair in Mammalian Cells"

Bernard Strauss, University of Chicago

Thursday, September 30, 1976

SESSION V: IMPLICATIONS AND PROSPECTS

Chairman, Donald F. Petersen, Alternate Health
Division Leader, Los Alamos Scientific Labor-
atory

"Extrapolation of Cellular and Molecular Level
Studies to the Human Situation"

Peter Magee, Fels Institute

"Extrapolation of Animal Studies to the Human Situa-
tion"

John A. Zapp, Jr., Consultant in Toxicology
and Industrial Hygiene

"The Scientific Bases of Environmental Carcino-
genesis and Cancer Prevention: Developing an
Interdisciplinary Science and Facing Its Implica-
tions"

Umberto Saffiotti, National Cancer Institute

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