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ABSTRACT

This report summarizes research and development activities of the Los Alamos Life Sciences Division's Biomedical and Environmental Research program for the calendar year 1981. The principal features of the program have been carried out by groups in the Life Sciences Division, supported by the United States Department of Energy. Previous annual reports in this series prepared under the auspices of the United States Atomic Energy Commission, the United States Energy Research and Development Administration, and the United States Department of Energy are LA-5633-PR (1973), LA-5983-PR (1974), LA-6313-PR (1975), LA-6898-PR (1976), LA-7254-PR (1977), LA-8023-PR (1978), LA-8577-PR (1979), and LA-8948-PR (1980).

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

OUTLINE OF PROGRAM

This report covers the activities of the Life Sciences Division of the Los Alamos National Laboratory for the period of January-December 1981. The activities reported are restricted to those supported by the Department of Energy and do not include the projects located within the Health Division which are being reported separately.

Further adjustments in the structure of the Life Sciences Division have taken place during 1981. These organizational changes are directed toward placing complementary research areas in closer administrative units to enhance the degree of interaction and to lower costs. Two major changes in administrative structure have resulted: 1) the Biophysics Group (LS-DOT), which was responsible for flow cytometry instrument development and maintenance, was dissolved and the programs and people were placed in the Experimental Pathology Group (LS-4) where most of the biology using flow systems is carried out; 2) the Organic Chemistry Group (LS-5) was also dissolved and the Stable Isotopes programs and people were placed in the Environmental Sciences Group (LS-6) with the Oil Shale Analytical Chemistry program moving to the Toxicology Group (LS-1). Along with these changes, two new Group Leaders have been named. For the Experimental pathology Group, Carleton C. Stewart became leader in August and for the Environmental Sciences Group, Michael D. Coburn became leader in November. As reported last year, the Division is now headed by Dr. Mark W. Bitensky, and in August 1981, Laurence M. Holland became Deputy Division Leader.

The format of this year's report follows that of recent years and consists of a brief summary of research accomplishments in each of the operating groups followed by short articles addressing pertinent findings in specific programs. The articles are brief and not intended to be complete renditions of the experiments involved in each project; however, we hope that the information

provided will serve to illustrate the breadth and sophistication of the programs. For further detail or discussion we encourage the reader to contact the principal investigators listed for each project.

As part of our multidisciplinary approach to the perceived needs of the Department of Energy we continue to encourage intergroup projects wherever possible. This atmosphere allows beneficial interactions between the scientists addressing fundamental questions and those studying more applied programs. Major programs such as oil shale and nuclear energy are approached on a broad basis utilizing teams of scientists with varied and complementary skills. We also continue our strong interactions with research activities in the Health Division and the Chemistry-Materials Science Divisions. Both Divisions have been helpful to us in strengthening our comprehensive approach to oil shale questions.

Much of the effort of the Division is focused on both fundamental and applied research related to the ultimate effect of energy technology on the health of man. As part of this research posture, we also spend considerable effort in studying the intrusion and transport of potential pollutants in the environment. The Environmental Sciences Group has the major responsibility in this area, and the research performed there is integrated into the overall program since changes in environment have a fundamental effect on the well-being of man.

Our program in what is generally termed basic science provides the foundation for nearly all of our applied energy-related programs. All of these projects address questions that are basic to understanding the fundamental interactions of toxic, mutagenic, and carcinogenic materials at the molecular and cellular level. Without this understanding, which is achieved in research that is conducted in close conjunction with more applied studies, the answers derived from applied research would be lacking in substance and ultimate meaning.

LIFE SCIENCES DIVISION*

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BIOPHYSICS GROUP (LS-DOT)

D. M. Holm, Ph.D., Acting Leader

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J. H. Jett, Ph.D.
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INTRODUCTION

Early in 1981, the Biophysics Group was reorganized, the agricultural functions being placed in the Environmental Sciences Group (LS-6) and the flow cytometry functions being placed under the Division Office. The main objective of this group continues to be the operation and maintenance of most of the flow cytometers, and further development of the instruments and the computer programs that support them. In addition, the group provides incidental instrumental and analytical support to other DOE programs in Life Sciences and Health Divisions.

In order to meet the requirements of various users, continual innovative improvements are made in available instruments. For example, a second laser with ultraviolet (UV) output capability has been added to Sorter II to make it possible to perform fluorescent polarization, dual laser excitation, and high-speed cell selection experiments.

The interest in analyzing and sorting chromosomes has prompted the evaluation of each instrument for chromosome analysis. All of them give quite good DNA resolution (about 2% for the largest chromosomes) but we are continually looking for further improvements. The effects of electronics, photon statistics, light intensity fluctuations, hydrodynamics, computations, and optics are being examined in considerable detail. It is anticipated that further improvements will be forthcoming but they will be obtained with considerable effort because we are approaching the limits of each technological and physical parameter that contributes to the coefficient of variation.

Significant quantities of specific chromosomes have been sorted on the Fluorescence Activated Cell Sorting (FACS) system with high purity (estimated to be 95+%) for support of DNA cloning experiments in Group LS-3 that are directed toward creating a "library" of DNA segments. To make a complete library of all individual human chromosomes will require improved DNA resolution. Radiation damage to chromosomes has been monitored routinely with the Flow Microfluorometer (FMF II) and is reported elsewhere in this document.

A new computer based data acquisition system, the Los Alamos Cell Analysis (LACEL), has been put into operation with new terminals and software programs. It is now possible to collect data with a common format and use common data analysis programs. The most important programs are now operational but further support will be needed to satisfy the various user needs. Considerable effort will be made to maintain compatible up-to-date LACEL systems in operation on each of the flow cytometers.

Up to eight individual parameters can be associated with each particle as a list of the data (called list-mode-data-collection). The list-mode-data-collection has proven to be a very useful addition to several of the systems. It is now routine to reprocess data to determine various correlations among parameters. The time parameter is also being used routinely for kinetic measurements and for analysis of specific time increments so that less than optimum data can be excluded (data taken when the instrument is not performing properly).

Resolution Improvements

[D. M. Holm, J. H. Jett, J. C. Martin, M. E. Wilder, M. F. Bartholdi (LS-4), L. S. Cram (LS-4), and R. D. Hiebert (E-5)]

Improved DNA resolution is important to many biological experiments, particularly for chromosome analysis where it is desirable to completely resolve different chromosome types. Because of this, a systematic analysis of all factors that contribute to DNA resolution measurements is underway. The work in the Biophysics Group is concentrating on instrumentation contributions and has been centered on the electronics, photon statistics, and laser light fluctuations. Optical and hydrodynamical effects will be considered later along with additional work on the effects of different methods of computation.

It is well known that the coefficient of variation (CV) should be an intrinsic variable and not subject to the modal channel in the histogram. However, we have observed that this is not always the case. Generally, the higher the modal channel of a specific peak, the smaller will be the coefficient of variation. Figure 1 illustrates the problem. It is more noticeable with biological samples and microspheres than when using light flashes from a light emitting diode (LED). We believe that variations in the trigger level in the LEDs cause some problems with the light

Fig. 1. Pulse height distribution of integrated fluorescent light signals from 10-micron spheres at four different gain settings. It illustrates the effect of a change in CV with gain setting. The cause of this effect is under investigation. The data were taken on FMF II at 800 mW.

flasher, so we have found that operation at about 10 volts and using neutral density filters to reduce the light output works best. Since it is desirable to collect the DNA histogram of the complete karyotype on a single plot and in the case of sorting, it is necessary to set windows for sorting decisions, this artifact needs to be minimized. Experiments have been conducted to determine the cause of this artifact but it has not been satisfactorily resolved. A slight error has been identified as being inherent in the digitalization process when the number of channels that defines a peak is less than five channels. But this error is smaller than that generally observed. Figure 2 illustrates the magnitude of the effect. Tests of the analog-to-digital converters indicate that they are performing properly. Some slight errors in the amplifier/integrator have shown that widely

Fig. 2. Theoretical (solid lines) and expected (dashed lines) values for peak full width at half maximum (FWHM) of Gaussian distributions show various coefficients of variation versus modal channel of the distribution. The theoretical curves were based on the continuous functional form of the Gaussian distribution. The dashed lines were calculated expecting FWHM values for digitized normal distributions.

differing pulse shapes and heights have a measurable error. A pretrigger integrator option has been constructed to minimize this problem by delaying the input signal until the integration gate has been opened.

Photon statistics has been recognized as an important contribution to broadening of the CV.¹ It has been assumed that on a log/log plot of CV vs laser power or its equivalent (increasing brightness of chromosomes), the slope would be minus one half if the effect was due to photon statistics. However, we have observed some results that lead us to doubt that this is a sufficient criteria. Figure 3 shows such a graph in which the straight line has the proper slope for photon statistics. The three data points at 720 mW were taken a few h apart and the modal channel did not change significantly. If the slope was only due to photon statistical effects there should not have been any data points below the line. Our best explanation is that there are other factors which have not been identified that have the same manifestation as photon statistics. Because of this observation, we have been making modifications to check the photon statistical effects with a LED light flasher whose output can be modified with ND

Fig. 3. A plot of CV vs laser power. The two data points below the line at 720 mW were taken a few h after the other data and indicates a significant improvement in CV. The straight line is the proper slope for photon statistical effects. However, if that was the principle effect, there should not be any data points below the line.

filters. The pulser peak is then put in the same modal channel as the peak in question (but with the same high voltage and amplifier settings) and the CVs compared. Any differences are due to effects other than photon statistics. For these experiments to be valid, the inherent pulser peak CV with no ND filters must be small ($\sim 0.4\%$).

The effects of laser light intensity have been presumed to be small but significant. Since it was desirable to minimize all of the contributors to CV, we purchased a "noise eater" (Coherent Radiation) that is designed to reduce laser power variations by modulating the laser power to compensate for fluctuations and maintain a constant light output. The manufacturer's specifications indicate that it should make a very significant improvement in laser stability. We have installed the unit temporarily on FMF II and have found that it seems to make some improvement in light stability but it did not make a significant improvement in CV. We are currently making a detailed analysis of the "noise eater" performance with an electrical signal "spectrum analyzer" to determine the magnitude and frequency distribution of the laser power fluctuations.

The complete analysis of the most important factors that contribute to the broadening of the CV will take considerable time but are very important for achieving the ultimate in small CVs. It will not be possible to resolve all of the individual chromosomes by DNA analysis in the human karyotype unless the instrumental contribution to CV is reduced to about 0.5%.

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Multiparameter Cell Sorter II

[J. C. Martin, M. E. Wilder, and J. H. Jett]

The Sorter II system is a multiparameter computer based cell sorter based on the Los Alamos style enclosed flow chamber.¹ The system was one of the earliest flow cytometers to have a computer based data acquisition system, and the first to operate with list mode data acquisition with four input parameters.¹¹ This instrument has been in operation for the past nine years and is in daily

use. Measurement capabilities include Coulter (cell) volume, two colors of fluorescence, light scatter, associated laser beam transit time durations (time-to-amplitude-conversion, TAC), and logarithmic amplification. The instrument data collection and sorting rates are 1,000 cells/sec.

The major improvement to the Sorter-II system during the past year has been the addition of a new Spectra-Physics model 164-05 argon-ion laser. This laser is enhanced for UV operation and has an output power in excess of 100 mW for the 352-361-nm lines. The old model 164-04 argon-ion laser, which has been in use for 9 yr, has been retained as a second laser for dual laser beam measurements.¹² The new UV laser capability has made it possible to begin making routine measurements using UV excitable dyes. For example, we are now able to measure DNA distributions using the viable stain Hoechst-33342 and to measure viable membrane properties using the probes 1,6-diphenyl 1,3,5-hexatriene (DPH) and 8-Anilino-1-naphthalenesulfonic acid (ANS). Additional membrane structure and function information can be obtained using the technique of fluorescence polarization with measurements of the probe DPH. Studies of fluorescence polarization in collaboration with Professor R.O. Kelley at the University of New Mexico have been initiated.

Measurements of the dynamics of changing fluorescence emission from populations of cells has continued to be a useful flow cytometry technique for studies of enzyme activities and studies of the kinetics of staining reactions.⁹ The ramp generator designed by Dick Hiebert (E-7) has been in routine use on Sorter-II as well as on other machines for making these kinetic measurements. The addition of the UV laser has allowed us to make simultaneous measurements of DNA and esterase activity in viable cells using dual laser excitation. A difficulty in the analysis of enzyme kinetics has been due to the natural variability in populations of cells giving rise to a broadening of the data. In addition changing data acquisition rates result in variations in the number of events at each time point which can also obscure the details of the kinetics being measured. The effect of these variations can be removed by normalizing the two-parameter histogram such that there is an equal number of counts at each time point. Figure 1 shows a contour plot of

Fig. 1. Normalized two-parameter histogram of time versus fluorescence intensity. Dots are placed in each channel containing at least 1% of the population in that time slice. Iso-percentile contours are plotted at the 2.5%, 5%, 10%, and 20% levels. Note the broadening of the contours that occurs as a function of time. The dashed line denotes the locus of the median fluorescence intensity determined by plotting the 50% points of the cumulative distribution function. The time scale ranges from zero to ten minutes.

changing fluorescence intensity versus time for the measurement of esterase activity in Chinese hamster cells. The data presented in Fig. 1 have been normalized such that the total number of cells along each time slice is equal to 10,000. Cells with no fluorescence and clumped cells have been removed from the distribution. Even after normalization, the contours shown are still rough and the median fluorescence values are hard to visualize at the later time points, any percentile contour can be plotted. In Fig. 1, the dashed line is drawn through the fluorescence 50 percentile points. Thus it represents the locus of the median fluorescence values versus time. Once the median locus has been determined, the kinetics of the fluorescence can be analyzed and many samples can be quantitatively compared.

Numerous additional improvements have been made on the system during the past year, most having to do with minor changes in the electronics. The addition of the second beam. Major maintenance problems this year were replacement of the plasma

tube in the old laser and computer disk malfunctions. The laser tube failure occurred after the new laser had arrived and resulted in minimal down time. The disks in use are CDC-Hawk drives which have been in constant use for six to eight yrs. The disk failures have been mechanical in nature. These problems will be eliminated when the new LACEL data acquisition system 2 is installed. In this new system RL-02 disk drives will be used with a PDP-11/23 minicomputer.

A list of machine usage the past year is tabulated in Table I showing a breakdown of both internal Los Alamos users as well as outside users. As can be seen from the table there is considerable interaction with outside investigators who use our facility. The average use rate for the second half of the year was 60%, which was a dramatic increase over the 33% rate for the first half of the year. This rise in usage reflects an increased use and a continuing interest in measurements with Sorter II by personnel in LS-4. Maintenance, both down time and preventive, amounted to 9% of the total use time

TABLE I

MACHINE USAGE

Organization	No. of Users	No. of Use Days
Los Alamos:		
LS-DOT	3	3.5
LS-1	2	18.5
LS-3	1	.5
LS-4	7	29.0
CNC-2	1	.5
G-7	1	1.0
T-10	1	1.0
(dev)	-	11.0
(maint)	-	10.0
University of New Mexico:	7	43.5
University of Arizona:	1	.5
University of Colorado:	1	2.5
M.D. Anderson:	1	1.5

Papers published or submitted during the past year in which Sorter-II was involved are being reported in references 2,3,4,5,6,7,8,10,13,14,15.

and is recorded in the table. Instrument development time, in which improvements to the system were made or new measurement techniques were tried out, amounted to 10% of the total use time and is also listed.

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Chromosome Sorting For Genomic Cloning Of Mammalian DNA

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

Cloned DNA from specific chromosomes will provide the means to answer a variety of fundamental and applied questions concerning the packaging of DNA in mammalian chromosomes and the relationships between packaging and gene expression. Once obtained in large quantities by recombinant techniques, the chromosome specific DNA will be used to analyze differences in DNA sequenced between normal and abnormal cells, to determine information content of specific chromosomes (J. Griffith and R. A. Walters, LS-3).

In order to build libraries of the genomic information in individual chromosomes, sufficient quantities of purified chromosomes must be obtained.

Fig. 1. Chromosome number versus fluorescence intensity (channel number). Chromosomes were obtained from metaphase blocked Chinese hamster line M-31 cells, stained with propidium iodide, analyzed and sorted by flow cytometry. The vertical tic marks bracketing the peaks due labeled 1 and 2 denote the sorting windows used.

Using the Chinese hamster cell line M-31 as a source of material, approximately 6 μ g of each of chromosomes number 1 and 2 have been obtained to construct the initial libraries. In the future, the quantity of chromosomes required to build a library will be less than 6 μ g as techniques for handling such small quantities of material are refined and cloning efficiencies improved. These quantities were sorted with a modified commercial flow cytometer (Becton-Dickinson FACS-II) using the Los Alamos developed LACEL data acquisition and sorting control system. Figure 1 shows a typical histogram of fluorescence intensity which is proportional to chromosome DNA content obtained while sorting M-31 cells. The chromosome analysis rate was approximately 1000/second. With the present instrumentation it will be possible to push the analysis rate up to 5000/second. This will be necessary to obtain sufficient quantities of the resolvable smaller chromosomes which appear at lower fluorescence intensities.

High-Speed Cell Selection

[J. C. Martin, C. Stewart (LS-4), and J. H. Jett]

A new technique for super high-speed cell selection is being investigated for sorting of viable cells. Instead of conventional charged droplet sorting, this selection method will be based on a technique of cell destruction. The goal is to analyze and select cells at rates of 100,000 cells per second, which is 20 to 100 times faster than sorting rates now available. A wide variety of biological problems are in need of such high-speed cell selection techniques. Any experiment in which the separation of a low-frequency population is necessary for subsequent reinjection of the selected cells into animals will be facilitated by high-speed selection. In cases where the frequency of occurrence of the desired cell type is very low (1 in 10^5), high-speed selection may be the only way to accomplish the desired results.

The new method will make use of an existing, conventional cell with the addition of a second high-power UV which can be rapidly switched on or off by an optical modulator (see Fig. 1). The modulated

Fig. 1. Block diagram of high-speed optical sorter showing the measurement laser beam (laser 1) and the irradiating laser beam (laser 2) within the flow chamber. Optical signals are detected by photomultiplier tubes (PMT), amplified, and displayed on a oscilloscope (SCOPE). The signals can be analyzed by an analog-to-digital converter (ADC) and multichannel analyzer (MCA). The ADC can be gated by single channel analyzers (SCA) in order to set selection windows. Selection conditions may be based on more than one measured parameter through the use of a coincidence circuit (COIN). The properly delayed sort signal is used to turn on and off laser beam 2 via the optical modulator (OM) driven by the modulator driver (MD).

high-power UV beam (Beam 2 in Fig. 1) will intersect the sample stream below the conventional interrogation beam (Beam 1 in Fig. 1). Selection decisions based on measurements made as the cell passes through the first beam will switch the UV laser beam off for those desired rare cells; the UV laser beam will remain on when the cells that are not wanted pass through it. Cell destruction is produced by irradiation with the second beam causing

photochemical reactions in the cells which are subsequently lethal to the cells upon return tissue culture.

To date, the most promising means of producing sufficient damage to the cell with the band of laser lines in the 350-360 nm region available with argon-ion lasers is by irradiating cells stained with the viable DNA specific dye Hoescht-33342. Sufficient power is not available at these wavelengths to directly inactivate the cells. Present indications are that for cells stained with Hoescht, the UV dose necessary to reduce the survival level to an acceptable level is cell type dependent. However, it is known that the survival of Chinese hamster cells (line CHO) is reduced to approximately 2% when they are exposed to 500 mW of UV in a flow system (Kissane, in preparation). Irradiation of cells by a triggerable pulsed laser which produces a 250-nm beam and thus directly damages the cellular DNA is being considered as alternative mode of operation.

Los Alamos Cell Analysis (LACEL) Flow Cytometry Data Acquisition System

[S. F. Wilkins, L-81]

The Los Alamos Cell Analysis (LACEL) data acquisition and processing system^{1,2} is now operational on four cell sorters at Los Alamos. The system can acquire up to 8 parameter list mode data and control left and right sorting on up to four parameters for each direction. The software has been distributed to several laboratories in the U. S. and elsewhere.

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TOXICOLOGY GROUP (LS-1)

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INTRODUCTION

The Toxicology Group consists of a number of programs which generate information of relevance in hazard assessment of energy technology associated material. A variety of analytical systems are utilized for these studies, ranging in complexity from cultured cells to intact animals. The primary objective of these studies is to identify products posing human health hazards at a very early stage in development so that appropriate engineering safeguards may be designed prior to construction of large-scale commercial facilities. A secondary objective is to delineate the underlying biochemical mechanisms of pollutant-induced alterations in metabolism; such information may suggest novel procedures for monitoring the status of individuals in the workplace and for counteracting problems arising from accidental exposure.

Intact animal studies employ a variety of rodent species which are commonly utilized in standard toxicology procedures. Various routes of exposure to test materials are used, with major

emphasis placed on inhalation studies, due in part to our close ties with the Aerosol Physics Section of the Industrial Hygiene Group (H-5). A second major route is via dermal exposure. Both short- and long-term exposure studies are carried out, utilizing different levels of test agents in an attempt to define dose-response relationships and to identify, where applicable, threshold levels of activity.

Major emphasis is directed toward examination of health effects associated with various materials generated during production of shale oil. Inhalation studies focus on effects of particulates such as raw or spent shale on lung tissue; multiple sources of raw shale are employed in these studies, including material from Estonia which is utilized in the Soviet oil shale effort. Liquid products are analyzed by means of topical application of oils to the skin. The major endpoints measured are acute toxicity and tumor formation, important considerations given the interrelationship of

the two processes which is documented in one of the reports comprising this section.

An experimental retort obtained from the Laramie Energy Technology Center became operational during the past year. Current plans call for an examination of the effects on animals of retort gases administered either alone or in combination with various technology-specific particulates during the early part of CY 1982. This retort, plus a second generation retort of Los Alamos design, should provide us with the capability of examining the effects of parameters such as temperature and gas flow on the chemical nature of the products and by-products generated, as well as on the biological effects associated with these materials. This approach has the advantage of providing generic information of relevance to a variety of technologies through elaboration of the complex relationship between retort conditions, product generation, and associated health effects. At the same time, ongoing and contemplated studies utilizing various shale oil materials, technologically relevant upgraded products, and reference standards should allow site- and process-specific health effects comparisons to be made among existing technologies.

The recent decision to incorporate the Life Sciences Division analytical chemistry effort into the Toxicology Group should enhance interactions of chemists and biologists associated with the various projects included in the multidisciplinary Oil Shale Project. To facilitate further such beneficial interactions, during the past year, attempts have been made to strengthen the Toxicology Group's ties with the Aerosol Physics Group in Health Division (H), Design Engineering Division (WX) (retort design and construction), and Chemistry-Materials Science (CMB), Chemistry-Nuclear Chemistry (CNC), and Earth & Space Sciences (ESS) Divisions (physical/chemical analyses of organic and inorganic constituents of shale oils, product waters, particulate by-products, etc.).

The lung tumorigenesis program is designed to delineate the role of internally deposited radiation in carcinogenicity of the respiratory tract. In response to DOE's reduced emphasis on such studies, the number of long-term inhalation studies has been drastically reduced, with a refocusing on more

mechanistic considerations which could provide explanations for some of the intriguing biological questions raised by the inhalation studies. This newer series of studies is addressing the role of lung cell turnover (stemming from exposure to radionuclide/tumor-enhancing agents) in carcinogenesis; an approach of this nature (which should provide baseline data of relevance to other areas such as the oil shale lung tumorigenesis effort) may allow identification of likely target cells and an assessment of the role of cell division and macrophage activity during the early stages of carcinogenesis.

A third animal study is investigating the effects of inhaling various manmade insulation fibers (e.g., microfibers, fiberglass, mineral wool, ceramic wool, etc.). This project is supported by the Thermal Insulation Manufacturers' Association, a consortium of companies which manufacture insulation products. A spinoff from this program, which is of relevance to DOE interests, is the ability to examine the role of particle geometry in tumor induction. Interest in particle geometry stems from observations made on the tumor-inducing properties of asbestos in which only those particles < 0.5 μm in diameter and > 10 μm in length appear to be able to induce mesotheliomas. [Details of this insulation-products program are not included in this package of DOE programs.]

Because of the accrual of large numbers of tissue samples resulting from these studies, a computerized data-handling system has been developed which provides a large and comprehensive repository of systemic pathology information. The repository also provides extensive information on the tissues of normal animals.

An immunology program maintains as a major goal the delineation of immunological events which are altered during conversion to the neoplastic state. Primary emphasis currently is directed toward monitoring immunological changes in animals enrolled in the oil shale program in addition to isolating and characterizing a class of cells which is involved in expression of delayed-type hypersensitivity.

Several cultured cell projects focus on pollutant-induced alterations in metabolism at the cellular level. Attempts have been made to define

biologically relevant conditions for solubilization of a series of pure compounds and particulates of complex composition which are suspected by-products of oil shale extraction procedures. Mechanistic studies are directed toward elaboration of cellular defense mechanisms and to interactions between pollutants and the cellular genome which alter the chemical composition/structure of chromosomal proteins. Information regarding this latter area may provide clues regarding the biochemical basis for altered gene expression and the induction of neoplasia.

The radiobiology and oncology project is concerned with the treatment of tumors. Cultured cells, spheroids, and intact animals are utilized for these studies. The two major goals of the program are directed toward increasing fundamental knowledge of the radiobiology of high-LET particles (π mesons,

heavy ions, plutonium alpha particles, etc.) and applying flow cytometric techniques in the design of more effective treatment schedules utilizing radiotherapy and chemotherapy.

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

Effects of Solubility on Tumorigenesis of Two Shale Crude Oils and Two Natural Petroleum

[J. S. Wilson and L. M. Holland (LS-DO)]

Conducting in vivo tumorigenesis assays with high-viscosity, complex hydrocarbon mixtures required that the mixtures be diluted. These mixtures were samples of process-specific crude shale oils or natural petroleum. Two problems arose: 1) several of the compounds were semi-solid at room temperature while others were liquid, and 2) none were equally soluble in several test solvents. Most of our current experiments use a mixture of 30% cyclohexane - 70% acetone as the solvent/vehicle. This vehicle was neither irritating to the skin nor tumorigenic when applied three times per week for 78 wk. However, the four test oils proved to be variably soluble in this vehicle, and the two shale oils were more soluble than either of the natural petroleum. Further, in dose-response studies, the shale oils had proven more tumorigenic and cytotoxic than the natural petroleum.¹ This study was conducted in order to assess the possible contribution of solubility to overall toxicity and tumorigenesis.

Each of the test oils (Table I) was mixed in each of three vehicles: acetone, cyclohexane, and a mixture of 30% cyclohexane - 70% acetone. All of the final samples represented 10% oil - 90% vehicle mixtures. The suspension solutions were deposited on the intrascapular area of C3Hf/Bd mice. Treatment groups were composed of 20 animals, 10 males and 10 females, per oil sample. The treatment area was clipped as necessary to remove hair. Animals were housed five per cage on aspen shavings with food and water ad libitum. The materials were

applied in 50- μ l doses, three times each week for 78 wk.

Based on physical appearance, PCSO II is apparently more soluble in acetone than is OCSO #6, which in turn is more soluble in acetone than is LCO, with KCO being the least soluble in this vehicle. All oils appear to be uniformly soluble in cyclohexane; however, after four days the KCO samples leave a coating deposited on the bottom and sides of the sample vials, and after seven days, LCO leaves a similar coating. As a result, new solutions were made weekly. An assay of weight-percent solubility showed these observations to be accurate (Table II).

The dose of oil applied in this study had been demonstrated in previous experiments with all oils to be tumorigenic to varying degrees. With the exception of OCSO #6, there was also little cytotoxicity or irritation accompanying epidermal exposure to these 10% concentrations. Control groups treated with 100% acetone failed to express any tumorigenesis or cytotoxicity (Table II). However, the cyclohexane alone caused a persistent low-grade dermatitis and one animal developed a papilloma after 268 days. Both shale oils expressed differing degrees of tumorigenesis and cytotoxicity (Table II). Cytotoxicity was expressed as epidermal degeneration (ED). Mice afflicted with ED had lesions at the site of application which appeared as denuded, non-healing, moist patches. Microscopically, these areas lacked epithelium and exhibited inflammatory, granulomatous responses in the dermis.

PCSO II was more tumorigenic than the OCSO #6 in all three solvents. In fact, each oil was

TABLE I
IDENTIFICATION OF THE TEST OILS

<u>Designation for this study</u>	<u>Repository designation</u>	<u>Process</u>	<u>Point of origin</u>
PCSO II	29 May 79.601	Paraho - above-ground retort	Paraho - Anvil Points, Colorado
OCSO #6	6 March 79.100	Modified <u>in situ</u> retort	Occidental - Logan Wash, Colorado
KCO	API #1	Natural petroleum	Kuwait
LCO	API #2	Natural petroleum	Louisiana

TABLE II
INCIDENCE OF TUMORS OR ED AFTER 500 DAYS OF APPLICATION
OF TEST OILS IN THREE SOLVENTS

<u>Oil</u>	<u>Wt. Percentage Dissolved</u>	<u>Percentage Mice with Tumors @ 500 Days</u>	<u>Percentage Mice with ED @ 500 Days</u>	<u>Earliest Tumor (days)</u>	<u>Mean Latency (days)</u>
<u>Acetone</u>					
PCSO II	83.6	95	5	130	282
OCSO #6	81.0	80	20	147	280
KCO	50.6	15	0	291	404
LCO	54.3	40	0	217	373
Solvent		0	0		
<u>Cyclohexane</u>					
PCSO II	85.9	90	5	114	257
OCSO #6	91.7	70	15	198	306
KCO	76.4	5	0	470	470
LCO	75.2	45	0	276	386
Solvent		5	10	268	268
<u>Mixture (30% cyclohexane - 70% acetone)</u>					
PCSO II	102.0	95.0	10.0	129	284
OCSO #6	91.1	82.5	37.5	145	302
KCO	76.6	7.5	0	346	473
LCO	74.1	42.5	0	168	378
Solvent		0	0		

relatively consistent in its tumorigenic potency regardless of the solvent: PCSO II dissolved in acetone caused tumors in 95% of the mice, while in cyclohexane or the mixture, 90% and 95% of the mice, respectively, developed tumors. LCO in acetone caused 40% of the mice to develop tumors, in cyclohexane 45% and in the mixture 42.5%. KCO in acetone, the solvent in which it is least soluble, caused 15% of the mice to develop tumors. This was twice the value for KCO in the mixture (7.5%) and three times the value when cyclohexane was the vehicle (5%). OCSO #6 behaved similarly to PCSO II.

Without exact chemical analyses of these samples it is difficult to speculate on the specific mobilization of tumorigenic components of each oil in each test combination. However, it is evident that there is sufficient suspension or solution of single or multiple tumorigenic components to produce a relatively stable response even with conditions of inhomogeneous mixing. These data demonstrate that the degree of solubility of the test oils in the carrier has minimal effect on the overall observations of tumorigenesis. Previous dose-response studies had shown PCSO II to be the most tumorigenic followed by OCSO #6, LCO and KCO, in that order. This was also evident in this study. The two individual solvents did not affect this

relative tumorigenesis among the four oils. However, there were changes in late cy and cytotoxicity.

When PCSO II is most soluble (in the mixed vehicle), cytotoxicity is increased. OCSO #6 is almost equally soluble in the mixture and cyclohexane alone, but again, cytotoxicity is most evident with the mixture. Tumorigenic potency for this oil is lowest in cyclohexane; however, cytotoxicity is also suppressed and time to earliest tumor is delayed, although mean latency is not significantly different. KCO is least soluble in acetone, yet in this solvent it has a higher tumor incidence than in the other vehicles and the shortest latency. It seems apparent that the solubility of these test oils in these vehicles has an effect on cytotoxicity and tumor latency but minimal effect on overall tumor incidence. More information is needed to explain whether these are synergistic effects of the oil-solvent complexes or whether there is selective mobilization by the respective vehicles.

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Dose-Response Study for Three Shale Crude Oils and Two Natural Petroleum

[L. M. Holland (LS-DO) and J. S. Wilson]

Historically, crude shale oils have proved to be irritating and carcinogenic.¹ While there is no single American commercial shale oil operation presently in production, several process-specific shale crude oils are available from pilot operations. One of these is an oil from the Paraho aboveground, gas-combustion retort process at Anvil Points, Colorado. Samples of this crude oil and its hydrotreated daughter product were obtained from the Oak Ridge National Laboratory Repository. The Occidental Oil Company also provided samples from their modified in situ process at Logan Wash, Colorado. One of these crudes is also available from the repository (Table I). The data presented here are for mice exposed by epidermal application to aliquots of these samples. For comparison to the crude shale oils, two natural petroleum and a known skin carcinogen, benzo(a)pyrene (BaP), were also tested.

The mouse epidermal carcinogenesis assay has been used extensively to evaluate carcinogenic potency for many compounds.²⁻⁷ Male and female C3Hf/He mice were individually identified with ear tags and were introduced into the experiment at 10 wk of age. The intrascapular area of each animal was clipped (Oster small-animal clippers, #40 blade) 1 to 2 days prior to the first oil application. Thereafter, the treatment area was clipped as necessary to remove excessive hair growth. The test

materials were delivered to the skin with a 50- μ l automatic, fixed-volume pipette. The solvent used was a mixture of 70% acetone - 30% cyclohexane. This solvent was applied to a group of control mice to test its carcinogenic-irritant properties. Additional control groups consisted of untreated colony animals and periodically shaved animals. The various doses applied were based on weight-volume dilutions of oil and solvent (Table II). Positive control groups with two doses of benzo(a)pyrene dissolved in the acetone-cyclohexane solvent were included. Because of variable solubility in the solvent, the oil samples were made fresh weekly and warmed gently at the time of application.

The parameters studied included tumor incidence and latency resulting from various concentrations of each test material. From these data, the relative tumorigenic potency of the shale oils and the natural petroleum was determined (Table III).

No epidermal tumors occurred in any of the control animals, and no epidermal tumors were observed in animals treated with any of the oils at concentrations less than 1% (0.5 mg) (data not shown). All the shale oils elicited tumorigenic responses at 2.5 mg (5% oil) per application (Fig. 1A). The two natural petroleum failed to induce tumors at doses less than 5 mg (10% oil). Figures 1B and 1D show the incidence and latency observed for each natural petroleum at two doses. Benzo(a)pyrene was 100% tumorigenic at the doses employed (2 μ g and 10 μ g) while exhibiting a dose-related difference in latency (Fig. 1E).

TABLE I
CHARACTERIZATION OF CRUDE SHALE OILS AND NATURAL PETROLEUMS

<u>Sample</u>	<u>Process</u>	<u>Origin</u>	<u>Repository Registration Designation</u>
PCSO II	Gas-combustion, aboveground retort	Paraho, Anvil Points, Colorado	PHRR CDOL 29 May 79.601
PCSO UP	Gas combustion, aboveground retort (hydrotreated)	Paraho, Anvil Points, Colorado	PHRR CDOL 29 May 79.502
OCSO #6	Modified <u>in situ</u>	Occidental, Logan Wash, Colorado	OCCO CDOL 6 Mar 79.100
KCO	Blended natural petroleum	Kuwait	API #1 reference oil
LCO	Blended natural petroleum	Louisiana	API #2 reference oil

TABLE II
PROTOCOL FOR DOSE-RESPONSE STUDY

Agent	mg	Dose Percent Oil	Total weekly Dose - mg
PCSO II	0.5	1.0	1.5
	2.5	5.0	7.5
	5.0	10.0	15.0
	7.5	15.0	22.5
	10.0	20.0	30.0
OSCSO #6	0.5	1.0	1.5
	2.5	5.0	7.5
	5.0	10.0	15.0
	7.5	15.0	22.5
	10.0	20.0	30.0
PCSO UP	2.5	5.0	7.5
	5.0	10.0	15.0
	7.5	15.0	22.5
	10.0	20.0	30.0
KCO (API #1)	0.01	0.02	0.03
	0.05	0.1	0.15
	0.5	1.0	1.5
	5.0	10.0	15.0
LCO (API #2)	0.01	0.02	0.03
	0.05	0.1	0.15
	0.5	1.0	1.5
	5.0	10.0	15.0
	10.0	20.0	30.0
Solvent	-	-	-
BaP	0.002		0.006
	0.010		0.030

The aberrant tumor incidences and latencies for the higher doses (7.5 mg and 10 mg) are due to the interference with tumorigenesis by cytotoxicity (Fig. 1 C,D). The three shale oils showed different degrees of cytotoxic potency on the epidermis of exposed animals, but the natural petroleum failed to demonstrate cytotoxicity except in one mouse (Table IV). Cytotoxicity, expressed as epidermal degeneration (ED), appeared as a complete denuding of the treated area. These lesions were non-healing during (and often after) cessation of treatment.

Comparing the tumorigenic potency of the samples among themselves clearly shows the shale oils to be more potent than the natural petroleum. The two process-specific, unaltered shale crudes also differ in tumorigenic and cytotoxic potency when compared with each other. The PCSO II appears to be more tumorigenic than the OCSO #6. The latter oil is, however, more cytotoxic. The hydrotreated daughter product of the PCSO, referred to as PCSO UP, is definitely less tumorigenic but more cytotoxic than the parent oil.

Among the more important results of these studies is the demonstration of a process-dependent difference among crude shale oils. Because several

TABLE III
TUMOR FREQUENCY AND LATENCY FOR THE OILS
TESTED AT FOUR DOSES

Oil	Dose	Frequency	Percent of Mice with Tumors @ 700 Days	Earliest Tumor (days)	Mean Latency (days)
PCSO II	2.5 mg	3x/wk	80.0 ^a	135	278 ± 97
PCSO UP	2.5 mg	3x/wk	27.5 ^a	241	232 ± 66
OCSO #6	2.5 mg	3x/wk	57.5 ^a	178	284 ± 74
PCSO II	5.0 mg	3x/wk	97.0	129	284 ± 78
PCSO UP	5.0 mg	3x/wk	60.0	115	433 ± 112
OCSO #6	5.0 mg	3x/wk	85.0	145	306 ± 97
LCO	5.0 mg	3x/wk	62.5	168	467 ± 112
KCO	5.0 mg	3x/wk	10.0	450	504 ± 49
PCSO II	7.5 mg	3x/wk	87.5 ^a	137	258 ± 80
PCSO UP	7.5 mg	3x/wk	20.0 ^a	66	236 ± 92
OCSO #6	7.5 mg	3x/wk	45.0 ^a	141	273 ± 96
PCSO II	10.0 mg	3x/wk	72.5	66	250 ± 77
PCSO UP	10.0 mg	3x/wk	10.0	161	242 ± 97
OCSO #6	10.0 mg	3x/wk	27.5	118	265 ± 99
LCO	10.0 mg	3x/wk	82.5	293	467 ± 112

^aData are only current to 535 days.

Fig. 1. Comparisons of latency and frequency of tumors for four doses of the test oils (A, B, C, D) and BaP (E). (● = OCSO #6, ■ = PCSO II, ▲ = PCSO UP, ○ = KCO, □ = LCO, △ = BaP @ 2 µg/3x/wk, ⋈ = BaP @ 10 µg/3x/wk).

TABLE IV
OCCURRENCE OF EPIDERMAL DEGENERATION (ED) FOR ALL OILS TESTED AT FOUR DOSES

<u>Oil</u>	<u>Dose</u>	<u>Frequency</u>	<u>Percentage of Mice with ED @ 700 Days</u>	<u>Earliest ED Lesion (days)</u>	<u>Mean Latency (days)</u>
PCSO II	2.5 mg	3x/wk	2.5	401	-
PCSO UP	2.5 mg	3x/wk	7.5	144	246 ± 98
OCSO #6	2.5 mg	3x/wk	2.5	304	-
PCSO II	5.0 mg	3x/wk	10.0	242	320 ± 53
PCSO UP	5.0 mg	3x/wk	15.0	298	454 ± 96
OCSO #6	5.0 mg	3x/wk	37.5	166	287 ± 51
KCO	5.0 mg	3x/wk	0	-	-
LCO	5.0 mg	3x/wk	2.5	508	-
PCSO II	7.5 mg	3x/wk	2.5	133	246 ± 81
PCSO UP	7.5 mg	3x/wk	50.0	193	323 ± 57
OCSO #6	7.5 mg	3x/wk	72.5	122	211 ± 98
PCSO II	10.0 mg	3x/wk	27.5	156	235 ± 79
PCSO UP	10.0 mg	3x/wk	92.5	105	209 ± 115
OCSO #6	10.0 mg	3x/wk	82.5	94	149 ± 59
LCO	10.0 mg	3x/wk	0	-	-

different retorting techniques are proposed for development in the United States, the range of this apparent process specificity remains to be defined. True definition can only come from comparison of results from highly defined oils produced under carefully controlled conditions with field samples from pilot and commercial scale retorts. These experiments also demonstrate the effectiveness of upgrading (hydrotreatment) in reducing the tumorigenic potency of one shale oil. Whether this reduction in potency is a uniform effect unrelated to the activity of the original crude oil remains to be demonstrated.

Although the mouse is not the usual model for studies of skin irritation, the differences in inflammatory actions of these oils suggests that benign dermatoses may be an important process-specific industrial hazard.

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Inhalation Studies

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The long-term, high-concentration inhalation studies of shale dusts that were reported in progress last year are nearing completion¹. These experiments utilize female Fischer 344 rats exposed to high concentrations of either Anvil Points raw shale, Paraho spent shale (150 mg/m³), or quartz (15 mg/m³) (Table I). Each exposure group included 62 animals exposed on 4 days each week for 6 h. Exposures were stopped after 24 months. Lower concentration (5 and 10 mg/m³) experiments are in progress and are now approaching 12 months of exposure. Most of the animals in these groups are still alive. Based on our previous experiments with Syrian hamsters exposed by inhalation and intratracheal instillation to the same dusts we anticipated severe pulmonary fibrosis with extensive lung scarring. In order to assess the degree of fibrosis in the current experiments, several of the weaker animals were sacrificed shortly after exposures were terminated. Histopathological examination of tissues taken at this time revealed multiple indications of fibrosis in animals from each exposure group. In addition to the anticipated fibrotic changes, lungs from several animals in each group contained primary tumors (Table II). Only one benign tumor has been observed in the untreated controls. The earliest tumors were observed after

17 months of exposure indicating the lateness of the tumorigenic phenomenon.

Histopathological analysis of all tissues from all animals is incomplete and the number of animals examined in each group varies. Because of this, the comparative incidence of lung tumor among the exposure groups cannot yet be established. The tumor type most frequently observed in all groups was adenocarcinoma with adenoma the next most frequent. Three squamous cell carcinomas have been observed so far with no apparent relationship to exposure group (Fig. 1). Adenomatous tumors are usually considered to arise in the peripheral portions of the lung and our observations are consistent with this conclusion. Many of the tumors occurring in this study were located near the pleural surface and usually in association with advanced fibrotic changes. The observations are consistent with the phenomenon of lung-scar cancer. Most of the animals examined showed evidence of moderate to severe fibrotic change (Fig. 2). This ranged from diffuse thickening of interalveolar tissues to advanced fibrosis with mature deposition of collagen. The fibrotic changes are described elsewhere in this report (see Halleck *et al.*)

The aerosol concentrations used in this study are clearly beyond any anticipated exposure in the industrial setting and were designed to achieve a maximum fibrotic effect. The concentration established for the quartz exposures was chosen to fall in the range of the assumed free silica content

TABLE I
INHALATION EXPOSURE PROTOCOLS^a

Exposure Material	Animals Female Fischer 344 Rats	Exposure Frequency	Aerosol Concentration	Duration of Exposure
Anvil Points Raw Shale	62	6 h/day 4 days/wk	150 mg/m ³	24 months
Paraho Spent Shale	62	6 h/day 4 days/wk	150 mg/m ³	24 months
Quartz	62	6 h/day	15 mg/m ³	24 months
Controls ^b	62	-----	-----	24 months

^aNose-only exposure.

^bNon-exposed.

TABLE II
PATHOLOGICAL CHANGES IN LUNGS OF EXPOSED RATS^a

Exposure Material	Number Examined Microscopically ^b	Number with Fibrotic Change	Number with Lung Tumors ^c	
			Benign	Malignant
Anvil Points Raw Shale	37	23	0	5
Paraho Spent Shale	50	26	3	5
Quartz	38	31	3	8
Controls	22	0	0	0

^aAll animals examined to date. Includes animals dying during exposure period and those sacrificed after exposures terminated.

^bComprehensive microscopic examination incomplete.

^cTumors observed in other tissues: Raw Shale, 5; Spent shale, 2; Quartz, 2; Control, 0.

Fig. 1 Photomicrograph of a section of squamous cell carcinoma in the lung of a Fischer 344 rat exposed to high concentrations of raw oil shale dust. Abundant keratin production (K) and dust laden macrophages (M) are adjacent to the stratified squamous epithelium. Fibrotic areas (F) are in close proximity to the tumor (H & E x 400).

Fig. 2 Photomicrograph of a section of lung from a Fischer 344 rat exposed to high concentrations of spent shale dust. The picture represents the edge of a pneumoconiotic lesion with consolidating fibrosis (F) intimately associated with prominent bronchiolar alveolar hyperplasia (BAH), which is considered by some to be a precursor to adenocarcinoma of the lung. The dark amorphous foci in alveoli are agglomerations of spent shale dust (H & E x 400).

of the shale materials. On this basis, at an assumed 10% free silica content for the shale materials, the quartz aerosol concentration was established at 15 mg/m³ (10% of the 150 mg/m³ used for the shale materials). These concentrations are at least 10-20 times the levels that might be expected in the industrial setting even for short-term exposures. Some of the animals in each exposure group are still alive; however, a planned sacrifice schedule is now being employed. Based on current data, we believe that these high exposure levels have led to a lung-scar cancer phenomenon. This conclusion is supported by the high incidence of lung tumors in the quartz exposed group. If this conclusion, which is based on partial data, is correct, the tumors observed in these experiments may be a general sequel of advanced pulmonary fibrosis rather than a result of exposure to specific carcinogens thought to be in shale materials. Lung-scar cancer is a largely anecdotal phenomenon frequently reported from observation of human autopsy material.^{2,3,4} A temporal relationship between the occurrence of fibrosis and lung carcinoma has not been established.⁵ Most of the evidence points to a pre-existing scar which becomes the focus of a neoplastic process; however, there is some evidence that suggests that pulmonary neoplasms, particularly adenocarcinomas, create a localized fibrotic response during active neoplasia. To our knowledge an animal model for lung-scar cancer has never been developed. If, indeed, the presence of active diffuse fibrosis is a factor in neoplasia of the lung due to a variety of environmental agents (e.g., organics, radiation, smoking), the approach used here could be employed in definitive studies of lung-scar cancer.

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The Effect of Shale Oil and Ultraviolet Light on Delayed Hypersensitivity in Mice

[P. A. Baron]

The topical application of gas combustion surface-retorted shale oil (PCSO II) to the shaved backs of C3Hf/HeBD mice has been demonstrated to result in tumorigenesis in 100% of the experimental animals within 360 days.¹ It has also been shown that mice painted with PCSO II express a delayed type hypersensitivity (DTH) reaction which is suppressed by 58% relative to the response of the normal, untreated animals.²

DTH, which is a T-cell function, may be used to assess certain aspects of the immunological competence of mice since those T-cells involved in the DTH reaction may also play a role in the control of infectious disease and surveillance of tumor cells. DTH T-cells accomplish this by concentrating sensitized lymphocytes and their secreted substances and also activated macrophages at the site of attack, thus providing a hostile environment for those invading agents. This report describes ongoing observations on the effects of shale oil alone or in combination with environmental factors such as ultraviolet light in the 280 - 320 nm range (UVB), and also the near-ultraviolet light in the 320 - 400 nm range (UVA).

UVB is known to be carcinogenic and capable of (a) altering the structure of biological molecules and their biosynthetic rates, (b) perturbing the number and function of skin cells, and (c) causing a suppression of the normal DTH response. UVA comprises a large percentage of the ultraviolet spectrum of sunlight penetrating the earth's atmosphere. While it is generally accepted that normal levels of UVA energy are not harmful, abnormally high levels, long-term exposure, and interactions with sensitizing drugs and chemicals may cause skin or eye irritations. The question

addressed in this study is whether ultraviolet light alters the dermal:PCSO II interaction and induces a change in the DTH response. The relevance of this question becomes apparent when one considers workers who may come in direct contact with the oil and then be exposed to the additional influence of sunlight in the normal course of the day.

The methodology for quantitating the DTH response has been described previously.² Briefly, sensitivity to 2,4-dinitro-1-fluorobenzene (DNFB) is established by applying the antigen topically to the oil- or UV-treated area on two consecutive days. After four days, the same antigen is applied as a challenge at a different site (ear), and 48 h later, the degree of swelling in the ear due to induration, edema, and clonal expansion is measured and compared to control (neither oil- nor UV-treated) values.

Figure 1 indicates that when an animal is painted with 1.25 mg of PCSO II on days 1, 3, and 5, and also exposed to 1.0 or 2.5 min of UVB (8.5 Joules/m²/sec) on days 1 - 5, the DTH manifested by these animals is very similar to PCSO II alone. This was the case in all experiments run on this regimen, even though the data acquired for UVA and UVB alone in repeated experiments were somewhat variable (i.e., the absolute degree of suppression varied from experiment to experiment).

UVA adapted with a blue filter to absorb practically all visible light caused a slight

erythematous response in animals exposed for 30 min. This was attributed to the small fraction of light in the 300 - 320 nm range. Figure 2 shows that while both UVA (6.2 joules/m²/sec) treatment for 30 min on five consecutive days and PCSO II (1.25 mg on days 1, 3, and 5) suppressed the normal response by approximately 30%, the effect when these two parameters were combined was not significantly changed ($p < 0.25$, student's T test).

Alterations of DTH competence may be due to changes in the number or function of antigen-presenting cells at the experimental site or to a compromised whole body T-cell function. Figure 3 indicates that sensitization through the belly (untreated area) resulted in some suppression of DTH response relative to the untreated control, although the magnitude of the suppression was greatly reduced relative to that obtained through back (treated area) sensitization. This observation indicates that mice painted with shale oil or exposed to UV light exhibit an alteration of immune function which may be due to a change in either priming or expression of T-cells.

The question addressed in this report is whether ultraviolet light acting on or in combination with PCSO II results in a greater suppressive influence of the DTH response. The results presented in this report suggest that the combination of either UVA or UVB with PCSO II under the conditions described does not result in an additional deleterious effect above that associated with the shale oil alone.

Fig. 1. DTH in mice treated with UVB and/or PCSO II. Delayed hypersensitivity in mice was determined by measurement of ear swelling in response to DNFB sensitization and challenge. Mice were exposed to 1.0 or 2.5 min of UVB on five consecutive days or to 1.25 mg PCSO II on days 1, 3, and 5. Other mice were exposed to the combination of PCSO II and UVB.

Fig. 2. DTH in mice treated with UVA and/or PCSO II. Conditions as in Fig. 1 except that UVA was substituted for UVB.

Fig. 3. DTH in mice sensitized to DNFB through a treated site (back) or untreated site (belly). Experimental mice had their backs treated with 5 mg PCSO II on days 1, 3, and 5 or 10 min of UVB on days 1-5. Their backs or their bellies were then sensitized with DNFB and a comparison was made of the ear swelling. Untreated control animals exhibited no significant difference in response when sensitized on the back or belly (data not shown).

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Tumorigenesis in the Lung

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One of the first accomplishments of this program was to disprove the hot-particle theory of pulmonary carcinogenesis: focal intense sources of internally deposited, alpha-emitting irradiation were thought to be far more tumorigenic than the same amount of irradiation distributed uniformly throughout the lung. Our data demonstrated that, regardless of how intensely focal areas of the lung are subjected to radiation, significant numbers of primary lung tumors cannot be induced by internally deposited radionuclides unless at least 20% of the total lung mass is irradiated. Ten-micron diameter ZrO_2 microspheres containing $^{238}PuO_2$ or $^{239}PuO_2$,

when injected intravenously (IV) in the Syrian hamster, lodge quantitatively in the pulmonary capillary bed and induce lung tumors in approximately 1% of the animals at risk. This is independent of the number of microspheres or total lung burden unless greater than 20% of the lung is irradiated.¹

Our next objectives were to: 1) delineate the role of enhancing agents in radiation-induced lung carcinogenesis; 2) examine the effect of the radionuclide matrix on carcinogenesis; 3) determine the effects of combined alpha- and beta-radiation exposures to the lung; and 4) establish underlying mechanisms as they relate to a) bronchial epithelial-cell kinetics and lung-cell DNA synthesis and b) alveolar macrophage numbers and function following exposure to radiation and during tumor development and growth in the respiratory tract.

Because such seemingly innocuous substances as iron oxide and physiological saline, when instilled intratracheally (IT) after the pulmonary deposition of alpha emitters, dramatically increase the induction of lung tumors,^{1,2} we initiated a series of experiments to determine whether ZrO_2 instilled IT following IV or IT injection of PuO_2/ZrO_2 microspheres affected tumor yields (Table I). When the PuO_2/ZrO_2 microspheres were administered IV with no additional treatment, 2.5% of the animals developed tumors, as anticipated from previous studies.^{1,3} Ten instillations IT of saline increased the tumor incidence to 16% while 10 instillations IT of 0.3 mg ZrO_2 suspended in saline resulted in a tumor yield of 12%. When the microspheres were injected IT (Table I), 10 doses of saline IT resulted in 52% of the animals having lung tumors, while 25% of the animals receiving the ZrO_2 IT had lung tumors. None of the animals receiving spheres IT only developed lung tumors. The enhancement of tumorigenesis with saline IT was consistent with our previous experience.² ZrO_2 given IT was not as effective in promoting lung tumor development. This strengthens our belief that ZrO_2 is relatively inert and a suitable matrix for examining the effects of "pure" irradiation on the lung (i.e., it has minimal chemical or physical effects on cells). It could be suggested that, since fewer tumors resulted in animals receiving the ZrO_2 IT compared to the saline alone IT, ZrO_2

TABLE I
EFFECT OF IT SALINE AND ZrO_2 ON PuO_2/ZrO_2 LUNG TUMOR

PM-EW	Group designation	Number of hamsters		Microsphere route ^a	Supplemental insult	Mds (DPE) ^d		Degenerative lesions of lungs			Lung Tumors		
		Male	Female			Male	Female	Pneumonia	Pneumonitis	Fibrosis	BAL ^e	Total	ACA ^f
12-108	-	14	28	IV	-	385	512	15%	21%	3%	3%	2.5%	1/3 (100%)
12-109	-	25	23	IV	Saline x 10 ^b	392	370	0%	13%	0%	29%	16.0%	5/6 (83%)
12-110	-	24	26	IV	ZrO_2 x 10 ^c	462	321	5%	19%	0%	5%	12.0%	5/5 (100%)
12-111	-	23	18	IT	-	468	402	9%	6%	21%	9%	0%	-
12-112	-	23	23	IT	Saline x 10 ^b	506	380	5%	15%	20%	25%	52.0%	21/22 (95%)
12-113	-	22	28	IT	ZrO_2 x 10 ^c	498	355	70%	3%	28%	28%	25.0%	13/13 (100%)
0-30	-	25	25	-	-	484	515	2%	9%	0%	2%	0%	-

LUNG TUMOR INDUCTION BY INHALATION

PM-EW	Group designation	Number of hamsters		Aerosols		Revised initial lung burden	Aerosol characteristics		Mds (DPE)		Degenerative lesions of lungs			Lung Tumors		
		Male	Female	²³² ThO ₂	²³⁸ Pu(NO ₃) ₃		AMAD ^h	LG ⁱ	Male	Female	Pneumonia	Pneumonitis	Fibrosis	BAL ^e	Total	ACA ^f
3-4	P	32	35	-	-	-	-	-	534	439	9%	0%	0%	1%	0%	-
13-11	Q	35	37	12.2 mg/m ³	0.85 nCi/m ³	180	0.7	2.0	147	144	0%	10%	76%	14%	12.5%	9/9 (100%)
13-12	R	33	29	12.2 mg/m ³	1.71 nCi/m ³	300	1.2	1.9	87	75	0%	15%	60%	0%	6.0%	1/4 (25%)
13-10	S	36	35	12.2 mg/m ³	1.20 nCi/m ³	40	1.0	1.7	283	227	3%	16%	63%	12%	9.5%	4/6 (67%)
13-14	W	27	45	12.2 mg/m ³	0.84 nCi/m ³	90	1.5	2.0	221	203	9%	14%	69%	12%	4.0%	3/3 (100%)

^a 30 000, lung burden = 56 nCi given when hamsters w100 days old.

^b 0.2 ml 0.15 M NaCl IT once a week x 10 weeks.

^c 0.3 mg suspended in 0.2 ml saline IT once a week x 10 weeks.

^d Mean life spans, days post exposure.

^e Bronchiolar adenomatoid lesion.

^f Adenomas.

^g Adenocarcinomas.

^h Activity median aerodynamic diameter.

ⁱ Sigma g.

particles may afford some protective factor against radiation carcinogenesis—perhaps by stimulating the reticuloendothelial and/or immune systems. Of course, this is only speculative and would need further study to be substantiated.

In another endeavor to look at the effects of particle matrix, Syrian hamsters were exposed to inhaled $^{232}\text{ThO}_2$ and $^{238}\text{PuO}_2$. These exposures mimicked possible releases during a reactor incident involving Th and Pu in the reactor core and also utilized an element (Th) resembling Zr chemically as a matrix for the plutonium. The thorium was a preparation of the oxide that had a concentration of 0.45 mg/ml. The plutonium was in dilute nitric acid. The colloidal ThO_2 and $\text{Pu}(\text{NO}_3)_4$ were mixed together in the aerosol generator and diluted to 4 ml with distilled water. The final ThO_2 and $\text{Pu}(\text{NO}_3)_4$ concentrations are given in Table I.

All of the Th-Pu exposed animals had dramatically shortened life spans compared to controls (Table I). The lung-tumor yields in the experimental groups ranged from 4% (group W) to 12.5% (group Q) and resemble those seen when hamsters are exposed to similar lung burdens of inhaled $^{239}\text{PuO}_2$.⁴ Fibrosis occurred in those animals at frequencies similar to those exposed to inhaled $^{239}\text{PuO}_2$.⁴ Group R (300-nCi lung burden) had two hamsters that developed malignant adenocarcinomas of the lung shortly after exposure; one died only 64 days post exposure. This latent period is much shorter than that associated with inhaled $\text{PuO}_2/\text{ZrO}_2$ or $^{239}\text{PuO}_2$ -induced tumors.^{1,4} The dramatic shortening of life spans and apparent shorter tumor latency periods in $\text{ThO}_2\text{-Pu}(\text{NO}_3)_4$ -exposed animals suggests that ThO_2 may also have a chemical or physical effect (unlike ZrO_2) that is synergistic with radiation effects.

A summary of experiments combining alpha- and beta-irradiation exposures to the lung is given in Table II. These studies are still in progress; however, it is already apparent that combining Pu exposures with Pm exposures shortens lifespans compared to unmanipulated controls and does result in lung-tumor induction. Undoubtedly, because animals that live longer have higher proclivities to develop tumors, the incidences of pulmonary neoplasms will increase in these experimental groups as more of the older animals are examined

microscopically. Combined Pu-Pm-induced pulmonary disease and the role each plays will be further illuminated when the aerosol experiments initiated in 1981² are completed and their results analyzed along with the results from the experiments in Table II. The 1981 experiments will be valuable because they include combinations of lower doses of both Pu and Pm.

Cell kinetics in bronchial epithelium following insult are being monitored by a [^3H]-thymidine DNA incorporation assay⁵ and via autoradiography. As a pilot experiment to test the system prior to working with internally deposited radionuclides, Syrian hamsters were randomized to one of three groups to receive either a) five instillations IT at weekly intervals of 5 mg 3-methylcholanthrene (3-MCA) suspended in 0.2 ml 0.1% gelatin; b) five instillation IT at weekly intervals of 0.2 ml 0.1% gelatin; or c) no treatment (controls) as shown in Table III. Animals from each group were then given [^3H]-thymidine parenterally at 7, 28, 60, or 90 days after the last instillation and the incorporation of the labeled nucleoside into lung cell DNA was measured. Control animals and animals instilled once with 0.1% gelatin were also given [^3H]-thymidine parenterally and the amount of label incorporated into lung-cell DNA was measured after the lungs had been either frozen or fixed in 2% glutaraldehyde (Table III).

Neither freezing lungs nor fixing them in 2% glutaraldehyde had any significant effect on [^3H]-thymidine incorporation into the DNA of lung cells (Table III). There were no gross differences in [^3H]-thymidine incorporation in the lungs of animals treated with 3-MCA at either days 7, 28, 60, or 90. This is not surprising since the latency period for this material to induce lung tumors is generally > 100 days, and the resolution of our assay may be insufficient to detect early, subtle changes in [^3H]-thymidine incorporation into DNA. Also, it was not possible to determine any significant differences in [^3H]-thymidine incorporation in 3-MCA treated hamsters compared to gelatin-instilled hamsters or control hamsters.

Considerable effort was spent in establishing methodologies to collect and culture hamster

TABLE II
1980 EXPOSURE SUMMARY AS OF 12 NOVEMBER 1981^a

PN-EN ^b	Cn ^c	To ^d	Pu(nCi)	Pm(nCi)	MdLs (DPE) ^e	De ^f	%S ^g	Degenerative lesions		Histology ^h % with	
								Pneumonitis	Fibrosis	BAL ⁱ	Tumors
0-37	C1	02 Jan 80	-	-	406	688	17	2%	2%	2%	0%
13-40	C2	15 Jan 80	60	35	266	675	4	13%	84%	37%	15%
13-41	C3	05 Feb 80	120	-	315	655	10	-----In progress-----			
13-42	C4	19 Feb 80	240	-	147	640	4	-----In progress-----			
13-43	C5	04 Mar 80	30	-	421	626	13	-----In progress-----			
13-44	C6	20 Mar 80	90	-	217	610	9	-----In progress-----			
13-45	C7	04 Apr 80	150	-	117	591	3	-----In progress-----			
13-46	C8	22 Apr 80	180	-	82	392	0	-----In progress-----			
13-47	C9	06 May 80	-	3500	518	563	43	20%	71%	24%	5%
0-38	C10	20 May 80	-	-	490	549	31	-----In progress-----			
13-48	C11	03 Jun 80	60	7000	245	535	6	9%	80%	40%	3%
13-50	C12	17 Jun 80	90	7000	170	521	11	15%	66%	17%	5%
13-49	C13	08 Jun 80	90	10000	150	500	4	-----In progress-----			
13-51	C14	22 Jun 80	-	10000	438	486	43	-----In progress-----			
13-52	C15	26 Aug 80	20	-	>452	452	56	-----In progress-----			
0-40	C16	16 Sep 80	-	-	>425	425	61	-----In progress-----			
13-53	C17	30 Sep 80	20	-	>411	411	61	-----In progress-----			
13-54	C18	13 Nov 80	15	-	>364	364	83	-----In progress-----			
13-55	C19	09 Dec 80	15	-	>347	347	77	-----In progress-----			

^a ~70 animals/group.

^b Project number, experiment number.

^c Group code.

^d Date of initiation.

^e Median life span (days post exposure).

^f Days elapsed.

^g Percent surviving.

^h Percentages reflect available data at time of analysis and may change when all animals enrolled in the project are examined.

ⁱ Bronchiolar alveolar lesions.

TABLE III
[³H]THYMIDINE CPM/MICROGRAM DNA

<u>Lung Tissue Treatment</u>	<u>CPM^a</u>
<u>Control animals</u>	
Fresh	12.8 (1.0)
Fixed in 2% glutaraldehyde	17.5 (7.7)
<u>Animals instilled IT once with 0.2 ml 0.1 % gelatin</u>	
Fresh	22.5 (11.8)
Frozen at -10°C	20.9 (17.6)
<u>Animals instilled IT 5x with 5 mg 3-MCA suspended in 0.1% gelatin</u>	
T ₇	16.1 (10.5)
T ₂₈	9.4 (5.3)
T ₆₀	11.2 (1.0)
T ₉₀	19.9 (2.2)
<u>Animals instilled IT 5x with 0.2 ml 0.1% gelatin</u>	
T ₇	8.4
T ₂₈	12.9 (6.3)
T ₆₀	9.0
T ₉₀	30.4
<u>Controls</u>	
T ₇	22.2
T ₂₈	15.1 (5.3)
T ₆₀	—
T ₉₀	14.9

^a Numbers in brackets represent standard deviations.

TABLE IV
NUMBERS OF ALVEOLAR MACROPHAGES OBTAINED
VIA BRONCHOPULMONARY LAVAGE

<u>Group Designation</u>	<u>Aerosol</u>	<u>Supplemental Insult</u>	<u>Lavaged cells (x10⁶)</u>			
			<u>T₀</u>	<u>T₇</u>	<u>T₃₀</u>	<u>T₆₀</u>
M1	⁵⁷ Co	—	5.3	5.5	11.4	14.5
M1	⁵⁷ Co	Saline x 10	5.3	5.5	12.1	18.5
M2	²³⁸ PuO ₂ /ZrO ₂ (100 nCi)	—	7.0	4.9	5.5	5.7
M2	²³⁸ PuO ₂ /ZrO ₂ (100 nCi)	Saline x 10	7.0	7.6	8.7	10.9
M3	²³⁹ PuO ₂ (90 nCi)	—	7.1	7.6	5.2	— ^a
M3	²³⁹ PuO ₂ (90 nCi)	Saline x 10	7.1	12.3	4.1	— ^a
M4	Control	—	4.2	6.2	8.7	8.3
M4	Control	Saline x 10	4.2	7.5	8.0	18.7

^a Experimental point not yet reached.

alveolar macrophages and monitor their function. This proved to be an unusually difficult task requiring many attempts and experimental manipulations. In our experience, hamster alveolar macrophages are readily collected via bronchopulmonary lavage ($\bar{x} = 7 \times 10^6$ from unmanipulated controls), but they are very difficult to maintain in a viable state in culture. Despite trying numerous culture media and experimental manipulations, we have not yet been successful in inducing them to divide and grow *in vitro*.

In an attempt to learn whether hamster alveolar macrophages respond differently to inhaled $^{238}\text{PuO}_2/\text{ZrO}_2$ particles (highly tumorigenic) compared to inhaled $^{239}\text{PuO}_2$ particles (poorly tumorigenic), groups of Syrian hamsters were exposed to inhaled ^{57}Co (a low-level gamma emitter used as a tag with Pu aerosols), $^{238}\text{PuO}_2/\text{ZrO}_2$ (100 nCi), or $^{239}\text{PuO}_2$ (90 nCi). One-half of the animals from each group were then given a series of 10 IT instillations of 0.2 mL of 0.15 M NaCl (Table IV). Animals from each group were anesthetized and alveolar macrophages obtained via lavage at days 0 (initiation), 7, 28, and 60. The numbers of alveolar macrophages harvested via lavage increased with time in the two groups of controls (M1 and M4). There were no apparent differences comparing yields from $^{238}\text{PuO}_2/\text{ZrO}_2$ -treated hamsters versus $^{239}\text{PuO}_2$ -treated hamsters, and the yields from these two groups did not increase with time post exposure. With only two exceptions, animals receiving saline IT instillations had higher macrophage numbers from lavage than their non-saline instilled counterparts (Table IV).

In the coming year, many of the tumor-enhancing studies will be completed and the data prepared for analyses and publication. Likewise, the matrix effect experiments will be continued along with the combined alpha and beta exposures. Our cellular-kinetic and DNA-synthesis work should start to provide assistance in understanding radionuclide-induced lung carcinogenesis, and it is expected that the macrophage studies will further elucidate the sequence of events resulting in tumor formation.

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Nickel Arsenide (Ni_3As_2): The Relationship Between Its Solubility and Its Toxicity and Mutagenicity

[L. R. Gurley, R. A. Tobey, J. G. Valdez, J. J. Miglio (CMB-1), S. H. Cox (LS-3), and S. S. Barham (Mayo Clinic/Foundation)]

Considerations of (1) oil shale retort operating conditions, (2) oil shale elemental composition, (3) nickel and arsenic physico-chemical properties, and (4) oil shale matrix structure have lead us to suggest that nickel arsenides may be formed during the oil shale retorting process.¹ Furthermore, crystal structure similarities between nickel subarsenide and the potent carcinogens nickel subsulfide² and nickel subselenimide have caused concern that nickel arsenides may have comparable effects on biological systems.¹ To determine whether fugitive nickel arsenides from an oil shale retort could pose a threat to personnel in the workplace or to other living organisms in the environment, a program to study the toxicity of nickel arsenides has been initiated. Six stable nickel arsenides (Ni_3As_2 , Ni_2As , $\text{Ni}_{11}\text{As}_8$, NiAs ,

NiAs_2 , and NiAsS) are possible species for consideration.¹

Studies have now been made on the toxicity of Ni_5As_2 . This metallic compound was synthesized by the J. Roy Gordon Research Laboratory of INCO Metals Company and ground to a fine powder having a modal size of $0.3\ \mu\text{m}$. Initial studies with this material indicated that, although it appeared to be essentially insoluble in water,³ Ni_5As_2 particles suspended with Chinese hamster (CHO) cells in culture were toxic to culture growth at suspension concentrations of $10\ \mu\text{M}$.⁴

Previous work in this Laboratory has shown that CHO cells will ingest both carcinogenic Ni_3S_2 particles and spent-shale particles having a size of $10\ \mu\text{m}$ or less.⁵ Thus, the possibility existed that the toxic effects of Ni_5As_2 on CHO cells might be associated with the phagocytosis of these particles. To determine whether this was the case, CHO cells were treated with Ni_5As_2 powder and then subjected to examination by electron microscopy. No Ni_5As_2 particles could be found in the thin sections of cells treated 8 h with 0-, 10-, 50-, or $100\text{-}\mu\text{M}$ Ni_5As_2 powder [Fig. 1 (A and B)], even though such concentrations were highly toxic.⁴ Thus, the toxicity of this metallic compound does not appear to result from the ingestion of these particles.

Particles were observed in cells treated with extremely high concentrations ($250\ \mu\text{M}$ and $500\ \mu\text{M}$) of Ni_5As_2 powder [Fig. 1 (C and D)]. However, the uniform size and shape of these particles compared with the irregular shape (Fig. 2A) and wide range of particle sizes ($0.18 - 1.40\ \mu\text{m}$ average diameter)⁴ in the Ni_5As_2 powder sample suggested some differences existed between the original particles and those observed in the cells. The electron microscopes used in these experiments were equipped with solid-state detectors which could measure the energy of x rays emitted from a sample when it is excited by the electron beam. An example of such a procedure is shown in Fig. 2, where a particle of Ni_5As_2 (Fig. 2A) is subjected to this microanalysis (Fig. 2B). When such x-ray microanalysis was applied to the particles inside the cells treated with high concentrations of Ni_5As_2 powder [Fig. 1 (C and D)], only nickel was measured; no arsenic was detectable.

Fig. 1. Transmission electron micrographs of CHO cells treated 8 h with particulate Ni_5As_2 . Samples of the treated cultures containing approximately 5×10^6 cells ($40\ \text{m}^2$) were centrifuged. The pelleted cells were washed once in saline-GM and fixed for 30 min in $2.5\ \text{m}^2$ of 1% glutaraldehyde/4% formaldehyde. The cells were removed from fixative by centrifugation, resuspended in phosphate buffer, and embedded for electron microscopy. Thin sections were cut and stained for transmission electron microscopy.⁷

- (A) $0\ \mu\text{M}$ Ni_5As_2 (control), 13,750 X.
- (B) $100\ \mu\text{M}$ Ni_5As_2 , 10,750 X.
- (C) $500\ \mu\text{M}$, intact cell, 22,500 X.
- (D) $500\ \mu\text{M}$, dead cell, 22,500 X.

Fig. 2. Example of the measurement of Ni and As in a nickel arsenide particle. (A) Scanning electron micrograph of an extracellular nickel arsenide particle recovered in the cell pellet following an 8-h treatment of CHO cells with particulate Ni_5As_2 . (B) Electron microscopy x-ray spectrum of the nickel arsenide particle shown in Fig. 2A showing the Ni- and As-emissions from the particle and the P-emissions from the phosphate buffer present in the culture medium.

Thus, these intracellular particles are not Ni_5As_2 particles, but rather some aggregate of metallic nickel or a nickel salt which has precipitated out of solution at nucleating centers in the cell when treated with high concentrations of Ni_5As_2 .

Ni_5As_2 particles exposed to F10 culture-growth medium or to cell cultures were also examined by

electron microscope x-ray microanalysis. From these extracellular particles, it was found that the surface of the particles was depleted in arsenic (Table I). Thus, the above observations (arsenic leaching in Table I, and independent nickel cellular deposition in Figs. 1C and 1D) suggest that both nickel and arsenic are solubilized in growth medium and one or both of their soluble products are responsible for the toxic effects of Ni_5As_2 .

To determine the true toxic level of the soluble product of Ni_5As_2 , culture medium was exposed to Ni_5As_2 powder for 25 h. The Ni_5As_2 particles were then removed and the soluble nickel and arsenic remaining in solution in the medium were measured by atomic absorption. The stoichiometry of nickel to arsenic remaining in solution was 5 to 2. The effect of the soluble Ni_5As_2 on CHO cell-culture growth was measured. Toxic effects were detectable below 1 ppm Ni_5As_2 (Fig. 3). Thus, even though this compound is not highly soluble, it is soluble enough to produce acutely toxic effects at very low concentrations.

Cell survival studies demonstrated that a 24-hr exposure of up to 5 μM (2.2 ppm) soluble Ni_5As_2 killed less than 20% of the CHO cells (Fig. 4A), even though these conditions had a dramatic inhibitory effect on the rates of cell division (Fig. 3). This caused us some concern, for if any genetic damage was caused by the Ni_5As_2 , it was sublethal and could be passed on to future cell generations.

To determine whether sublethal genetic damage (expressed as an increase in mutation frequency) was produced by Ni_5As_2 , CHO cells were exposed to soluble Ni_5As_2 for 24 h and then subjected to the Hypoxanthine-Guanine Phosphoribosyl Transferase (HGPRT) mutagenicity assay.⁶ We found that Ni_5As_2 did not cause any mutations at the HGPRT locus (Fig. 4B). The mutation frequency at a variety of loci must be measured before it can be conclusively stated that Ni_5As_2 is not mutagenic.

It is concluded from the results to date that the soluble produce of Ni_5As_2 is acutely toxic to cells at low concentrations (less than 1 ppm) but not mutagenic. These results will provide a comparison for similar studies of the toxic effects of nickel subarsenide ($\text{Ni}_{11}\text{As}_8$) which is a suspected carcinogen.¹ $\text{Ni}_{11}\text{As}_8$ has recently been synthesized by INCO Metals Co. for this study.

Fig. 3. Growth of CHO cells in the presence of the Ni_5As_2 -soluble product. F10 culture-growth medium was exposed to Ni_5As_2 powder for 24 h. The powder was removed by centrifugation and the concentration of Ni and As remaining in solution was determined by atomic absorption. F10 growth medium containing known concentrations of Ni and As were then prepared by dilution with untreated F10.

Fig. 4. Survival and mutation frequency of CHO cells following a 24-h exposure to the Ni_5As_2 soluble product. (A) Cell survival expressed as the ratio of surviving treated cells (S) to surviving untreated cells (S_0). Cells treated for 24 h with Ni_5As_2 were resuspended in fresh, untreated medium and plated for colony formation to measure cell survival. (B) Mutation frequency in CHO cells. Cells treated for 24 h with Ni_5As_2 were resuspended in fresh, untreated medium. The frequency of mutants in the surviving cells was measured at the HGPRT locus.

TABLE I
ELECTRON MICROSCOPIC X-RAY MICROANALYSIS OF
NICKEL ARSENIDE PARTICLES

Sample No.	Nickel Arsenide Treatment	EM method of Microanalysis	Number of Samples	Average Ni/As Atomic Ratio
1	Dry Ni_5As_2 powder (untreated)	Transmission EM	(2)	2.595
2	Ni_5As_2 powder carried through the EM preparation process	Transmission EM	(2)	2.605
3	Ni_5As_2 particles exposed to cell cultures	Scanning EM	(2)	3.02
4	Dry Ni_5As_2 powder (untreated)	Transmission EM	(5)	2.55
5	Ni_5As_2 powder H_2O -washed	Transmission EM	(5)	2.41
6	Ni_5As_2 treated 24 h with F10 and H_2O -washed	Transmission EM	(5)	3.21

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Histone Variants and Histone Modifications Associated with Constitutive Heterochromatin

[L. R. Gurley, M. S. Halleck (LS-4), and J. G. Valdez]

In mammalian cells, chromatin is organized in a hierarchy of structures which condenses nearly two meters of DNA to such a short length that it can be packed into a cell nucleus only a few microns in diameter. This hierarchy consists of first wrapping the DNA around a core of histones to form nucleosomes, and then packing these nucleosomes together to form a nucleofilament. This nucleofilament is then coiled to form a solenoid structure. These structures can be further condensed by aggregating them into heterochromatin (see review of this organization by Gurley *et al.*¹). Histone metabolism studies in our laboratory have led us to propose that certain histone modifications (phosphorylation, acetylation, and amino acid variation) are involved in these various stages of chromatin condensation.¹⁻⁴ The results described here provide further support for one aspect of this concept: the involvement of unacetylated arginine-rich histones and the involvement of a specific H2A variant in condensed constitutive heterochromatin structure.

The extent of histone modifications was examined in a variety of cultured cell lines of the deer mouse (*Peromyscus*) which contain differing amounts of highly condensed, constitutive heterochromatin (Fig. 1). It was found⁴ that, while histone H1 phosphorylation was the same in those various cell lines, the phosphorylation of histone H2A was much higher in the greater-heterochromatin-containing cells (Fig. 1A).

Histone H2A has now been fractionated into two different protein variants (Fig. 2) having different hydrophobic properties: a less-hydrophobic H2A (LHP-H2A) and a more-hydrophobic H2A (MHP-H2A).⁵ The MHP-H2A is phosphorylated more than twice as much as the LHP-H2A (Table I). Recent studies with the two *Peromyscus* cell lines shown in Fig. 1 have demonstrated that, while the MHP-H2A to LHP-H2A phosphorylation ratio is the same in both cell lines (Table I), the greater-heterochromatin-containing

Fig. 1. Constitutive heterochromatin is stained darkly by the Giemsa C-band method (A) Interphase nucleus and mitotic chromosomes of *P. eremicus*. Most chromosomes have short arms composed of darkly staining C-band positive heterochromatin. Nucleus contains large clumps of constitutive heterochromatin. (B) Interphase nucleus and mitotic chromosomes of *P. boylii*. Note the absence of C-banded short chromosome arms. Nucleus shows small C-banded clumps of heterochromatin. *P. eremicus* cells (A) contain 34% more condensed constitutive heterochromatin than the *P. boylii* cells (B).

cell line (*P. eremicus*) contains 29% more of the MHP-H2A than does the lesser-heterochromatin-containing cell line (*P. boylii*) (Table I). This excessive amount of MHP-H2A in *P. eremicus* is similar to the amount of excessive heterochromatin

Fig. 2. Acid-urea-detergent preparative electrophoresis profile of histones H2A, H3, and H4 showing histone phosphorylation in exponentially growing cells. (A) *Peromyscus eremicus* cells; (B) *Peromyscus boylii* cells. Individual histone fractions are indicated by the incorporation of [^3H]lysine for 1.42-generation (—). Phosphorylation of each histone fraction is indicated by the 2-h incorporation of $^{32}\text{P}_4$ (---). Electrophoretic migration proceeds from right to left.

in *P. eremicus* (Table I). Thus, the increased level of H2A phosphorylation in the greater-heterochromatin-containing cells is due to a greater proportion of the more highly phosphorylated MHP-H2A, rather than due to an increase in H2A phosphorylation rate. These interspecies comparisons suggest that the more highly phosphorylated MHP-H2A may be involved in the structure of highly condensed constitutive heterochromatin.

In other studies, the acetylation of histones in various *Peromyscus* cell lines was compared.⁶ It was demonstrated that the arginine-rich histones (H3 and H4) are less acetylated in cells containing excessive amounts of constitutive heterochromatin (*P. boylii*) (Table II). These comparisons suggest that condensed constitutive heterochromatin may contain greater amounts of unacetylated histones than other forms of chromatin.

In order to confirm (by direct measurement) our suggestion that a relationship exists between these histone modifications and condensed constitutive heterochromatin, it would be necessary to fractionate the chromatin of one cell type into euchromatin and heterochromatin and then compare the histone modifications in each fraction. While such distinct fractions are presently very difficult to obtain in pure form, we have succeeded in fractionating the chromatin of *P. eremicus* cells into a rapidly sedimenting (RS) fraction which is

TABLE I
H2A MASS AND PHOSPHORYLATION IN
EXPONENTIALLY GROWING *PEROMYSCUS* CULTURES

	Phosphorylation ($^{32}\text{P}_4$ /H-lysine)	H2A H4	Mass Ratios (^3H -lysine/ ^3H -lysine)		Relative Amount of Heterochromatin ^a
	MHP-H2A LHP-H2A		H2A H3	MHP-H2A LHP-H2A	
<i>P. boylii</i>	2.20	1.20	1.93	1.04	1.00
<i>P. eremicus</i>	2.31	1.24	1.85	1.34	1.34
Percent Difference	5	3	4	29	34

^a Determined by flow cytometry measurements and confirmed by arm-length measurements of C-banded chromosomes.

TABLE II
HISTONE ACETYLATION RELATIVE TO HETEROCHROMATIN CONTENT^a

<u>Cell Line</u>	<u>Histone</u>	<u>Number of Acetylations</u>	<u>Percent of Total H3 or H4</u>	<u>Rel. Amount of Unacetylated H3 or H4</u>	<u>Relative Amount Heterochromatin</u>
<u>P. boylii</u>	H3	0	41.3	1.00	1.00
		1	37.7		
		2	13.8		
		3	7.2		
<u>P. eremicus</u>	H3	0	53.3	1.29	1.34
		1	25.0		
		2	17.8		
		3	3.7		
<hr/>					
<u>P. boylii</u>	H4	0	37.1	1.00	1.00
		1	34.2		
		2	15.2		
		3	8.9		
		4	5.1		
<u>P. eremicus</u>	H4	0	50.0	1.35	1.34
		1	25.0		
		2	11.6		
		3	7.6		
		4	5.8		

^a Quantified from electrophoretic separations of acetylated histones.

enriched in heterochromatin, and into a slowly sedimenting (SS) fraction which is depleted in heterochromatin (Fig. 3). The amount of heterochromatin in these fractions has been determined from measurements of their relative proportions of satellite DNA, while the content of phosphorylated histones, acetylated histones, and amino-acid variants of H2A in these fractions has been determined by electrophoresis.

A calibration curve was constructed relating the quantities of MHP-H2A and H₄₀ (unacetylated H4) with the percentage of heterochromatin using the values of these parameters measured in the unfractionated chromatin of low-heterochromatin-containing P. eremicus cells (Fig. 4). When the values of the MHP-H2A and H₄₀ contents of the RS and SS fractions were placed on this calibration curve, it was predicted that the SS fraction should contain 25% - 28% heterochromatin and the RS fraction should contain 41% - 45.5% heterochromatin (arrows on curves, Fig. 4) if a correlation exists between

heterochromatin content and histone modifications. The heterochromatin content of the SS and RS fractions estimated by satellite DNA measurements was 26.9% and 41.7%, respectively (Table III). The close quantitative agreement of estimates of heterochromatin content in chromatin fractions by direct satellite DNA measurement, MHP-H2A measurement, and H₄₀ measurement (Table III) strongly support our proposal that constitutive heterochromatin is enriched in these unacetylated and more-hydrophobic histones.

Reports from other laboratories have suggested that tri- and tetra-acetylated H3 and H4 histones are concentrated in template-active euchromatin.⁷⁻⁹ In a complimentary sense, our results suggest that unacetylated H3 and H4 are concentrated in constitutive heterochromatin. From these observations, one is led to consider a model of chromatin structure in which H3 and H4 are: a) unacetylated in non-genetic, constitutive heterochromatin; b) mono- and di-acetylated in

Fig. 3. Fractionation of *P. eremicus* chromatin labeled with [^{14}C]thymidine. Nuclei were homogenized at 4°C for 1 min at 15,000 rpm in 3 ml 0.001 M Tris (pH 8.0) in a Virtis 60 homogenizer. The sheared chromatin was layered on a 0.4 M - 2.8 M sucrose gradient and centrifuged for 30 min at 24,400 rpm in a SW 27 Beckman Spinco® rotor. One-ml fractions were collected from the bottom (left) of the tube. The rapidly sedimenting (RS) and slowly sedimenting (SS) peaks were collected as shown and the chromatin in the peaks recovered by centrifugation for 4 h at 50,000 rpm in a 60 Ti Beckman Spinco® rotor. Histones from these chromatin fractions were subjected to electrophoresis and then analyzed as shown in Fig. 4.

Fig. 4. Estimation of the heterochromatin content of *P. eremicus* rapidly sedimenting (RS) and slowly sedimenting (SS) chromatin fractions based on histone modifications. The MHP-H2A variant:total H2A ratio (based on [^3H]lysine incorporation) is plotted versus the constitutive heterochromatin content (based on C-banding) of *P. boylii* and *P. eremicus* whole cells. The unacetylated H4 (H4_o):total H4 ratio (based on stained electrophoresis bands) is plotted versus the constitutive heterochromatin content (based on C-banding) of *P. boylii*, *P. crinitus*, *P. eremicus* whole cells. Location of the MHP-H2A:total H2A ratio for each chromatin fraction on the solid line (arrows) predicts that the RS fraction is composed of 41% heterochromatin while the SS fraction contains 25% heterochromatin. Similarly, location of the percentage of unacetylated H4 obtained for each chromatin fraction on the dashed line (arrows) predicts that the RS fraction is composed of 44.5% heterochromatin while the SS fraction contains 28% heterochromatin.

TABLE III

DISTRIBUTIONS OF CONSTITUTIVE HETEROCHROMATIN
IN THE CHROMATIN FRACTIONS OF *P. EREMICUS* CELLS
ESTIMATED BY THREE DIFFERENT MEASUREMENTS

	Heterochromatin Content Estimated from Satellite DNA ^a	Heterochromatin Content Estimated from MHP-H2A in Fig. 4	Heterochromatin Content Estimated from H4 in Fig. 4
RS fraction	41.7%	41%	44.5%
Whole cells	35.0%	35%	35.0%
SS fraction	26.9%	25%	28.0%

^a Based on percentage contribution of GC-rich satellite DNA ($\rho = 1.703$ g/cc) to total DNA determined by neutral CsCl buoyant density gradient analysis.

genetically inactive euchromatin; and c) tri- and tetra-acetylated in genetically active euchromatin.

Our observation that the greater H2A phosphorylation in heterochromatin is due to an enrichment of a particular variant H2A species leads us to suggest that the cell may permanently install a more highly phosphorylated H2A into the nucleosomes of constitutive heterochromatin rather than try to modulate the H2A phosphorylation in such a permanently condensed chromatin structure.

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Zinc-Induced Enhancement of Resistance to Alkylating Agents

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A major goal of our cultured-cell toxicology studies is to identify and characterize protective responses which are mounted to counteract the effects of toxic inorganic and organic energy-related pollutants. This type of information ultimately might be utilized to identify individuals of high susceptibility to given classes of toxic agents and to devise procedures to modulate human protective responses in order to enhance naturally occurring resistance to these agents.

Over the past several years, we have been interested in the induction of metallothionein (MT), a low-molecular-weight, thiol-rich protein which plays a role in modulation of zinc and copper metabolism and which is also involved in the detoxification of energy-related pollutants such as cadmium and mercury. We were interested, therefore, in the report by Kotsonis and Klaassen¹ which suggested that the MT content of hepatic tissue is elevated in rats treated with alkylating agents. In view of the thiol-rich nature of metallothionein and the propensity for alkylating agents to interact with sulfhydryl moieties, it seemed reasonable to assume that induction of MT in alkylating-agent-treated animals represented a potential detoxification mechanism. If correct, it would indicate that this protein species plays a role in the detoxification of both organic and inorganic agents.

A major prediction of this hypothesis is that cells which differ in their capabilities to induce synthesis of metallothionein should exhibit a corresponding variability in sensitivity to alkylating-agent-induced cytotoxicity. We were able to determine the validity of this notion directly using a series of cadmium-resistant cultured cell variants of line CHO Chinese hamster cells, which differ greatly in their capacity to synthesize MT following exposure to high levels of $ZnCl_2$ (Fig. 1). All three cadmium-resistant variants responded to treatment with zinc by elaborating synthesis of metallothionein while the zinc treatment was without effect on MT synthesis in CHO cells.

Fig. 1. Metallothionein induction kinetics in CHO cells and in a series of derived, cadmium-resistant variant cells as a result of exposure to zinc. Zinc chloride (100 μM) was added to each culture at the start of the experiment and maintained throughout. Relative rates of metallothionein synthesis were determined in the manner described previously.⁵

If MT plays a major role in reducing alkylating agent cytotoxicity, then the zinc-pretreated variant cells (30F9, 20F4, and 2C10), which possess high levels of MT at the time of addition of alkylating agent, should exhibit enhanced resistance relative to their non-zinc-treated counterparts. CHO cells should not benefit from zinc pretreatment since the metal fails to enhance the endogenous level of MT synthesis. Any variation from that pattern (such as a lack of enhancement of survival of zinc-pretreated 30F9, 20F4, or 2C10 cells or an increase in resistance of zinc-treated CHO cells) would indicate that MT does not play a major role in the detoxification of alkylating agents.

Figure 2 presents results obtained when the four types of cells were treated with the alkylating agent iodoacetate. It is readily apparent that the survival of 30F9 and 20F4 cells was greatly increased as a result of pretreatment with $ZnCl_2$. Note, however, that there was: a) no enhancement in survival of zinc-pretreated 2C10 cells (which possessed a high level of synthesis of MT at the time of addition of iodoacetate) (Fig. 2C); and b) significantly increased survival of $ZnCl_2$ -treated CHO cells (which failed to synthesize MT) (Fig. 2D). Taken together, these observations indicate a lack of correlation between MT inducibility and protection against alkylating-agent-specific auto-toxicity.

Fig. 2. Survival of iodoacetate-treated CHO and cadmium-resistant variants in the presence and absence of zinc pretreatment. $ZnCl_2$ was added to a final concentration of $100 \mu M$ in the zinc pretreated cultures (solid figures) and maintained for 9 h prior to addition of iodoacetate. Non zinc-treated cultures served as controls (open figures). Iodoacetate was added to all cultures at a concentration of $100 \mu M$, and aliquots were removed from each culture at varying times thereafter for plating in a drug-free medium. Following a seven-day incubation period, colonies containing 50 or more cells were scored as survivors. The cells utilized were Cd³⁰F9 (A), Cd²⁰F4 (B), Cd²C10 (C), and CHO (D).

Generally similar results were obtained (Fig. 3) when cells $\pm$ zinc pretreatment were exposed to the anticancer alkylating agent, melphalan (L-phenylalanine mustard). The only difference between the two studies is enhanced survival of 2C10 cells which had been pretreated with zinc prior to exposure to melphalan (Fig. 3C).

The results presented in this report indicate the existence in cultured cells of a novel, non-MT-dependent, zinc-inducible protective response which grossly decreases alkylating-agent toxicity. While we have not yet identified the mechanism responsible for this zinc-induced response, several speculations may be proposed for consideration.

One possibility is that zinc induces an increase in the intracellular level of glutathione. Although we have not yet measured the level of glutathione in zinc-treated and control cultures in our cells, there was no evidence for significant enhancement of glutathione levels in kidney and liver tissue from rats exposed to zinc,^{2,3} suggesting that zinc does not appear to represent a likely candidate for the response observed in this

Fig. 3. Survival of melphalan-treated CHO and cadmium-resistant variants in the presence and absence of zinc pretreatment. Zinc pretreatment conditions and symbols used are those described in Figure 2. Cells were exposed to $8 \mu M$ melphalan for variable periods prior to plating for survivors.

report. Among other possibilities are zinc-induced non-MT proteins which might be capable of binding and inactivating the alkylating agents or enhancing their catabolism. We can not yet rule out the possibility that zinc affects transport of alkylating agents into the cells.

Finally, the zinc may cause an enhancement of DNA repair capacity analogous to the situation observed by Samson and her colleagues. Specifically, Samson and Schwartz⁴ demonstrated an enhancement of resistance to high levels of the alkylating agent, N-methyl,N'-nitro-nitrosoguanidine (MNNG), in CHO and cultured human skin fibroblasts which were chronically exposed to sub-toxic levels of MNNG prior to challenge with normally toxic concentrations of the drug. The degree of protection observed was roughly equivalent to that observed in our zinc enhancement studies. The protection did not appear to be attributable to changes in cell-cycle distribution or altered drug permeability or detoxification, leading Samson and Schwartz to conclude that low levels of MNNG induced an adaptive DNA repair system. While the precise nature of the mechanism responsible for the observed resistance may be open to question, it is important to note that both the studies of Samson and Schwartz and the present report describe an inducible (adaptive) cellular response which increases the resistance to alkylating-agent-specific toxicity.

These similarities perhaps suggest a similar or identical domain of responses induced by zinc and low levels of alkylating agents.

Studies into the mechanism responsible for this interesting and potentially important response are in progress.

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Synthesis of Histone H1^o in Synchronized Chinese Hamster Cells

[J. A. D'Anna, L. R. Gurley, R. A. Tobey, R. J. Sebring, J. G. Valdez, R. J. Kissane (LS-4), and J. Tesmer (LS-3)]

Previous studies from several laboratories have shown that the cellular content of a minor histone, called H1^o,¹ is inversely proportional to the rate of DNA synthesis or cell division. This inverse relationship has been observed during tissue degeneration and regeneration, during development, with aging in some animal tissues, and following overgrowth of some cultured cell lines (see reference 2 for review). Recently, we have shown that treatment of CHO cells with butyrate causes an increase in the proportion of G₁ cells in culture and an increased cellular content of histone H1^o.^{2,3} These and other observations have led to suggestions that H1^o may play a functional role in the control of DNA synthesis, in condensation of chromatin, in the appearance of a G₁ phase, and in cellular regulation by glucocorticoids (see reference 2 for

review). Since H1^o also is found in a number of rapidly growing cell lines (H1^o comprises approximately 7% of the H1 class proteins in CHO cells), it may also serve regulatory functions during the cell cycle as well as in non-proliferating cells.

Although the inverse relationship between the cellular content of H1^o and DNA synthesis strongly suggests that the synthesis of H1^o can be uncoupled from that of DNA, only a few studies pertaining to H1^o synthesis or H1^o enhancement have been reported. To gain insight into the possible functions of H1^o, we investigated H1^o synthesis during G₁ in butyrate-treated cultures and during traverse of G₁ and S phases in synchronized Chinese hamster (line CHO) cells.

Table I shows the effects of prolonged G₁ block and the effects of butyrate concentration upon the H1^o:H1 molar ratio in cultures arrested in G₁ by isoleucine deprivation. Figure 1 shows time-dependent changes of the H1^o:H1 molar ratio in: (1) cultures blocked in G₁ by isoleucine deprivation; (2) cultures blocked in G₁ by isoleucine deprivation, but containing 15 mM butyrate; and (3) cultures released from isoleucine-deprivation G₁ block into 15 mM butyrate (which maintains approximately 85% of cells in G₁). These data show that: (1) the H1^o:H1 ratio is increased in cells blocked in G₁ (by isoleucine deprivation) in the absence of butyrate (Table I and Fig. 1); (2) butyrate greatly elevates the H1^o:H1 enhancement observed in G₁-blocked cells (Table I and Fig. 1); (3) the enhancement is even larger in cultures released from isoleucine deprivation into complete medium containing butyrate (Fig. 1); and (4) the initial time dependence of the H1^o:H1 ratio (Fig. 1) exhibits a time lag which suggests a delay in the entry of butyrate into the cells or a delay in the enhancement process itself. Although the H1^o:H1 ratio reflects changes in the chromatin content of both H1 and H1^o, measurements of H1^o and H1 contents relative to that of the nucleosomal core histone H4 (not shown) indicate the H1^o:H1 molar ratio does reflect changes in the net H1^o content in these experiments. We conclude, therefore, that the synthesis and accumulation of H1^o are uncoupled from DNA synthesis in the butyrate-treated cultures.

To determine whether H1^o synthesis also occurs during G₁ in traversing cells (in the absence of

TABLE I
EFFECTS OF BUTYRATE CONCENTRATION ON THE $H1^O:H1$ MOLAR RATIO
IN CELLS BLOCKED IN G_1 BY ISOLEUCINE DEPRIVATION (ile^-)

Time in ile^-	Butyrate	Time in butyrate	Percent Cells ^a in G_1	Molar ratio ^b $H1^O/H1$
0 ^c (h)	0.0	0 (h)	58	0.08
36	0.0	0	92	0.11
60	0.0	0	92	0.15
60	2.5	24	95	0.19
60	5.0	24	94	0.29
60	15.0	24	92	0.37

^a Measured by flow cytometry.

^b Histones were extracted with perchloric acid by the first method of Johns^{5,6} from the chromatin of blended cells. The $H1^O:H1$ absorbance ratio, which closely approximates the molar ratio, was measured at 630 nm from cylindrical (0.5 x 25 cm) 2.5 M urea - 15% acrylamide - 5.2% acetic acid electrophoretic gels stained with amido black.

^c Exponentially growing control cultures.

Fig. 1. Time dependence of $H1^O/H1$ enhancement in G_1 cells. Cultures were grown for 36 h in isoleucine-deficient medium, then: (Δ) one culture was left in isoleucine-deficient medium (the control); ($\circ$) a second culture was left in isoleucine-deficient medium, but the culture was made 15 mM in butyrate; ($\bullet$) the third culture was resuspended in complete F-10 containing 15 mM butyrate (which retains most cells in G_1). During the next 24 h, aliquots were taken from the cultures for extraction and analysis of histones as described in Table I.

butyrate), we blocked cells in G_1 by the isoleucine deprivation method of Tobey and Ley⁴ and then released them into complete medium for resumption of cell-cycle traverse. Prior to release and at 1.0 - 1.5 h intervals after release, aliquots were taken and pulse-labeled for 1 h with [3H]lysine (1 μ Ci/ml). Figure 2 shows (1) the percentage of the maximum specific activity for histones $H1^O$, $H1$, and $H4$ and (2) the percentage of S phase cells plotted as functions of time after release from G_1 block. These data show that the specific activities of $H1$ and $H4$ during G_1 are much less than their values during S phase, in agreement with earlier reports; however, the specific activity of $H1^O$ during G_1 is approximately 60% of its maximum value which occurs when cells enter S phase. We conclude, therefore, that $H1^O$ is synthesized and deposited on chromatin during G_1 , as well as during S phase, in the absence of butyrate.

To compare the syntheses of $H1^O$ and $H1$ in more detail, we plotted (1) the $H1^O:H1$ molar ratio (Fig. 3a), (2) the ratio of rates of deposition of newly synthesized $H1^O$ and $H1$ onto chromatin (Fig. 3b), and (3) the ratio of rates of deposition onto chromatin of newly synthesized $H1^O$ and $H1$ per mole of the respective histone isolated from chromatin by PCA

Fig. 2. Specific activities of histones H1⁰, H1, and H4 (1.0-h pulses with [³H]lysine) during traverse of G₁ and S. The percent of maximum specific activity for H1⁰ (○), H1 (△), and H4 (□) (from the chromatin of blended nuclei) and the percentage of S-phase cells (●) are plotted as functions of time after release from G₁ arrest (isoleucine deprivation). The maximum specific activities of H1⁰, H1, and H4 are 1.2-, 2.2-, and 0.8 × 10⁶ cpm/μg, respectively. Following release from G₁ arrest, 450-ml aliquots were labeled with [³H]lysine for 1.0 h at a concentration of 1.0 μCi/ml. Histones were extracted from blended nuclei and they were separated as described in Table I. After absorbance profiles were measured, the gels were sliced into 2.2-mm pieces which were dissolved in H₂O (1.0 ml for 18 h at 55° - 60°). [³H]lysine incorporation was determined by scintillation spectrometry. The percentages of S-phase cells were determined by autoradiography.

Fig. 3. Comparisons of molar ratios and rates of synthesis between histones H1⁰ and H1 following release from G₁ arrest. The H1⁰/H1 absorbance (and molar) ratio (a), the ratio of rates of deposition of new molecules of H1⁰ and H1 onto chromatin (b), and the ratio of rates of deposition of new H1⁰ and H1 per mole of those histones obtained by perchloric acid extraction (c) are plotted as functions of time after release from G₁ arrest (isoleucine deprivation). The percentages of S-phase cells (—, ---) are also plotted in (b) and (c). The open symbols and solid lines represent quantities from experiments employing blended cells; the closed symbols and dashed lines represent quantities from experiments employing blended nuclei.

extraction (Fig. 3C). These data show the following: (1) the $H1^0:H1$ ratio is modulated during the traverse of G_1 and S so that it is maximal near the G_1/S boundary; (2) on an equimolar basis, the rate of deposition of new $H1^0$ onto chromatin per mole of $H1^0$ is 2.8 times greater than that of $H1$ at approximately 2.0 h - 2.5 h when most cells are in G_1 , but as cells enter S phase, the rate of deposition of new $H1^0$ per mole declines to only 0.5 that of $H1$; (3) while the rate of deposition of new $H1^0$ per mole of $H1^0$ is greater than that of $H1$ during G_1 , only 0.4 - 0.5 molecule of $H1$ is actually deposited onto chromatin for each molecule of $H1$ synthesized; as cells enter S phase, only 0.06 molecule of $H1^0$ is deposited for each molecule of $H1$.

These data show that synthesis of $H1^0$ is uncoupled from that of DNA in both butyrate-treated and traversing cells. They suggest that: (1) newly synthesized $H1^0$ is not simply a packaging agent for newly synthesized DNA; and (2) newly synthesized $H1^0$ may alter chromatin structure and gene expression during G_1 and, perhaps, other phases of the cell cycle in proliferating cultures.

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Pion Radiobiology

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Physical characteristics of negative pions depend on the pion stopping volume. Therefore, the biological effects of pions may be affected by the width of the Bragg peak, range modulations, and the size of the treatment volume. As a part of pretherapeutic radiobiology, attempts were made to provide: (1) biological dosimetry to verify that range-shifter functions used to spread peaks are optimal for therapy in producing uniform cell killing across the region of interest; and (2) data on cell killing by pion beams of different peak widths or sizes.

The results of Phase I studies using V79 cells suspended in gelatin indicated: (1) no significant differences in cell killing between pion beams of different ranges but of the same peak width; (2) no significant differences between pion beams of different sizes but of the same peak width; (3) a slight decrease in biological effectiveness with increasing peak width; and (4) satisfactory range-modulation functions to produce uniform cell killing for intermediate-range pion beams (Tune 16B).¹

Figure 1 shows the depth dose distribution and cell survival as a function of depth for pion beam, Tune 16B, 10-cm peak width, for different sized collimators. The data indicate that the cell killing is uniform across the peak for the three different collimators and that no significant differences are observed between the cell survival data of different sized collimators except for the beam entrance regions.

Since the clinical program employs fractionated schemes for therapy, Phase II studies were carried out using one and four fractions of pions on the mouse jejunum. The results indicated that the beam modulation used for the 14-cm peak (Tune 16B) also produced uniform cell killing and that no signifi-

Fig. 1. Depth dose distribution (top) and cell survival (bottom) as a function of depth for a pion beam, Tune 16B, 10-cm peak width, for different sized collimators.

Fig. 2. Intestinal crypt cell survival data for four pion dose fractions plotted as a function of proximal peak dose. The error bars indicate the 95% confidence limit.

cant differences in biological effect were observed between the beams of 14-cm and 6-cm peak width.²

Figure 2 shows the intestinal crypt cell survival data for four fractions plotted as a function of the proximal peak dose indicating that the beam modulation used for this tune, 16B, is appropriate to produce uniform cell killing after four pion fractions.

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Cytometric Analysis of Irradiation Damaged Chromosomes

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Investigations were begun to determine what differential effects, if any, could be seen in the chromosomes of x- and α -irradiated cells by flow microfluorometry. Exponentially growing M3-1 (Chinese hamster fibroblast) cells were exposed to various doses of X or α irradiation. The doses of each modality were matched on the basis of cell survival. For example, 760 rad of x rays were compared with 180 rad of α irradiation since both produce a 10% survival rate in M3-1. Chromosomes were produced in free suspension from the cells after irradiation by the method of Aten *et al.*¹ Briefly, after irradiation and after a suitable delay to correspond with the cell-cycle progression delay induced in the cells by irradiation, cells were blocked in mitosis by treatment with Colcemid for several hours. Mitotic cells were isolated from interphase cells by shaking the culture vessels and removing the mitotic cells which were suspended in the medium. The cells were then swollen in hypotonic KCl solution and stripped of cell membrane material by addition of detergent. Free individual chromosomes were thus liberated into the surrounding fluid. They were stabilized by the presence of propidium iodide which is also the fluorochrome used in the flow analysis.

Typical results are seen in Figs. 1 and 2, which represent chromosomes from cells at two isosurvival radiation dose levels. The peaks correspond to individual chromosome types or, in some cases, to several chromosome types having nearly the same DNA content. All the distributions have the same total number of counts. The most striking feature of the data is the appearance of a new peak (E) at channels 80 - 85 and the rapid disappearance of peaks A and B. A possible interpretation of this result is the splitting of chromosomes A and B into roughly equal-sized smaller fragments. Examination of chromosomes from similar experiments by classical cytogenetic methods should confirm or deny this conclusion. Another aspect of the data is that at the 50% survival dose (Fig. 1), x rays perturb the normal distribution more than α irradiation, whereas at the 10% survival level, the reverse is true. The apparent slope of increasing damage is greater for α than for x rays. Finally, there is some skewing to the left of the peaks of the larger chromosomes (A and B) in the case of x rays that is not noticeable in the case of α exposure. This may mean that x rays induce more breaks along the length of the chromosome than a comparable dose of α irradiation. That the skewing is not so obvious in the peaks of the smaller chromosomes is likely to be a reflection of the relative target size of the chromosomes. Again, cytological examination will help resolve this question.

We have confirmed the findings of others^{1,2,3} that irradiation of cells in interphase results in dose-dependent damage to DNA which is discernable by flow-cytometric analysis of chromosomes. We have found, in addition, that the quantity (and possibly the quality) of chromosomal changes is different in survival-matched doses of X and α irradiation. It may, therefore, be possible to use these methods for analysis of dose and type of exposure in unknown cases.

Fig. 1. Chromosome DNA content distributions from control and irradiated M3-1 cells. α = 60 rad, X = 400 rad (50% survival level).

Fig. 2. Chromosome DNA content distributions from control and irradiated M3-1 cells. α = 180 rad, X = 760 rad (10% survival level).

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INTRODUCTION

The Genetics Group (LS-3) examines the characteristics of environmental pollutants as assayed in biological systems. Our work seeks to define and quantify both the potential hazard to human health that exposure to pollutants may entail and the physiological modes by which eucaryotic cells respond to such exposure. As a consequence, our staff has acquired or is developing a battery of test systems which detect and measure genotypic and/or accompanying phenotypic change induced by selected pollutants.

If we assume that DNA is the critical target, then the study of pollutant-induced cellular change becomes the study of gene structure and regulation of gene function. In this context, one can define a minimum of two modes of metabolic response to pollutant exposure.

1. Selective induction of gene function. In selected cases, an agent may actually induce the synthesis of a protein product which either binds to or enzymatically modifies the inducing agent. It is likely that such systems evolved to regulate cellular homeostasis rather than to specifically respond to the inducing agent per se. Consequently, the induced response may be either beneficial or harmful to the organism. Inactivation or detoxifi-

cation of the agent is certainly a beneficial response while metabolic activation of an initially innocuous agent would be harmful.

2. Alteration of gene function. The selected agent may act either directly or via a reactive intermediate to alter the DNA. Although the alteration may be DNA specific, it may not necessarily be site specific. Thus, one can expect changes ranging from a single nucleotide change to deletions or rearrangements of large portions of the genome. Agents which induce such DNA specific alterations are considered to be potentially mutagenic and/or carcinogenic.

The selective induction of gene function by a specific agent provides the means for examining genetic protective mechanisms and metabolic activation of promutagens and/or procarcinogens, and there are programs within our group devoted specifically to each of these two topics. It is apparent that inducible cellular functions also provide the means of examining the regulation of gene expression, and our studies in this area have centered about cadmium (Cd⁺⁺)-induced synthesis of protein products which bind and detoxify the metal. This response is a very interesting one in a number of respects. Not only does it provide one example of a naturally occurring protective mechanism which

has been conserved during evolution, it also serves as an example of a homeostatic mechanism for the control of metal (primarily zinc) balance within the cell. In addition, this system has a number of unique features which make it most useful in the study of gene expression in eucaryotes: Cd^{++} -induced synthesis of proteins is comparatively very rapid and of large magnitude; metallothionein, the only induced protein product thus far characterized, has a highly conserved amino acid sequence in a number of animal cells; induction of protein synthesis requires RNA transcription. A number of cadmium-resistant Chinese hamster cells have been isolated by growth in increasing concentrations of Cd^{++} . It is primarily from examination of these variants that most of what is known of the properties of cadmium induction has been obtained. Although we now have a description of the induction kinetics of metallothionein, very little is known of the features which regulate the synthesis of induction-specific products. It is to the study of these problems that our effort is now being directed. We have evidence that RNA products, in addition to metallothionein mRNA, may be induced by metal treatment, and we have begun to search for additional induction-specific proteins. It may be that such products modulate the synthesis of metallothionein (e.g., activators, repressors) or participate in other metal related processes. We have also begun experiments which will enable us to examine the nature of genes encoding the induction-specific products. Using recombinant DNA techniques, cDNA clones have been constructed in plasmid vectors, and genomic DNA libraries of Chinese hamster DNA have been constructed in Charon 4A λ phage vectors. We are currently using state-of-the-art techniques to determine the primary nucleotide sequence of the genes encoding metallothionein, to identify and isolate recombinant cDNA clones complementary to other induction-specific RNAs, to examine the control of transcription of the induction-specific RNAs, and to examine the molecular mechanisms by which a cell acquires a cadmium-resistant phenotype. Using analysis of induction-specific protein products, recombinant nucleic acid probes of induction, specific nucleic acid products, and somatic cell hybrids, our group has also begun to investigate the

role of metallothionein in the etiology of metal-related human disease. It is our expectation that the selective combination of technologies acquired in our group will permit a more complete description of the regulation of Cd^{++} -induced gene expression, the nature of genetic protective mechanisms, and the relationship of metal metabolism to human disease. A description of the results of this work follows.

A substantial part of the group's effort is devoted to assessment of the cytotoxicity and mutagenicity of the products and by-products of developing oil shale technologies. Cytotoxic and mutagenic activities are assayed in Salmonella (the Ames test) and in rodent and human cells. To these assays have been added those which test for induction of chromosome damage, for the ability of an agent to induce tumorigenic transformation of Syrian hamster embryo cells, and for ability to induce tumor formation in animals.

Cytotoxicity and mutagenicity of natural and synthetic oils as well as shale oil retort process waters have been examined in the Ames assay. The results, reported below, show that a good deal of care must be exercised in the use of the Ames test. It is clear that simply reproducing the conditions known to maximize the response to model carcinogens and/or mutagens is inappropriate.

One of the problems associated with estimating the potential hazard to man posed by some agents is the fact that, in many cases, the ultimate reactive product is a derivative of the original agent. Many known carcinogens are chemically unreactive and require metabolic (i.e., cellular) activation before they can exert their biological effect. Metabolic activation is known to occur via cellular enzymatic detoxification processes, and most in vitro assays used to evaluate the mutagenic/carcinogenic potential of an agent employ cells incapable of metabolic activation. Since potentially mutagenic agents must be assayed after treatment with enzymatic activating extracts, any difference in activation potential will alter the results and introduce an additional source of variability. Studies currently in progress are assessing both the qualitative and quantitative differences in the products formed during the metabolic activation process and the way these differences are reflected in the yields of in vitro assays.

As metabolic activation can change an unreactive compound into a reactive one, so can near-ultraviolet (NUV) irradiation form reactive photoproducts via photooxidation of complex organic mixtures. Formation of mutagenic derivatives from NUV-irradiated oil shale retort process waters and natural and synthetic petroleum has been measured, and the degree of mutability of different genetic loci has been compared. The results are reported below.

As noted above, both the kind and amount of metabolic activation products vary with the individual preparation and species origin of the

activating extract. Since one wishes to reduce the uncertainties involved in extrapolating the results of in vitro assays to effects in humans, both metabolic activation and assay of mutagenesis should most properly be performed with human cells. Such an assay has been developed in which feeder layers of human epithelial cells are co-cultured with human keratinocytes. Thus metabolic activation and induction of mutation occur within the same culture preparation. This assay has given reproducible results and promises to be a most useful addition to the battery of in vitro tests used in determining the toxic and carcinogenic potential of energy-related pollutants.

Genes Coding for Metal-Induced Synthesis of RNA Sequences Are Differentially Amplified in Mammalian Cells

[R. A. Walters, M. D. Enger, C. E. Hildebrand, B. Griffith, and J. K. Griffith]

In order to define the role(s) played by cellular processes in cadmium detoxification,¹⁻³ the molecular events associated with cadmium exposure have been examined in Cd⁺⁺-resistant and Cd⁺⁺-sensitive Chinese hamster cells. Three cadmium-resistant variants have been selected by stepwise culture in increasing Cd⁺⁺ concentrations. Compared to the parental cell (line CHO), each of the resistant variants (i) has a higher cadmium toxic threshold, (ii) can synthesize more inducible metallothionein (MT), and (iii) can accumulate more inducible translatable MT mRNA.⁴⁻⁵ While these results are consistent with a role for MT in the acquisition of a cadmium-resistant phenotype, they reveal very little of the underlying molecular mechanisms responsible for Cd⁺⁺ resistance. We have initiated studies designed to probe cellular responses to Cd⁺⁺ treatment and report here that Cd⁺⁺ treatment of the resistant variants induces the synthesis of a highly abundant poly A⁺ RNA class, a major portion of which is metallothionein mRNA. Not only is this RNA class differentially regulated in resistant variant cells but the structural genes encoding these inducible RNAs are differentially amplified in the resistant variants. However, there is not a direct correlation between the degree of gene amplification and resistance.

The conditions of cell culture, derivation of resistant variants, and properties of the three resistant variant Chinese hamster cell lines Cd^r30F9, Cd^r20F4, and Cd^r2C10 have been described elsewhere.^{3,5} In none of the variants derived from the parental CHO cell was cadmium resistance due to a failure of cells to transport cadmium from the extracellular medium. In each of the experiments reported here, Cd⁺⁺ induction conditions were those previously shown to result in maximum production of translatable metallothionein mRNA: Cd^r30F9 cells - 40 μ M CdCl₂ for 4 h; Cd^r20F4 cells - 20 μ M CdCl₂ for 4 h; Cd^r2C10 cells - 2 μ M CdCl₂ for 8 h; CHO cells - 2 μ M CdCl₂ for 11 h.⁵

We recently showed that the poly A⁺ RNA from Cd⁺⁺-induced Cd^r20F4 cells contains a highly

abundant RNA class undetectable in uninduced CHO cells.⁹ We have exploited this difference in abundance to isolate from total induced Cd^r20F4 cDNA a tracer (cDNA_a) complementary to induction-specific RNA sequences. A comparison of the hybridization to induced Cd^r20F4 RNA of cDNA_a and total Cd^r20F4 cDNA is shown in Fig. 1. The cDNA_a hybridized, with an ERot_{1/2} of 0.04, to the most abundant RNA class which comprises ~5% of the total induced Cd^r20F4 poly A⁺ RNA. Comparison of the data in Fig. 1 with that measured earlier⁶ for the hybridization of the kinetic standard chicken globin cDNA with its template (ERot_{1/2} of 0.0018, 1820NT complexity) indicates that the total complexity of the induced class is ~2000NT. It will be noted that, at an ERot of 1, more than 80% of cDNA_a is hybridized to induced Cd^r20F4 RNA while less than 4% is hybridized to uninduced CHO RNA and that the concentration of RNA sequences complementary to cDNA_a is at least 10 000 fold higher in induced Cd^r20F4 than uninduced CHO RNA.

One way by which the resistant variant cells could attain an increased capacity for the synthesis of induction-specific RNA sequences is by amplification of their respective structural genes. We tested for this possibility by annealing cDNA_a to genomic DNA isolated from nuclei of each of the cell types.^{7,8} To be absolutely certain that the results could not be compromised by different rates of

Fig. 1. Hybridization of cDNA to poly A⁺ RNA. Hybridization of Cd⁺⁺-induced Cd^r20F4 RNA to total Cd^r20F4 cDNA (solid line) and cDNA (open triangles). Also shown is hybridization of cDNA_a to RNA from uninduced (open circles) CHO cells.

annealing of driver DNA, each hybridization mixture contained, as an internal standard, ^{14}C -labeled genomic DNA; thus, the rates of annealing of both driver genomic DNA and cDNA_a were assayed simultaneously. As shown in Fig. 2A, the rates of hybridization of genomic DNA from each of the cell types were experimentally indistinguishable. cDNA_a was hybridized to genomic DNA from CHO, Cd^R2C10 (Fig. 2A), Cd^R20F4, and Cd^R30F9 cells (Fig. 2B). The kinetics of reaction of cDNA_a to CHO DNA and Cd^R2C10 DNA were experimentally indistinguishable and were similar to that of the single copy component of genomic DNA (Fig. 2A). However, cDNA_a reacted with Cd^R20F4 DNA and Cd^R30F9 DNA 10 fold and 4 fold faster, respectively, relative to CHO DNA (Fig. 2B). These data show that, relative to CHO, the structural genes coding for induction-specific RNA sequences are amplified 10 fold and 4 fold, respectively, in the genomes of Cd^R20F4 and Cd^R30F9

Fig. 2. Amplification of genes encoding induction-specific RNA sequences. ¹⁴(A) Closed symbols represent annealing of ¹⁴C-labeled genomic DNA to driver DNA isolated from Cd^R30F9 (circles), Cd^R20F4 (squares), Cd^R2C10 (triangles), and CHO cells (diamonds). Open symbols represent annealing of cDNA to genomic DNA from Cd^R2C10 (circles) and CHO cells (triangles). (B) cDNA_a was annealed to genomic DNA from Cd^R20F4 (circles) and Cd^R30F9 cells (triangles). For comparison, the dashed line shows the annealing of cDNA_a to DNA from Cd^R2C10 and CHO cells and is reproduced from panel A.

and are unamplified in the genome of Cd^R2C10. Since cDNA_a annealed to CHO DNA with kinetics similar to that of single-copy DNA, the respective structural genes must be present at no more than a few copies per genome in cadmium-sensitive CHO cells from which the cadmium-resistant variants were derived. Gene amplification is likely not the result of formation of high heteroploid cells during variant selection since the total DNA content of resistant variant cells is the same as that of parental CHO cells based on flow microfluorometric DNA analysis (data not shown).

Chronic exposure of sensitive cells to increasing concentrations of particular toxic agents can result in the acquisition of specific resistant phenotypes. Alt *et al.*¹⁰ and Wahl *et al.*¹¹ have shown that mammalian cells resistant to methotrexate and N-(phosphonacetyl)-2-aspartate could be derived by continuous culture in progressively increasing concentrations of the drugs. In each case, resistance was accompanied by (i) an increased production of the enzyme which ameliorated the cytotoxic effects of the drug, (ii) an increased production of the mRNA coding for the enzyme, and (iii) amplification of the structural gene encoding the enzyme to an extent sufficient to completely account for the increased production of the respective mRNA. In some respects, the scenario developed for the acquisition of a cadmium-resistant phenotype displayed by the Chinese hamster variants studied here is similar to that outlined above for acquisition of resistance to methotrexate and N-(phosphonacetyl)-2-aspartate. In each case, the cadmium-resistant variant cell can synthesize more of the protein, metallothionein, putatively associated with amelioration of cadmium cytotoxicity⁵ and can accumulate a minimum of 100 fold higher concentrations of poly A⁺ RNA sequences (which include the metallothionein mRNAs) induced by Cd⁺⁺ treatment when compared to the sensitive parental CHO cell (Fig. 1); in two of the three resistant variants, the structural genes encoding the induction-specific RNAs have been amplified.

However, for each of the properties the cadmium-resistant variants share with methotrexate and N-(phosphonacetyl)-2-aspartate resistant variants, there are other important properties that are quite different. These include the observations that:

(i) each of the cadmium-resistant variants synthesize approximately the same amount of induction-specific sequences when maximally induced (Fig. 2); (ii) while the induced concentration of these sequences in each of the resistant variants is ~ 2000 fold higher than the constitutive level, the structural genes encoding these RNA sequences are not amplified in Cd²⁺2C10 (Fig. 2A); (iii) differences in the extent of gene amplification, 10 fold and 4 fold, in Cd²⁺20F4 and Cd²⁺30F9 cells, respectively, are not accompanied by similar differences in the ability to synthesize Cd²⁺-induced RNA sequences or in resistances to Cd²⁺; (iv) although sensitive CHO and resistant Cd²⁺2C10 cells each have the same number of structural genes, CHO cells can synthesize only a subset of the induction-specific RNAs (Fig. 1) and then to a level < 1% of that in Cd²⁺2C10 cells.

Since sensitive parental CHO cells can tolerate < 0.2μM Cd²⁺ while the independently derived Cd²⁺30F9, Cd²⁺20F4, and Cd²⁺2C10 cells are resistant to 40μM, 26μM, and 2μM Cd²⁺, respectively, it would appear that neither gene amplification nor the ability to synthesize induction-specific RNAs is directly responsible for the acquisition of a Cd²⁺-resistant phenotype.

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Molecular Cloning and DNA Sequence of the Chinese Hamster Metallothionein-II Gene

[B. B. Griffith, R. A. Walters, and J. K. Griffith]

Studies in this laboratory have focused on the genetic mechanisms which regulate the concentrations of essential and toxic trace metals in normal and abnormal mammalian cells. In this regard, we recently reported that cadmium-sensitive and cadmium-resistant Chinese hamster cells differ significantly in their ability to induce a class of low-molecular-weight proteins, which includes the metal-binding proteins, metallothionein I and II.^{1,2} In order to study the genetic basis of such differences, we have constructed recombinant DNA-containing plasmids from the mRNA that encodes these differentially induced proteins. We describe here the details of the construction of these recombinant DNA species and based on DNA nucleotide sequence data, identify one component of this induced battery as metallothionein II.

Incubation of Chinese hamster cells with non-toxic concentrations of the essential trace metal Zn²⁺, or the toxic trace metal Cd²⁺, results in the induction of an abundant, low-complexity RNA class.¹⁻³ This RNA contains sufficient genetic

information to encode 4-5 low-molecular-weight proteins, has a modal length of 400 nucleotides (NT), and comprises 3-5% of the total poly (A+) mRNA in metal-treated Chinese hamster cells.^{1,4} Owing to the unusual size and abundance of this mRNA, we have been able to isolate this RNA class with 30-50% purity using preparative methyl mercury agarose gel electrophoresis.^{1,4}

The purified 400 NT RNA class was used to construct recombinant cDNA plasmids (Fig. 1). Briefly, a single-stranded complementary DNA (cDNA) was synthesized from the mRNA template using the enzyme, avian myeloblastoma virus reverse transcriptase. After RNase hydrolysis of the original mRNA template the second DNA strand was synthesized using the enzyme *E. coli* DNA polymerase I (Pol I). The newly synthesized DNA was trimmed and the 5'-hairpin loop was cleaved with the single-stranded DNA specific nuclease *S*₁. Finally, the double-stranded DNA was inserted into the BamHI restriction endonuclease site of the plasmid cloning vehicle pBR322 using dA-dT tailing.⁵ The recombinant DNA-containing plasmids were introduced into *Ca*⁺⁺ permeabilized, cold shocked *E. coli* cells⁶ and those bacteria which acquired a recombinant plasmid were selected on the basis of their resistance to the antibiotic ampicillin (amp) which results from the amp^r gene provided by the plasmid DNA cloning vehicle. Once introduced into the bacteria, the recombinant DNA-containing plasmids replicate their DNA autonomously and do not harm the bacterial cells. Since each bacterium initially acquires only one recombinant DNA-containing plasmid, growth of bacterial clones from single bacterial cells provides means to purify and greatly amplify individual DNA sequences. Using this approach, we have isolated 206 recombinant DNA-containing plasmid species whose insert DNA was derived from the 400-nucleotide RNA class.

We identified a metallothionein gene containing plasmid using the technique of hybrid enhanced translation. This was accomplished by finding a plasmid whose insert DNA could specifically hybridize to mRNA, which was subsequently identified as metallothionein mRNA by the protein that it made in a cell-free translation system.⁷ DNA fragments from this plasmid (CHO pMT1) were subsequently used to find identical sequences in other recombinant DNA-containing plasmids. The plasmid containing the

longest cDNA insert, CHO p25, was further characterized by DNA sequencing.¹⁰

Beginning with the initiation codon, AUG, the cloned Chinese hamster cDNA insert decodes a 61-amino-acid-long protein followed by a non-translated 3' tail (Fig. 2). The amino acid sequence of purified Chinese hamster metallothionein protein has not been experimentally determined. However, as shown in Fig. 3, the amino acid sequence decoded from the cloned CHO p25 is virtually identical to the several mammalian metallothionein II proteins where amino acid sequence is known.⁹ We conclude, therefore, that CHO p25 was synthesized from the mRNA encoding Chinese hamster metallothionein II.

We have used the CHO p25 DNA to search for other related DNA sequences — the genes for metallothionein I for example. Since the amino acid sequence of metallothionein I and II are very similar (not shown), we expect that there are also

Fig. 1. Scheme for constructing recombinant cDNA plasmids. mRNA highly enriched for metallothionein mRNA was isolated from a 400-nucleotide-long RNA class and used for the synthesis of cDNA-containing plasmids as described in the text.

significant similarities in the nucleotide sequences of the amino acid coding regions of their genes. However, the heterogeneity of the 3' non-coding regions of other sets of related mRNA species suggests that the 3' non-coding sequences could provide a means of identifying and separating the metallothionein I or II genes. Using amino acid coding and 3' non-coding portions of the metallothionein II gene as probes (Fig. 2), we have isolated putative metallothionein I gene-containing plasmids. Accordingly, while the amino acid coding sequences of the two DNAs are very similar, hybridization data suggest that the 3' non-coding portion of these two genes are indeed quite different.⁸ We are now sequencing the putative metallothionein I gene in order to confirm these preliminary observations. Other members of the metal-induced 400 NT RNA class have also been cloned as recombinant DNA plasmids and are currently being isolated and characterized.

The cloned DNA fragments such as those described here permit us to quantify the flow of genetic information from individual genes each of whose products are involved in regulating cellular metal homeostasis. These will be particularly valuable in our effort to define the underlying lesions which

p25 DNA Sequence.

15	30	45	60
ATG GAC CCG AAT TGT TCT TGC GCT ACA GAT GGA TCC TGC TGC TGC GCT GGG TTT -GC AAA			
MET ASP PRO ASN CYS SER CYS ALA THR ASP GLY SER CYS SER CYS ALA GLY SER --- LYS			
5	10	15	20
75	90	105	120
TGC AAA GAG TGC AAA TGC ACA -CC TGC AAG AAA AGC TGC TGC TGC TGC CCG GTG GGC			
CYS LYS GLU CYS LYS THR --- CYS LYS LYS SER CYS CYS SER CYS CYS PRO VAL GLY			
25	30	35	40
135	140	145	150
-GC GCG AAG TGC -CC CAG GGC TGC GTC TGC AAA GAG GCT TCG GAC AAG TGC --- TGC			
--- ALA LYS CYS --- GLN GLY CYS VAL CYS LYS GLU ALA SER ASP LYS CYS --- CYS			
45	50	55	60
GCG TGA AGTGGGATTCGCCCTTMAAGCTGCCCTGTAATAAGACATGTTGATGAACGATATTTTATTTTACACCC			
ALA STOP			

Fig. 2. DNA Nucleotide Sequence of CHO p25 DNA. The recombinant DNA plasmid CHO p25 was synthesized as described in Fig. 1 and isolated as described in the text. The DNA sequence of the cDNA insert was determined by the method of Maxam and Gilbert.¹⁰ The amino acid sequence decoded from the cloned DNA sequence (lower line) is nearly identical to that of mouse metallothionein II (Fig. 3). Dashes denote positions where a nucleotide assignment has not yet been made.

MDPNCSEAAAGDSCTCAGSCKCHCKCTCKKSCSCCPVGEARCAAGGICAGASDKSCCA	HUMAN MT 2
WUPNCSVAGISCTCAGSCKCHCKCTCKKSCSCCPVGEARCAAGGICAGASDKSCCA	EQUINE MT 2
MDPNCSEAAAGDSCTCAGSCKCHCKCTCKKSCSCCPVGEARCAAGGICAGASDKSCCA	RABBIT MT 2
MDPNCSEAAAGDSCTCAGSCKCHCKCTCKKSCSCCPVGEARCAAGGICAGASDKSCCA	MOUSE MT 2
MDPNCSEAAAGDSCTCAGSCKCHCKCTCKKSCSCCPVGEARCAAGGICAGASDKSCCA	CLONED HAMSTER MT

Fig. 3. Comparison of the amino acid sequences of mammalian metallothionein II. The amino acid sequences of several mammalian metallothionein II proteins are compared to the amino acid sequence decoded from the cloned CHO p25 DNA sequence. "X" denotes position in which a nucleotide assignment has not yet been made. Each letter denotes a specific amino acid as defined by Kagi and Nordberg.

are responsible for abnormal metal metabolism in particular human genetic diseases.

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Differential Induction by Cadmium of a Low-Complexity Ribonucleic Acid Class in Cadmium-Resistant and Cadmium-Sensitive Mammalian Cells

[J. K. Griffith, M. D. Enger, C. E. Hildebrand, and R. A. Walters]

The Chinese hamster ovary (CHO) cell subline Cd^r20F4 is a stable, cadmium-resistant variant derived from the CHO line. It is distinguished from the parental CHO line by a 100 fold higher cadmium toxic threshold, a 50 fold or greater cadmium-inducible metallothionein synthesis rate,^{1,2} and a 50 fold or greater level of cadmium-inducible translatable metallothionein messenger ribonucleic acid (mRNA).^{3,4}

Molecular hybridization of cDNA with its template poly (A+)RNA indicates that cadmium-induced Cd^r20F4 cells contain an abundant RNA class not found in uninduced CHO cells.⁵ As shown in Fig. 1B, these sequences comprise approximately 5% of total Cd^r20F4 cDNA and reacted within a range of E_{Rot} values of 10⁻²-10⁻¹. There is no comparable transition in the reaction of CHO cDNA with its template RNA (Fig. 1A).

We determined the size distribution of the Cd^r20F4 RNA species which were both highly abundant and inducible by cadmium. A mixture of [³H]uridine-labeled poly(A+) RNA from cadmium-induced Cd^r20F4 cells and [¹⁴C]uridine-labeled poly(A+) RNA from uninduced Cd^r20F4 cells was resolved by coelectrophoresis in methyl-mercury-agarose gels.⁶ Highly abundant, cadmium-induced RNA species were identified by the increase in the ratio of ³H:¹⁴C radioactivity. As shown in Fig. 2, a single peak of high ³H:¹⁴C radioactivity was resolved from the RNA mixture and migrated with RNA whose modal chain length was 400 nucleotides. This RNA encodes four inducible proteins, including metallothionein.^{6,7}

We have utilized the data in Fig. 2 to independently determine the portion of the total poly(A+) RNA which represents cadmium-induced

Fig. 1. Hybridization of cDNA with poly(A+) RNA. Homologous hybridization of cDNA with its poly(A+) RNA templates. (A) Uninduced CHO cell RNA; (B) RNA from Cd^r20F4 cells induced 4 h with 20 μ M CdCl₂.

Fig. 2. Methylmercury-agarose gel electrophoresis of Cd^r20F4 RNA. A mixture of 10 μ g each of ³H-labeled poly(A+) RNA from induced Cd^r20F4 cells and ¹⁴C-labeled poly(A+) RNA from uninduced Cd^r20F4 cells was electrophoresed at 50V in a 100-mm 2% methyl-mercury-agarose gel. The relative migration of defined RNA species is shown in the upper portion of the panel. The 400-NT peak of ³H-radioactivity comprised 9% of the total [³H]RNA.

abundant sequences in Cd^R20F4 cells. As shown for the resolution of the RNA mixture in Fig. 2, the highest ³H:¹⁴C ratio of radioactivity in the 400-NT peak is about twice that of the other RNA size classes. Thus, the abundant, inducible RNA sequences comprise about half of the newly synthesized RNA mass in the 400-NT RNA fraction. Calculating from this fraction (50%), the total ³H radioactivity loaded onto the gels, and the fraction of the total ³H radioactivity which migrates in the 400-NT RNA fraction (see legend to Fig. 2), we conclude that about 4% of the total newly synthesized RNA from cadmium-induced Cd^R20F4 cells represents cadmium-induced abundant RNA sequences. This value is very close to the 5% mass estimate independently derived by the cDNA:RNA molecular hybridization analyses presented in Fig. 1 and indicates that the majority of the cadmium-induced abundant RNA sequences are contained in a single 400-NT size class.

In order to determine the kinetic complexity of the 400-NT RNA class, we prepared a cDNA tracer (cDNA_a) which contained the sequences complementary to those present in high abundance in cadmium-induced Cd^R20F4 cells but which are not abundant in CHO cells. As shown in Fig. 3, 80% of the cDNA_a tracer was hybridized by cadmium-induced Cd^R20F4 cell RNA by an ERot of 1.0 while only 4% of the cDNA_a was hybridized by uninduced CHO cell RNA at an ERot of 1.0. We calculate from these data that the cDNA_a tracer represents RNA sequences whose concentration is 10⁴ higher in the cadmium-induced Cd^R20F4 RNA than in CHO RNA.

The kinetic complexity of the cDNA_a was determined by comparing the rate of its hybridization with cadmium-induced Cd^R20F4 cell RNA to that of the hybridization of cDNA synthesized from chicken globin mRNA and hybridized with its template (not shown).⁸ Calculating from the ERot_{1/2} of these two reactions (0.04 and 0.0018 for cDNA_a and chicken globin, respectively), and from the 5% of the total poly(A+) RNA estimated from Figs. 1 and 2 to represent the inducible, abundant Cd^R20F4 sequences, we conclude that the total kinetic complexity of this class is about 2000 NT's and is sufficient to encode only a few low-molecular-weight proteins.

Fig. 3. Hybridization of cDNA_a with poly(A+) RNA from induced and uninduced Cd^R20F4 (A) and CHO (B) cells.

We next compared the regulation of this RNA class in induced and uninduced cadmium-sensitive and -resistant cells. In contrast to the reaction obtained with the cadmium-induced Cd^R20F4 cell RNA, uninduced Cd^R20F4 RNA hybridized with cDNA_a at a rate at least 2 x 10³ slower (Fig. 3A). Only 55% of the cDNA_a was in hybrid at an ERot of 5 x 10³ when the reactions were terminated due to the thermal instability of the poly(A+) RNA. Thus, a 4-h exposure of the cadmium-resistant Cd^R20F4 cell to 20 μM CdCl₂ increases the concentration of those RNA sequences complementary to cDNA_a by at least 2 x 10³.

The induction of CHO cells with cadmium increases the concentration of only a subset of those sequences induced in Cd^R20F4 (Fig. 3B). The reaction of cDNA_a with poly(A+) RNA from induced CHO cells is described by a biphasic curve spanning more than 3.5 decades of ERot to achieve a 70% hybridization. The reaction with induced Cd^R20F4 cell RNA, on the other hand, is qualitatively

different. It is described by a monophasic curve in which greater than 90% hybridization can be achieved in slightly more than 2 decades of ERot, the same span of ERot required to hybridize completely the chicken globin cDNA. These data indicate that there is little or no difference in the relative concentrations of the individual sequences induced by cadmium in Cd²⁺20F4 cells. However, there is a significant difference in the relative concentrations of the individual sequences comprising the two distinct abundance classes defining the reaction of cDNA_a with induced CHO cell poly(A+) RNA.

Our estimates from hybridization kinetics and cell-free translation indicate that the metal-induced RNA class has sufficient complexity to encode five metallothionein-sized mRNAs. The finding that the individual components of this mRNA class are differentially regulated in cadmium-resistant and cadmium-sensitive cells is further evidence for a specific role of these mRNAs in cadmium detoxification.^{1,2,9} However, the complexity of the response also suggests that acquisition of a cadmium-resistant phenotype may involve factors other than (or in addition to) an enhanced capacity for metallothionein synthesis.^{1,6}

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Somatic Cell Genetics Approaches to the Analysis of Regulation of Metallothionein Gene Expression

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In our laboratory, several tools of somatic cell genetics are being employed to delineate the mechanisms regulating genes involved in the metabolism of trace metals. These genes encode the low-molecular-weight, high-affinity metal-binding proteins (metallothioneins or MTs) which are implicated in a dual capacity in heavy metal (Cd, Hg) detoxification and essential trace metal (Cu, Zn) homeostasis.¹ In most eukaryotic cell types that have been examined, MT genes are induced by exposing cells to subtoxic levels of Cd or excess Zn.^{1,2} However, studies in our laboratory of the responsiveness of the CHO cell to either Cd²⁺ or Zn²⁺ indicated that this cell line was deficient in expression of MT genes at the phenotypic level.³ Consequently, it was possible to employ Cd²⁺ as a selective agent to obtain clonal Cd²⁺-resistant, MT-induction proficient sublines of the CHO cell for more detailed analysis of the regulation of Cd²⁺- (and other metals) induced responses.

Several stable Cd²⁺-resistant variants have been derived from the Cd²⁺-sensitive CHO cell.⁴ These variants designated Cd²⁺2C10, 20F4, and 30F9 show Cd²⁺-toxic thresholds of 2, 26, and 40 μ M CdCl₂, while the CHO cell has a toxic threshold of 0.2 μ M

Cd^{2+} .⁴ In all of the Cd^{2+} -resistant variants, induction of MT gene expression (measured both by MT synthesis and by expression of MT genes using cloned MT structural gene probes and molecular hybridization analyses) is a major factor in conferring the Cd^{2+} -resistant phenotype. In addition to induction of MT genes, several other metal-inducible factors have been identified with the Cd^{2+} -resistant phenotype.⁵

To further analyze the genetic regulation of the Cd^{2+} -resistant phenotype, intraspecific somatic cell hybrids have been constructed between CHO and the Cd^r 2C10 variant. For these studies a spontaneous double mutant of the CHO cell, selected for resistance to ouabain (Oua^r , 1 mM) and 6-thioguanine (6-TG, 10 μM) was fused using the polyethylene glycol procedure⁶ with the Cd^r 2C10 cell and hybrids were selected for growth in HAT medium (containing hypoxanthine, aminopterin, and thymidine) with ouabain. This selection procedure kills both donor cell types while allowing only hybrid cells or revertants of the Oua^r , 6-TG^r CHO cell to grow. Comparison of frequencies of the appearance of the Oua^r , HAT^r clones ($\sim 0.1\%$) from fused cells is at least a factor of 100 greater than the frequency of

TABLE I
CHARACTERISTICS OF CHO x Cd^r 2C10 HYBRIDS

Hybrid	Growth in CdCl_2	Growth in HAT	MT Inducibility
LA2C6	-	+	-
LA2C25	-	+	-
LA2C26	+	+	+

appearance of Oua^r , HAT^r revertants. Six hybrid clones were isolated and characterized further for Cd^{2+} sensitivity, MT inducibility, stability, and DNA content.

The data in Fig. 1 indicate that two classes of hybrids arise. Ten Cd^{2+} -resistant (Oua^r , HAT^r) hybrid clones were tested for stability of the Cd^r phenotype during continuous cultivation in the absence of selective pressure (no Cd^{2+} , no HAT, no Oua) for 5 months and all 10 retained the Cd^r , HAT^r, Oua^r phenotype. In all hybrids examined, the cellular DNA content determined by flow cytometry indicated a subtetraploid G1 DNA content, suggesting the loss of DNA (possibly whole chromosomes) in the process of cell fusion or post-fusion segregation.

The properties of three of the CHO x Cd^r 2C10 hybrids are summarized in Table 1. These comparisons indicate a close correlation between Cd^{2+} -resistance and MT inducibility. Since the Oua^r , 6-TG^r CHO cell contains a mutant, nonfunctional HPRT locus (and therefore is HAT-sensitive), the observation that all hybrids examined exhibit HAT resistance (requiring X-chromosome-linked, functional HPRT locus) indicates they have retained the X chromosome contributed by Cd^r 2C10. The lack of correlation between MT inducibility (Cd^r) and HAT^r suggests that MT inducibility is not X-linked in the CHO cell. Further studies are required to confirm this suggestion. In addition it will be necessary to distinguish between structural and regulatory components in the analysis of chromosomal linkage of MT inducibility.

Fig. 1. Cd^{2+} -sensitivity of CHO (Oua^r , HAT^r) x Cd^r 2C10 hybrids. Different symbols represent different hybrid clones. Two hundred cells of each type were plated in 60-mm culture dishes in Ham's F-10 plus 15% newborn calf serum containing indicated concentrations of CdCl_2 . Cloning ability was determined after one-wk incubation in a CO_2 incubator.

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Inducibility of Metallothionein Gene Expression as an Indicator of DNA Damage and Repair Proficiency in Human Cells

[C. E. Hildebrand, G. F. Strniste, and J. L. Hanners]

Previous studies of repair of various types of lesions in cellular DNA have utilized direct measurements of lesion removal or DNA repair synthesis in the damaged region, recovery of cell survival, or host cell reactivation of damaged viral DNA.¹⁻³ Repair of damage to specific gene functions in mammalian cells has been studied only in the context of repair of lesions which lead to mutation induction at the HPRT locus in human cells.⁴ In the present report, evidence is presented to suggest the general utility of a ubiquitous inducible gene function in studies of DNA damage and repair both in cultured cells and *in vivo*.

The specific genes of interest in this study code for low-molecular-weight, metal-binding proteins, metallothioneins.⁵ These proteins have been studied extensively in various eukaryotic systems in relation to the role of the proteins' metal-binding capacity in heavy metal detoxification^{5,6} and in essential trace element metabolism.⁷⁻⁹ In mammals, metallothioneins (MTs)

generally contain 61 amino acids (of which 20 are cysteine), bind group IIB metals (Zn, Cd, Hg) as well as Cu, with high affinity, and are synthesized in response to exposure of cells *in vivo* or in culture to Zn, Cd, or Cu.⁵ Recently, several laboratories have reported on studies of the regulation of metal-mediated induction of MT synthesis in cultured mammalian cells.¹⁰⁻¹² In general, those studies indicated that induction of MT synthesis by Cd or Zn is regulated at the level of transcription of thionein (the apoprotein of MT) mRNA.^{10,12} Further, the response is not tissue-specific and has been observed in human cell explants.¹³⁻¹⁷

In this study we have characterized the Zn²⁺-mediated induction of metallothionein in normal human skin fibroblasts and in xeroderma pigmentosum (XP) cells having a defect in the ability to repair lesions introduced into DNA by ultraviolet (UV) light. Evidence is provided indicating that this inducible system may have value in detecting repair deficiency conditions for a specific class of DNA lesions (transcription-termination events) in human populations.

Metallothionein was induced maximally in both NF and XP cells during exposure to the maximum tolerable level of ZnCl₂ (200 μ M) in monolayer culture.¹⁸ During exposure to 200 μ M ZnCl₂ the MT synthesis rate rose within 7 h from a low basal rate to a maximal rate at least 50 fold more than basal rate in both cell types. Induction of MT synthesis was inhibited by 5 μ g/ml actinomycin D suggesting that induction is regulated at the level of MT mRNA transcription. Irradiation of both NF and XP cells with UV light resulted in a single-hit exponential survival of MT synthesis measured 8.5 h postirradiation and postinduction with Zn²⁺.¹⁸ The D₀ for inactivation of induction of MT synthesis was $\sim$ 7 Jm⁻² in XP and $\sim$ 12 Jm⁻² in NF cells.¹⁸ The apparent difference in the sensitivity of MT induction could be due to differential repair capacities of NF and XP cells with consideration of kinetics of repair of UV-induced lesions superimposed on kinetics of MT induction.¹⁸

The striking difference between XP and NF cells in their capacities to repair UV-induced transcription-terminating lesions is illustrated by "liquid holding" experiments.^{5,19} Results of these

measurements (Fig. 1A) showed that the recovery of MT inducibility was very rapid in NF cells (complete by 4 h of liquid holding) while recovery in XP cells was negligible. These results are consistent with the differential recovery of cloning ability shown in Fig. 1B.

The major findings of these studies are summarized in the model shown in Fig. 2. While this simplified model is consistent with our results, it is possible that the regulatory mechanism(s) controlling MT induction is more complex than depicted here.¹⁸ Definitive analyses of the

regulation of MT induction in human cells is essential for a complete understanding of the UV sensitivity of this inducible gene system.

Several features of the inducible MT system in human cells suggest the utility of this genetic function (a) for identifying genotoxic agents whose primary mode of action is via transcription-terminating lesions, (b) for quantitating the relative contribution of transcription-terminating lesions introduced by complex mixtures of genotoxic agents, and (c) for detecting repair deficiency conditions in human populations. In the case of the latter potential application of MT inducibility, induction of MT synthesis can be measured readily in mononuclear cells obtained from a small sample of human peripheral blood (e.g., 10 ml) such as that taken for routine clinical laboratory testing.^{17,21} Ongoing studies will address these three major areas of application of MT inducibility as a functional assay for the mode of action for genotoxic agents and for interindividual variation in human repair capacity for specific classes of genotoxic lesions.

Fig. 1. Liquid holding recovery of (A) MT induction or (B) cloning ability of NF and XP cells. For MT induction experiments, confluent monolayers were held as controls or irradiated with 10 Jm^{-2} UV. Conditioned medium was added to culture dishes immediately after irradiation. At various times postirradiation, ZnCl_2 was added to cultures to a final concentration of $200 \mu\text{M}$ and MT synthesis was determined by ^{35}S -Cys pulse labeling from 7.5 h to 8.5 h following addition of Zn^{2+} . The liquid holding period is defined as the time between UV irradiation and addition of ZnCl_2 to cultures. For colony survival, confluent monolayers were held as controls or UV irradiated to ~50% surviving fraction (i.e., 0.5 and 10 Jm^{-2} for XP and NF, respectively). Assays were performed as described previously.¹⁸

Fig. 2. Proposed model for MT induction, UV inactivation of MT induction, and reactivation of MT induction. In the preinduction state MT genes are transcriptionally inactive as indicated by the dashed line surrounding putative promoter (P) and initiator (I) regions. (The termination signal is indicated by T.) Addition of Zn^{2+} causes activation or derepression of the MT structural genes and RNA polymerase (solid circles) produces primary transcripts (pre-MT mRNA) which are processed to form MT mRNA. Translation of MT mRNA is tightly coupled to the appearance of cytoplasmic MT mRNA. MT is indicated by the star-shaped symbol with associated Zn^{2+} (solid triangles). If the MT gene sustains a pyrimidine dimer within the transcription unit prior to Zn^{2+} addition, incomplete transcripts are formed following initiation of transcription by Zn^{2+} addition. No MT mRNA is produced and consequently no MT is synthesized. However, in cells proficient in excision repair, the transcription-terminating pyrimidine dimers can be removed and transcription can proceed normally.

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Quantitation of Mutations Induced by Metabolically Activated Mutagens in Normal Human Fibroblasts

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

Many chemical mutagens such as polycyclic aromatic hydrocarbons (PAH), aromatic amines, nitrosamines, and aflatoxins require metabolism by mixed-function oxidases and related enzymes to induce such biological endpoints as cell cytotoxicity and mutagenicity. However, most mammalian cells grown in culture cannot metabolize these chemicals.¹ A number of reports have shown both the toxic and mutagenic effects of PAH to mammalian cells by using either cell-mediated co-cultivation systems or liver microsomal activation systems. We have adapted a rat-liver microsomal S9 activation system both for cultured Chinese hamster ovary (CHO) cells² and normal human skin fibroblasts.³ We have been able to show that metabolic activation of three known promutagens/procarcinogens results in mutations at the HGPRT locus in normal embryonic human skin fibroblast cells (GM10).

At the concentration tested, benzo(a)pyrene [B(a)P], 3-methylcholanthrene (3MC), and dimethylnitrosamine (DMN) were not toxic or mutagenic to the diploid human cells without metabolic activation. When GM10 cells were treated with each of these chemicals in the presence of rat-liver S9 fraction² cytotoxic and mutagenic effects were observed. The dose response induction of 6-thioguanine resistant (6-TG^R) mutants by the three promutagens was determined under the optimum conditions described elsewhere.³ The results are shown in Fig. 1 (bottom). The mutation frequencies induced by B(a)P and 3MC at 5 $\mu\text{g}/\text{ml}$ were 18×10^{-5} and 10×10^{-5} , respectively. The background mutation frequency was

Fig. 1. Induction of cytotoxic and 6-TG^R mutational events in GM10 cells following metabolic activation of 3MC, B(a)P, and DMN. Cells were treated with 3MC, B(a)P or DMN in the presence of rat-liver microsomal homogenate (S9) for 2 h. After 8 days expression period, 5×10^4 cells were plated in 100-mm dishes in DMEM medium containing 30 μM 6-TG and 10% FCS. Dishes were incubated for an additional 21 days before staining and scoring of colonies. For cytotoxicity, dishes were incubated for 12 days before staining. Symbols used are: B(a)P (Δ, ●), 3MC (○, ●), and DMN (□, ■). Open symbols and closed symbols represent cytotoxicity and mutagenicity, respectively. Control dishes containing S9 mixture were treated with 0.5% DMSO or distilled water instead of promutagens. The background mutant frequency ranged from $0.25 - 0.32 \times 10^{-5}$.

$2.5-3.2 \times 10^{-6}$. Thus, 6-TG^R mutants induced by B(a)P and 3MC at 5 $\mu\text{g}/\text{ml}$ were 60 and 30 times the background mutation frequency. Frequencies of 6-TG^R mutants induced by DMN were lower than those observed for those induced by B(a)P or 3MC even though the concentrations of DMN were considerably higher. At 5 mg/ml , the DMN induced-6-TG^R mutation frequency was 25 fold over the background rate. Mutations induced at the HGPRT locus in GM10 by the three

promutagens are plotted versus survival in Fig. 2. In an attempt to compare the mutagenic potential of the three promutagens, we chose to relate 6-TG^R mutants induced at 50% survival. For 3MC, B(a)P and DMN there were 10, 8, and 4.4 6-TG^R mutants induced per 10⁵ cells, respectively.

In order to determine the optimum S9 protein concentration for the metabolic activation of promutagens tested, varying concentrations of S9 were used in the presence of fixed B(a)P concentrations. Figure 3 shows both the cytotoxic and mutagenic effects of B(a)P GM10 cells at various S9 protein concentrations. When 3 µg/ml B(a)P was used during the activation period, the number of 6-TG^R mutants increased to a maximum at 0.3 mg/ml S9 protein. Additional S9 levels (above 0.3 mg/ml) resulted in a decrease in mutation frequency. A similar pattern was observed when GM10 cells were treated with 5 µg/ml B(a)P with varying S9 concentration. However, the maximum number of 6-TG^R mutants were induced at 0.5 mg/ml S9 instead of at 0.3 mg/ml S9 protein seen with the lower B(a)P concentration. Cytotoxicity of B(a)P in GM10 was

Fig. 2. Induced 6-TG^R mutants as a function of logarithm of survival. The curves are derived from the data in Fig. 1 and Fig. 3. Symbols used are: 3MC (●), B(a)P (△, ▲), and DMN (■). Closed symbols are the data obtained from dose response experiments in Fig. 1. The open symbols are the data obtained from Fig. 3.

Fig. 3. The effect of S9 protein concentration on cytotoxicity and mutagenicity induced in GM10 human fibroblasts by B(a)P. Cells were treated with 3 µg/ml (□, ■) or 5 µg/ml (○, ●) B(a)P in the presence of varying concentrations of S9 for 2 h. Closed symbols represent cytotoxicity, whereas open symbols are mutagenicity.

also measured with varying S9 protein concentrations (upper two curves in Fig. 3). Maximum induced 6-TG^R mutation frequency is achieved when survival is at a minimum.

The validity of results obtained by S9 mediated mutation assays has been questioned by others because of the uncertainties introduced by the detoxification phenomena.⁴ Attempts were made in this report to determine if mutagenic potency is indeed altered by the detoxification and deactivation processes. This was accomplished by replotting the mutation frequency data as a function of the logarithm of the surviving fraction. The information in Fig. 2 indicates that the mutagenic potency of three promutagens relates as follows: 3MC > B(a)P > DMN. More significant for this discussion, with one of the chemicals, B(a)P, the mutation frequencies as a function of log survival remained linear, even when the mutation and survival data were collected from a) dose-response curves and b) experiments where increasing S9 concentrations

were resulting in maximum detoxification. These results imply that the mutagenic potency of activated chemicals when plotted as the number of induced mutations versus log survival is unaffected by the deactivating capacity of S9 proteins. The generality of such a statement is being tested in our laboratory employing other promutagens including 3MC and DMN.

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Mutagenicity of Various Oil Shale Retort Process Waters in the Ames/Salmonella Bioassay¹

[J. W. Nichols and G. F. Strniste]

Using Salmonella tester strains TA98 and TA100 we have examined for mutagenic activity a variety of process waters from the Paraho facility (aboveground retort), the modified in situ retort facilities of Occidental and Rio Blanco, the true in situ retort of Geokinetics and waters obtained from the Fischer retorting of an Estonian oil shale sample. The results are presented in Table I. Mutagenic activity was seen only in those reactions which contained S9 fraction. That is, we were unable to detect any direct acting mutagenic activity in these waters when assessed with this in vitro test. Although each water sample was examined over a dose range of up to 300 μ l, the dose-response curves for mutagenically active waters were generally linear only up to sample volumes of < 100 μ l. Deviations from a linear dose response can be partially accounted for by cytotoxic properties of these

active waters at higher concentrations. Employing the standard plate assay the only waters determined to be mutagenic were the highly active Paraho process water (PRHO 005) and the weakly mutagenic Occidental room 6 boiler blowdown water. Our definition for positive mutagenicity in either TA98 or TA100 is a reproducible response which is linear over some portion of dose range tested and when the maximum number of histidine (his^+) revertants induced per plate over the dose range tested equals or is greater than 1.7 X our historical background mutation frequencies for either strain, which can be shown to be a statistically significant result ($p < 0.01$). As reported previously,² the employment of the modified Ames procedure,³ which incorporates a preincubation step, has yielded positive mutagenic results for certain waters tested with TA98 which were declared negative in activity when tested in the standard plate assay scheme. In Table I (bottom) we show an example of positive activity for the Geokinetics retort room 17 process water which was negative in activity in the standard plate assay. Other process waters obtained from this particular retort burn and an Occidental process water (room 4) were negative in this modified assay in agreement with that seen in the standard plate assay.

Due to the high mutagenic activity of the Paraho water (PRHO 005) we have done initial chemical fractionation of this sample in an attempt to determine the chemical species present which account for the genotoxic activity. A standard acid/base fractionation scheme was employed and each fraction (after extraction with methylene chloride and redissolving in distilled water to the original sample volume) was assayed for mutagenic activity in both TA98 and TA100. The results are presented in Table II. The activity of the original process water as determined in TA98 is $\sim 100 his^+$ revertants/ μ l and $\sim 50\%$ of this activity partitions into the base/neutral fraction. The acid fraction and residual water contain < 2% of the original activity. Approximately 48% of the activity has been lost; however, two precipitate fractions which form when the water is adjusted to either pH extreme have not yet been tested for possible mutagenic activity. It is interesting to note that when the assay utilizes strain TA100, most of the mutagenic

TABLE I
MUTAGENICITY OF OIL SHALE RETORT PROCESS WATERS IN THE SALMONELLA BIOASSAY^a

Code	Registry# ^b	Description	rev/ μ l ^c	TA 98	rev/plate ^e H.B.	rev/ μ l ^c	TA 100	rev/plate ^e H.B.
				Sample Volume ^d (μ l)			Sample Volume ^d (μ l)	
PRHO 005	6 SEP 78.005	Product water from holding tank	103	0-300	77	11	0-30	3.8
OCCD-4	30 AUG 79.1	Process water, room 4	0.1	0-100	1.1	0.1	0-100	1.2
OCCD-5	30 AUG 79.2	Process water, room 5	0.1	0-100	1.3	0.1	0-100	1.0
OCCD-6-1	7 MAR 79.106	Process water, room 6	0.2	0-100	1.1	0.6	0-100	1.6
OCCD-6-2	6 MAR 79.104	Room 6, heater treater water	0.4	0-100	1.7	ND	ND	ND
OCCD-6-3	7 MAR 79.105	Room 6, steam make-up	0.1	0-300	1.6	0.2	0-300	1.6
GEOK-17-4	15 AUG 79.104	Process water, room 17	0.2	0-300	1.1	0.4	0-300	1.0
RIO-4A	10 MAR 81.2	Process water, burn zero	0.1	0-100	1.3	0.2	0-100	1.3
RIO-C1	1 APR 81.3	Sour water, burn zero from tank 102 to underground separator	0.1	0-100	1.1	0.1	0-100	1.3
RIO-C2	1 APR 81.4	Sour water, burn zero from pond #4	0.1	0-100	1.4	0.1	0-100	1.2
ESTON-1	23 APR 81.1	Fischer retorted Estonian shale process water	0.9	0-100	1.3	0.1	0-100	1.2
ESTON-2	23 APR 81.2	Shake leach, Estonian raw shale	0.1	0-100	1.4	0.1	0-100	1.2
ESTON-3	23 APR 81-3	Shake leach, Estonian spent shale	0.1	0-100	1.3	0.1	0-100	1.2
-modified procedure- ^f								
OCCD-4	see above	see above	0.1	0-100	1.4	0.7	0-100	1.2
GEOK-17-4	see above	see above	0.5	0-100	2.1	0.7	0-100	1.5
GEOK-17-1	15 AUG 79.101	Room 17, outlet demister	1.5	0-100	1.2	1.4	0-100	1.2
GEOK-17-2	15 AUG 79.102	Room 17, XAD concentrate	0.1	0-100	1.2	0.6	0-100	1.5
GEOK-17-3	15 AUG 79.103	Room 17, impinger water	0.1	0-100	1.0	0.7	0-100	1.3

- a Data represents results of 2-6 separate experiments in which the average number of his⁺ revertants formed was determined from three plates per dose per experiment. S9 fraction was obtained from the livers of Aroclor 1254-induced male Sprague/Dawley rats and 50-μl (2350 μg protein) was added to each plate.
- b Registry number used for particular sample in the Los Alamos National Laboratory Synfuel Repository.
- c Slope of response (revertants per μl sample) determined by linear regression analysis.
- d Range of sample volumes tested.
- e Ratio of the maximum number of his⁺ revertants induced per plate compared to the historical background (H.B.) for the range of sample volumes tested. For TA 98, the H.B. is 41 ± 9 and the experimental diagnostic mutagens 2-nitrofluorene (1.5 μg/plate) and benzo[a]pyrene (1.8 μg/plate) induce 327 ± 10 and 395 ± 54 revertants, respectively. For TA 100, the H.B. is 136 ± 28 and the experimental diagnostic mutagens MNNG (1 μg/plate) and benzo[a]pyrene (1.8 μg/plate) induce 285 ± 50 and 348 ± 38 revertants, respectively.
- f Samples were assayed for mutagenic activity using the modified (preincubation) Ames/Salmonella test.³

TABLE II
MUTAGENIC ACTIVITY OF ACID/BASE FRACTIONATED PRHO-005 PROCESS WATER^a

Sample	$\mu\text{l}/\text{plate}$	Histidine Revertants Induced in TA98 & TA100			
		TA98 (+S9) ^b		TA100 (+S9)	
		Rev/plate	Rev/ μl ^c	Rev/plate	Rev/ μl
PRHO 005 (6 SEPT 78.005)	1 μl	77 $\pm$ 10		187 $\pm$ 122	
	5	286 $\pm$ 23		212 $\pm$ 6	
	10	643 $\pm$ 128		270 $\pm$ 15	
	15	1165 $\pm$ 173		298 $\pm$ 40	
	30	3137 $\pm$ 115	(103)	518 $\pm$ 22	(11)
Base/Neutral Fraction	1 μl	65 $\pm$ 4		195 $\pm$ 11	
	5	177 $\pm$ 10		204 $\pm$ 4	
	10	302 $\pm$ 11		258 $\pm$ 25	
	15	582 $\pm$ 58		295 $\pm$ 16	
	30	1643 $\pm$ 136	(53)	495 $\pm$ 26	(10)
Acid Fraction	1 μl	44 $\pm$ 5		168 $\pm$ 7	
	5	52 $\pm$ 15		203 $\pm$ 11	
	10	53 $\pm$ 9		193 $\pm$ 4	
	15	53 $\pm$ 10		180 $\pm$ 9	
	30	71 $\pm$ 5	(1)	171 $\pm$ 7	(0)
Residual Water	1 μl	41 $\pm$ 4		163 $\pm$ 9	
	5	67 $\pm$ 10		182 $\pm$ 15	
	10	59 $\pm$ 9		198 $\pm$ 8	
	15	53 $\pm$ 6		196 $\pm$ 11	
	30	64 $\pm$ 11	(0.6)	185 $\pm$ 10	(0)

^a Numbers of his⁺ revertants per plate were obtained from two independent experiments in which each data point represents the average from three plates. Data is recorded $\pm$ one standard deviation. Each fraction was adjusted to the original volume of process water with distilled water.

^b S9 fraction was obtained from the livers of Aroclor 1254-induced male Sprague/Dawley rats, each plate contained 50 μl (2350 μg protein).

^c Numbers of revertants per 1 sample determined by linear regression analysis. Experimental background mutation frequencies for TA 98 and TA 100 were 39 ± 11 and 193 ± 7 , respectively.

activity ($\sim 90\%$) of the original water partitions into the base/neutral fraction. These preliminary data suggest that since each tester strain has been developed for sensitivity towards certain types of mutagens (TA98 is sensitive to frame shifters whereas TA100 is sensitive to base pair substitutions) it is possible that those mutagens responsible for inducing base substitutions have a much larger partition coefficient for the base/neutral fraction than does the class(es) of mutagens responsible for frameshifts. Currently, this active base/neutral fraction is being further defined using reverse phase HPLC and MS/GC methodologies.

We are also attempting to refine the conditions of the standard plate and modified (preincubation) procedures in order to optimize their responses

toward complex mixtures. As shown in Fig. 1, for the classical promutagen/procarcinogen benzo(a)pyrene (B(a)P), maximal his⁺ revertants induced are obtained for increasing B(a)P concentrations tested only when the volume of S9 fraction is also increased (upper curves). However, maximal his⁺ revertants induced by increasing amounts of Paraho process water (PRHO 005) added are achieved at the constant volume of 15 μl S9 fraction per plate. Different kinetic responses are seen for other complex mixtures (synthetic oil extracts) and in the modified Ames procedure.⁴ Thus, conditions which have been established to maximize the response of this *in vitro* screening bioassay toward model promutagen/procarcinogen compounds may not necessarily be optimal for assessing genotoxic potential of complex mixtures.

Fig. 1. His⁺ revertants per plate for Salmonella TA 98 incubated in the standard plate assay with varying amounts of benzo(a)pyrene (B(a)P) (top) or Paraho (PRHO 005) aboveground oil shale retort process water (bottom) as a function of amount of S9 mixture added. The S9 fraction obtained from Aroclor 1254-induced adult male Sprague/Dawley rats was 47 mg/ml protein. The numbers designating the upper curves represent amount (μg) of B(a)P added per plate whereas the numbers for the lower curves represent volume (μl) of PRHO 005 process water added per plate. Each data point represents the average number of revertants obtained from three plates.

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Genotoxicity of Natural and Synthetic Oils in the Ames/Salmonella Test

[J. W. Nichols and G. F. Strniste]

Dimethyl sulfoxide (DMSO) extracts of a variety of natural and synthetic oils and upgraded petroleum products were tested for both cytotoxic and mutagenic activities in the Ames/Salmonella bioassay.¹ We employ both TA 98 and TA 100 bacterial strains with and without the addition of Aroclor 1254-induced rat hepatic S9 fraction in our routine screening strategy. The data presented in Table I, however, include results for TA 98 only. In agreement with our previous observations,² natural petroleum are only marginal in activity or nonmutagenic. Our criteria for mutagenicity is a response for the induction of maximum number of histidine (his⁺) revertants over the dose range tested equaling 1.7 X or greater the historical background mutation frequency, indicating significant activity in the sample ($p < 0.01$). The various crude oils from different *in situ* processes (Occidental, Geokinetics, and Rio Blanco) all show weak but significant mutagenic activity. The 150 ton simulated MIS (LETC) oil, the coal derived medium distillate blend (SRCII) and the aboveground retort composite crude oil (PRHO 601) are strongly mutagenic, yet this activity in the latter oil can be completely eliminated by hydrotreatment (see Table I).

Several variables exist in the assessment of the genotoxic potential of these complex mixtures by this *in vitro* testing scheme which must be addressed before making even qualitative comparisons in the mutagenic responses seen. The cytotoxic effects of these various oil extracts can be considerable. For example the WILM (natural California crude oil) is 100% toxic to TA 98 at 100 μl sample per plate, whereas the other two natural crude petroleum (KCO and LCO) are minimally cytotoxic. The various shale oils tested are generally strongly cytotoxic; upgrading of the (PRHO 601) through hydrotreatment had little effect in reducing its cytotoxic potential. The SRC II medium distillate blend was extremely toxic. The toxicity of these oils can be modified (detoxification effect) by the addition of

TABLE I
GENOTOXICITY OF VARIOUS NATURAL AND SYNTHETIC OILS IN SALMONELLA TA 98^a

Code	Registry ^b	Description	Percent Extractable ^c (into DMSO)	Rev/ μ l ^d	Range ^e (μ l)	Precipitate volume (μ l) ^f	rev/plate ^g	Dose (μ l) ^h	Cytotoxicity ^j		
									rev/plate H.B. ⁱ	-S9	+S9
SAE90	10CT78.2	90 wt refined oil	11.4	1.4	10	ND	57 $\pm$ 9	10	1.4	99	97
TSC0113	15NOV78.1	pilot run, AGR ^k shale oil	34.5	1.4	10	ND	58 $\pm$ 3	100	1.4	90	ND
TAR	10CT78.1	roofing tar	8.1	1.5	10	ND	78 $\pm$ 6	100	1.9	95	97
WILM	24FEB81.1	Wilmington CA, crude API oil	32.1	1.5	6.7	3.3	72 $\pm$ 6	33.3	1.6	0	0
PRHO602	29MAY79.602	Hydrotreated PRHO601 crude oil	16.2	1.6	10	ND	63 $\pm$ 7	10	1.5	20	60
LCO	3FEB78.2	Louisiana crude, API oil	13.4	3.1	6.7	ND	77 $\pm$ 13	100	1.9	86	87
KCO	3FEB78.1	Kuwait crude, API oil	5.0	3.8	6.7	ND	74 $\pm$ 5	100	1.8	83	90
OCCD3	1JAN76.100	Occidental MIS crude oil (room 3)	8.6	4.5	6.7	ND	71 $\pm$ 3	10	1.7	41	89
RIO#A	10MAR81.1	Rio Blanco retort zero crude oil	10.3	5.5	10	10.0	102 $\pm$ 4	100	2.5	44	100
OCCD185	20CT78.1	Occidental MIS crude oil (room 5)	28.7	7.1	6.7	3.3	96 $\pm$ 10	10	2.3	18	31
OCCD120	6MAR79.100	Occidental MIS crude oil (room 6)	17.0	7.4	6.7	3.3	111 $\pm$ 6	33.3	2.7	10	67
ESTON	23APR81.1	Fischer retorted Estonia oil shale	100	7.9	3.3	3.3	78 $\pm$ 15	6.7	1.9	ND	ND
GEOK188	5MAR80.3	Geokinetics TIS crude oil (room 17)	42.7	10.0	3.3	3.3	80 $\pm$ 7	10	2.0	19	92
GEOK191	19DEC80.2	Geokinetics TIS crude oil (room 24)	32.0	14.9	3.3	3.3	115 $\pm$ 17	6.7	2.8	9	86
LETC	24FEB81.2	150-ton simulated MIS crude oil	27.6	68.1	3.3	6.7	430 $\pm$ 5	33	10.5	15	84
PRHO601	29MAY79.601	Paraho AGR composite crude oil	44.9	200	0.75	3.3	355 $\pm$ 26	3.3	8.7	1	46
SRCII	24FEB81.3	Solvent refined coal, medium distillate blend	100	410	1.0	3.3	2315 $\pm$ 193	10	56.5	0	0

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- a Data represent the results of two or more independent experiments. For mutagenicity, the number of his⁺ revertants induced per plate for a minimum of 4 sample doses with Aroclor 1254 rat hepatic S9 fraction was determined. S9 fraction volume was 50 μ l equaling 2350 μ g protein per plate. For cytotoxicity, the ability of treated TA 98 cells to form colonies was determined.
- b Number used for particular sample in the Los Alamos National Laboratory Synfuel Repository.
- c Amount of oil extracted into DMSO (weight percent) determined by isotope dilution methodology.
- d Slope of the response determined for each sample as determined by linear regression analysis.
- e Range of sample volumes in which a linear dose response for induction of his⁺ revertants is obtained.
- f Volume of sample at which visible precipitate in dish was observable.
- g For dose range tested (usually 0.1-100 μ l DMSO extracted material), the maximum number of his⁺ revertants induced is reported $\pm$ one standard deviation.
- h Dose (μ l) of sample at which maximum number of his⁺ revertants per plate was achieved.
- i Ratio of maximum number of his⁺ revertants per plate to the historical background (H.B.). For TA 98 the H.B. is 41 ± 9 . Experimental diagnostic mutagens: 2-nitrofluorene (1.5 μ g/plate) = 296 ± 94 , benzo(a)pyrene (1.8 μ g/plate) = 354 ± 105 .
- j Cytotoxicity determined by colony formation assay in which 1000 plated cells were exposed to 100- μ l DMSO extracted material $\pm$ S9 mixture. Surviving fraction was determined after 48-h incubation.
- k Abbreviations used: AGR, aboveground retort; MIS, modified in situ retort; TIS, true in situ retort; API oil, American Petroleum reference oil; DMSO, dimethyl sulfoxide; ND, not determined.

S9 mixture. Furthermore, different cytotoxic effects can be observed when TA 100 test strain is used (data not shown).

All oils tested to date require metabolic activation to induce mutagenicity in the Salmonella test. The dose response curves obtained for the mutagenic oils show initial linearity between his⁺ revertants induced and sample size tested followed by declines in these responses to varying degrees. The range of sample sizes necessary to induce a linear dose response is generally 1-10 µl. We have noted precipitates forming with many of these samples for volumes tested > 3 µl. Thus, these decreases in the dose responses could be attributed to 1) unavailability of test sample due to its precipitation out of the test medium, and 2) cytotoxicity.

Another factor which could influence the mutagenic potential of an oil is its extractability into DMSO, a feature which varies by 20 fold for the oils tested (see Table I). In addition the complex role of S9 in optimizing the mutagenic responses of complex mixtures must be considered. All the data reported in Table I is the result of reactions (standard plate assay) containing 50 µl S9 fraction (2350 µg protein/plate). Matrix experiments incorporating up to 10 different S9 concentrations (0-100 µl per plate) and seven sample volumes have indicated that different conditions are necessary to maximize the mutagenic response for different oils, for example, comparing SRC II distillate blend with PRHO 601 composite shale oil.³ Thus, established conditions which optimize the response of the Ames bioassay using promutagen standards [e.g., benzo(a)pyrene] may not necessarily be optimal for assessing mutagenic potential of complex mixtures.

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Light Activation of Oil Shale Retort Process Waters

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

1. Comparison of Mutagenic Effects at Five Genetic

Loci. We have previously shown that near ultra-violet light (NUV) activates oil shale retort process waters which induce genotoxicity in cultured Chinese hamster ovary (CHO) cells.¹⁻⁵ Since the degree of mutability of different genetic loci as well as the type of mutation damage induced may vary considerably for different mutagens, a mutagenesis assay system employing a variety of markers on a single cell line may be more effective in detecting the broader spectrum of mutagens or carcinogens present in complex mixtures. We have employed five drug-resistant markers to monitor mutagenesis induced in CHO cells by process water plus NUV including: (1) resistance to 6-thioguanine (6TG^R) to measure forward mutation at the X-linked hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus; (2) resistance to 8-azaadenine (8AA^R) with presumptive alterations in the autosomal recessive adenine phosphoribosyl transferase (APRT) gene; (3) resistance to ouabain (OUA^R), a codominant marker affecting membrane Na⁺/K⁺ ATPase; (4) resistance to emetine (EMT^R); and (5) resistance to diphtheria toxic (DIP^R), where lesions in these latter two markers affect protein synthesis.

Our results indicate that the qualitative responses of these various markers in CHO after exposure to two different retort process waters and NUV are in agreement with the results for these two process waters' mutagenic potential as assessed at the HGPRT locus only as previously reported.^{2,4} That is, the sample of an aboveground retort process (ARP) water was strongly mutagenic at all loci, whereas the true *in situ* retort process (TIS) water was a weaker mutagen when assessed under the same conditions. However, there is a quantitative difference in the magnitude of the mutagenic response for the various markers tested compared to natural background mutation frequencies. For example, at 50% surviving fraction of cells, the ARP water plus NUV-induced mutations in CHO at the OUA^R, 8AA^R, 6TG^R, DIP^R, and EMT^R markers at about 66,

31, 23, 24, and 10 fold over background mutation frequencies, respectively. This same order for mutagenic response was seen for the weaker, mutagenic TIS water.

When the induced mutation frequencies (at the five different markers) are plotted versus NUV dose a nearly linear relationship results (see Fig. 1). This would indicate that the various genes examined are affected by the mutagenic components in these light-activated complex mixtures (retort process waters) in a similar fashion;⁶ however, the sensitivity of the response varies considerably as noted above with $OUA^R > 8AA^R > 6TG^R > DIP^R > EMT^R$. A complete account of this work has been submitted for publication.⁷

Fig. 1. Dose-response relationships for various genetic markers in CHO cells after exposure to oil shale retort process waters plus NUV. Background mutation frequencies for the various markers are indicated at dose equal to 0. Symbols used are: $8AA^R$ ($\blacktriangle$, $\triangle$), $6TG^R$ ($\blacklozenge$, $\lozenge$), OUA^R ($\bullet$, $\circ$), DIP^R ($\blacktriangledown$, $\triangledown$), and EMT^R ($\blacksquare$, $\square$). Closed symbols are for ARP water plus NUV, and open symbols are for TIS water plus NUV.

2. Sunlight Activation of Retort Process Waters.

Since significant amounts (2-4%) of solar radiation reaching the earth's surface is NUV, there exists a concern about the environmental impacts resulting from solar activation of discharged, industrial waste streams. We have examined both cytotoxicity and mutagenicity induced in CHO cells after their exposure to a particular aboveground oil shale retort process water and natural sunlight and we briefly summarize in this report our published results.^{5,8}

CHO cells were pretreated with dilutions of ARP water for 1 h in the dark and subsequently exposed to sunlight on the rooftop of the Health Research Laboratory at the Los Alamos National Laboratory in Los Alamos, NM. The survival data in terms of colony forming ability are shown in Fig. 2 (right panel). For comparison, the results of an experiment in which artificial NUV was used as the radiation source are also shown (left panel). The exposure time of sunlight necessary to reduce cell survival to 37% (D_{37}) in the treated population is about 50 sec, whereas nontreated CHO cells are inactivated to 37% surviving fraction only after 6 min exposure to sunlight. This represents a 7-fold increase in the photosensitivity of CHO cells to sunlight when pretreated with the process water as compared to untreated cells. The measured

Fig. 2. Cytotoxicity induced in CHO cells by oil shale retort process water and artificial NUV or sunlight. Open symbols represent cells exposed to light only whereas closed symbols represent cells exposed to process water (1:270 dilution) 1 h prior to irradiation. Artificial NUV incident fluence averaged $5.6 \text{ J per m}^2 \text{ per sec}$.

fluence for sunlight (200–2500 nm wavelength) in Los Alamos, NM, during January 1981 (when the experiments were performed) averaged 900 J per m² per sec of which we estimate ~3% is NUV (300–400 nm). Thus, the dose of NUV from sunlight necessary to reduce survival to 37% is about 1400 J per m², strikingly similar to the dose of artificial NUV (5 min or 1680 J per m²) necessary to elicit a similar cytotoxic response.

In Fig. 3, we show the number of HGPRT deficient mutants induced per 10⁵ viable cells as a function of cell survival for CHO cells pretreated with oil shale process water and subsequently exposed to either artificial NUV (left panel) or sunlight (right panel). At 37% survival, there is about a 7 fold increase in the number of mutants induced by sunlight for cells pretreated with process water compared to untreated cells after subtraction of the background mutation frequency (~1 per 10⁵ cells). It should be noted that no genotoxic effects were seen in CHO cells exposed to artificial NUV only (up to 30 min exposure).

To examine the role of the NUV component of natural sunlight in this photoactivation process, an experiment was performed in which special "cut-off" light filters were placed between the source (sun) and the target (plated cells). The results using a

colorless glass, sharp-cut UV filter (< 0.1% transmission for light of 320 nm or less) showed similar cytotoxic and mutagenic effects when compared to treated and nonfiltered, sun-exposed cells. However, when a sharp-cut yellow glass filter was employed, which transmits < 0.1% light at 400 nm or less, cell survival increased and cell mutagenicity decreased substantially (data not shown). These results suggest that the component of sunlight responsible for photoactivation is primarily NUV although a measurable portion of this photoactivating potential resides at wavelengths > 400 nm.

The exact magnitude of the potential environmental and health-related risks resulting from photoactivation of retort process waters is unassessed at this time. Our demonstration that a significant rate of mutation occurs in cultured mammalian cells exposed to high dilutions of these waters and nominal exposures to natural sunlight suggest that such an assessment is a prerequisite to minimal risk development of our oil shale resources.

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Fig. 3. Mutagenicity induced at the HGPRT locus in CHO cells by oil-shale retort process water and artificial NUV or sunlight. The numbers of mutants induced at each dose point have been corrected for plating efficiencies determined at time of selection. Open symbols represent cells exposed to light only whereas closed symbols represent cells exposed to the process water (1:270 dilution) 1 h prior to irradiation.

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Light Activation of Natural and Synthetic Petroleum

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

Photooxidation of complex organic mixtures by sunlight after their discharge by industry or by accidental spillage presents an environmental hazard of unassessed magnitude. Recent reports on the formation of toxic photoproducts in a No. 2 fuel oil exposed to near ultraviolet light (NUV)^{1,2} and several reports from this laboratory concerning genotoxic components induced in oil shale retort process waters exposed to NUV³⁻⁷ substantiate this

concern. Of equal importance is the assessment of potential health risks associated with occupational and public exposure to industrial effluents and/or waste streams plus light. In this report we summarize data concerning NUV-induced cytotoxicity and mutagenicity in cultured Chinese hamster ovary (CHO) cells after treatment with dilutions of various natural or synthetic petroleum. Our data indicate that significant mutagenicity occurs in cells exposed to high dilutions of certain synthetic crude oils and nominal exposure to NUV.

Due to the non-miscible properties of the oils with aqueous medium, dimethyl sulfoxide (DMSO) extracts of the natural and synthetic oils were used throughout this study. Listed in Table I are selected properties of the tested oils including the amount of oil extracted into DMSO (weight percent) as determined by isotope dilution methodology. For each oil sample, the minimum tolerated dilution

TABLE I

NATURAL AND SYNTHETIC CRUDE OILS TESTED

Code	Registry # ^a	Definition	Percent Extractable ^b	A ₃₅₀ ^c	MTD ^d
KCO	3FEB78-1	Kuwait natural crude oil	5.0	50	1:500
LCO	3FEB78-2	Louisiana natural crude oil	13.4	48	1:1000
WILM	24FEB81-1	Wilmington (CA) natural crude oil	32.1	68	1:1000
SAE 90	10CT78-2	90 wt refined transmission oil	11.4	30	1:1000
PRHO 601	29MAY79-601	Paraho AGR composite crude oil ^e	44.9	275	1:2250
PRHO 602	29MAY79-602	hydrotreated PRHO 602 crude oil	16.2	68	1:2250
MIS 3	1JAN76-100	modified <u>in situ</u> crude oil (room c)	8.6	175	1:3000
MIS 185	20CT78-100	modified <u>in situ</u> crude oil (room e)	28.7	184	1:3000
LETC	24FEB81-2	NBS reference MIS crude oil	27.6	164	1:1500
SRCII	24FEB81-3	solvent refined coal, distillate blend	100	52	1:3000

^a Number used for particular sample in the Los Alamos National Laboratory Synfuel Repository.

^b Amount of oil extracted into DMSO (weight percent) determined by isotope dilution methodology.

^c Absorptivity of sample at 350-nm wavelength (units per ml in DMSO-extracted sample) determined with dilutions made with glass-distilled methanol and spectra recorded with a Unicam model SP800 ultraviolet spectrophotometer.

^d Minimum tolerated dilution for non-cytotoxic effect in CHO cells for 1-h exposure in the dark.

^e Abbreviations used: AGR, aboveground retort; MIS, modified in situ retort; NBS, National Bureau of Standards.

(MTD) for a noncytotoxic effect in CHO cells was established in a colony formation assay for cells exposed to various oil dilutions in alpha-MEM medium (GIBCO) supplemented with 1% fetal calf serum (FCS) for 1 h in the dark (37°). Absorptivities at 350 nm wavelength equaled 0.02–0.12 for the various oils used at their respective MTD.

NUV-induced cytotoxicity in CHO cells pretreated with dilutions of various natural or synthetic petroleum is shown in Fig. 1. In all examples except for the refined SAE 90 wt oil, substantial cell killing resulted with D_{37} values (doses of NUV necessary to reduce colony forming ability to 37% of control, nontreated cells) of 1300–2500 J per m^2 . No cytotoxic effects were seen in cells treated with NUV only up to the highest dose used in this study (3000 J per m^2). Dilutions of natural petroleum from three different sources are as photoactive in inducing a toxic response in CHO cells as are the variety of synthetic oils tested. Hydrotreatment of a particularly active composite shale oil (PRHO 601) did not reduce the phototoxic properties of the oil; in fact, we

routinely have observed a slight enhancement in the phototoxicity of this upgraded oil (PRHO 602).

In Fig. 2 we show the number of mutations induced at the HGPRT locus in CHO cells by combinations of natural or synthetic oil plus NUV plotted vs cell survival. At the D_{37} for cell survival the natural petroleum plus NUV show only marginal (1.5–2 times background mutation frequency for the LCO and KCO oils, respectively) or negative activity as is the case for the WILM oil. No mutagenicity was observed for cells exposed to NUV only for doses up to 3000 J per m^2 .

On the contrary, two shale oils were potentially photomutagenic (10–12 times background mutation frequency for the PRHO and LETC oils at their D_{37} for cell survival, respectively). These two oils have been shown to be exceptionally mutagenic in the Ames/Salmonella bioassay employing TA98 tester strain with rat hepatic S9 activation (Nichols and Strniste, elsewhere in this report). Two other shale oils from a modified *in situ* retort (MIS 3 and MIS 185) were slightly mutagenic (~ 3 times background frequency) and the solvent refined coal distillate blend (SRC II) was only marginally photomutagenic. This latter synthetic petroleum, however, shows high mutagenic activity when metabolically activated in the Ames/Salmonella test (see Nichols and Strniste, this report). The

Fig. 1. Inactivation of the colony forming ability of CHO cells after one hour incubation with dilutions of DMSO extracts of various natural or synthetic oils and subsequent exposure to NUV. Incident NUV fluence averaged 7.5 J per m^2 per sec. Oils were diluted to established MTD as listed in Table I. Symbols used are: (■) SAE 90; (●), LCO; (▲), KCO; (◆), WILM; (◇), LETC; (⊕), MIS 3; (⊗), MIS 185; (○), PRHO 601; (△), PRHO 602; and (□), SRC II. Codes for various oils are described in Table I.

Fig. 2. $6TG^R$ mutants induced per surviving fraction of CHO cells after exposure to dilutions of DMSO extracts of various natural and synthetic crude oils and NUV. Mutation frequencies have been corrected for plating efficiencies observed at time of selection. The background $6TG^R$ mutation frequency in control nontreated cells averaged 10 per 10^6 cells. Symbols used are: (●), LCO; (▲), KCO; (◆), WILM; (◇), PRHO 601; (⊕), LETC; (⊗), MIS 3; (⊗), MIS 185; (□), SRC II; and (△), PRHO 602. Codes for various oils are described in Table I.

refined SAE 90 wt oil showed no photo-induced mutagenicity in CHO cells. Hydrotreatment of the photoactive PRHO 601 shale oil essentially abolished the photo-induced mutagenic properties of this oil.

From these data it has been shown that NUV can activate natural and synthetic crude oils resulting in the formation of genotoxic products to cultured mammalian cells. The doses of NUV light necessary to kill cells (D_{37}) or induce significant mutations in certain shale oil treated cells ranged between 1300-2500 J per m^2 , which is equal to the amount of NUV in 1-2 min exposure of natural sunlight reaching the earth's surface. It is apparent that the contribution of phototransformation of complex mixtures into genotoxic agents must be accounted for in assessing potential health-related and environmental risks of complex industrial waste streams.

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Benzo(a)pyrene Metabolism and Mutation Induction in CHO Cells Using Either Rat Liver Homogenate or Syrian Hamster Embryonic Cell-Mediated Activation [R. T. Okinaka, G. F. Strniste, and D. J.-C. Chen]

Many carcinogens are chemically nonreactive and require metabolic activation by cellular enzymes before they can exert their biological effects. Many cell types are incapable of activating such procarcinogens. Among these are Chinese hamster ovary (CHO) cells, which are routinely used for studies involving mutagenesis. Mutagenesis in CHO cells has been studied by the addition of an enzymatically active liver homogenate (S9) fraction.¹ However, the metabolism of certain procarcinogens, such as benzo(a)pyrene [B(a)P], by liver homogenates has been shown to differ from that elicited by intact cellular systems.² Consequently, the biological effects induced by B(a)P metabolism may show considerable variation depending upon the activation system utilized.

In this study we have attempted to compare B(a)P metabolism by rat liver homogenates (S9) and in Syrian hamster embryonic (SHE) cells. In addition, a CHO mutation assay incorporating both of these activation systems is being used to measure both cytotoxic and mutagenic effects.

Using ethyl acetate extraction methods, the S9 fraction shows rapid water solubilization of B(a)P (> 80% after 2 h incubation) whereas the SHE cells show less than 50% solubilization even after prolonged incubation (24 h) (data not shown). Elution profiles of B(a)P metabolites from high pressure liquid chromatographic separations are shown in Fig. 1. The results indicate that B(a)P metabolites formed in the S9 preparation differ quantitatively and qualitatively from those formed in SHE cells. For SHE cells (Fig. 1, bottom panel), the major B(a)P metabolite is the 9, 10-diol. The 4,5- (or 7,8-) diols and the 7,8,9,10-tetrols (hydrolysis products of the ultimate carcinogen 7,8-diol-9,10-epoxide) are also present in lesser amounts in the medium. In addition to the variety of metabolites mentioned above, quinones (primarily the 1,6- and 3,6-) and very hydrophilic derivatives of B(a)P are produced in the S9 preparation (Fig. 1, top panel).

The pattern of B(a)P metabolites produced by x-ray treated (4000 r) SHE cells does not change

Fig. 1. HPLC elution patterns of B(a)P metabolites formed by rat hepatic S9 fraction (top) and SHE cells (bottom). The S9 reactions containing [^3H]-B(a)P were extracted with ethyl acetate at various times up to 2 h after the reaction was started. Dishes containing SHE cells were incubated with [^3H]-B(a)P for 48 h. The resulting metabolites were extracted into ethyl acetate at various times. Samples of standards of B(a)P metabolites were gifts from the NCI chemical repository.

qualitatively as a function of passage number (up to passage 10). This pattern in nonirradiated cells, however, does change with the disappearance of the 9,10-diol and the appearance of more hydrophilic species including tetrols (data not shown).

Chromatographic separations of conjugated B(a)P species using alumina columns and a 4-step gradient³ indicate that the two metabolic systems appear to differ significantly in their ability to conjugate B(a)P to sulfates, glucuronic acid, and glutathione. As shown in Fig. 2 (panel C), SHE cells conjugate B(a)P to products which have been presumptively identified as being sulfate containing. The S9 reaction appears to contain all three forms of

Fig. 2. Separation of conjugated B(a)P metabolites on alumina columns. The first peak contains primarily non-conjugated B(a)P and some sulfate conjugates. The metabolites in the 2nd, 3rd, and 4th peaks have been presumptively identified as conjugated sulfates, glucuronides, and glutathiones, respectively. Panel A: B(a)P with no activation; panel B: S9 activation (2 h); and panel C: SHE cell activation (48 h).

conjugated products (Fig. 2, panel B). These data suggest that the S9 preparations possess a greater potential in removing cytotoxic and mutagenic species by conjugation mechanisms. This notion is consistent with our previous results which showed that at a fixed B(a)P concentration increasing S9 concentrations are followed by the detoxification of cytotoxic and mutagenic events.⁴

Figure 3 shows the increase in frequency of induced mutations as a function of cell survival after treatment with B(a)P activated by S9 preparation or irradiated SHE cells. At 50% survival SHE cell mediated B(a)P induced mutation frequency is approximately 20 fold higher than that observed with rat liver S9 activation. We have also observed that the rate of induction of mutations in CHO is independent of passage number of the x-ray treated feeder layer SHE cells (data not shown).

Fig. 3. 6TG^R mutants induced in CHO cells by B(a)P activated by rat liver S9 (■) or SHE cells (●) as a function of the surviving fraction of cells.

This is consistent with the HPLC patterns observed for these x-ray treated cells.

SHE cells are clearly more efficient at inducing mutations in CHO than is the S9 preparation. Our results would suggest two explanations for this difference in mutation induction potential. S9 preparations, for one, appear to convert B(a)P into a wider variety of moieties than does the intact hamster cell. Some of these moieties, such as quinones, are probably cytotoxic to CHO cells without being able to exert significant mutagenic effects. In addition, the liver homogenate appears to possess more extensive conjugation mechanisms which would preferentially remove the more mutagenic oxide molecules.

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INTRODUCTION

The Los Alamos Life Sciences Division has maintained a world leadership role in instrumentation development and biological application of flow cytometry. Our group's major responsibility to this effort is providing the biological application support. Our inhouse programs include: studies to understand tumorigenic potential and the role that karyotypic instability plays in its expression, analysis, and sorting of chromosomes; study of lymphocyte and mononuclear phagocyte differentiation by analysis and sorting based on membrane antigen differences; evaluation of tumor heterogeneity; sorting fused cells from both hybridomas producing monoclonal antibodies and tumor cells for cloning particular genetic traits.

Our national outreach program includes ongoing collaborative studies with Dr. Jack Dean at the National Institute of Environmental Health Sciences, Dr. William Walker at St. Jude's Children's Research Hospital, Drs. Anderson, Standifer, Lanier, Walker, and Lewis at the University of New Mexico, Dr. George Olsen at the University of Arizona, and Dr. Noel Warner at the Becton-Dickinson Monoclonal Research Center. We expect our outreach program in

flow cytometry to increase to an even greater extent by providing sabbatical and short-term visiting scientist opportunities for noted scientists to solve biological problems which can only be done at Los Alamos. In this way we can provide for them advanced instrumentation while they provide to us a new dimension, application, and experience for flow cytometry, thus insuring our continued leadership role in this field.

We also have a strong commitment towards understanding the mechanisms involved in lung damage from environmental pollutants. We feel the most important question to answer is: How long and at what level can a population be exposed to a noxious substance after which time normal repair mechanisms can no longer prevent the onset of degrading disease, e.g., fibrosis? Are some individuals more susceptible than others? Our philosophy in answering these questions is to provide objective criteria for identifying high risk individuals. In this way the energy industry will be better able to prevent injury before it occurs while effectively obtaining our natural resources safely. This effort includes our lung biochemistry program, and tumor heterogeneity and cytogenetics programs.

A most important program deals with the understanding of the neoplastic process. This includes studies involving identification of transformed cells using phenotypic markers and subsequent karyotypic analysis to recognize possible chromosomes involved in progression to aneuploidy. In addition, we now recognize that several growth regulating substances (e.g., epidermal growth factor, platelet derived growth factor, macrophage growth factor) as well as inhibitors are involved in exquisitely regulating cell proliferation. The

isolation, characterization, and identification of the mode of action of these substances on normal and neoplastic cells is of paramount importance to our understanding of normal cell growth and neoplastic transformation. Thus, as we face a difficult economic period we are encouraged by the tremendous scientific progress and state-of-the-art technology which exists at Los Alamos, some of which is briefly described below. We look forward to our continued productivity in each of these areas as we continue to strive to be at the forefront of scientific discovery in Life Sciences.

An Assay for Collagenase Using Peroxidase-Labeled Collagen as a Substrate

[E. H. Tate]

The chronic lung diseases, fibrosis and emphysema, are characterized by abnormalities in the biosynthesis and degradation of collagen and elastin, the major connective tissue components in the lung. Abnormal collagen deposition is indicative of fibrosis, while abnormal destruction of elastin characterizes emphysema. We are currently conducting studies to determine what biochemical changes occur in rat lungs after the animals are exposed to various doses of raw and spent shale oil particulates by inhalation. Determinations of lung collagen and elastin contents and the activity of several degradative enzymes found in fluid lavaged from rat lungs are being made on treated and control rats.

Measurement of the degradative enzyme activities necessitated the development of enzyme assays. This report describes our newly developed assay for collagenase using horseradish peroxidase (HRP) conjugated collagen as the substrate. Enzyme activity is measured according to the following scheme:

1) Collagen - HRP $\rightarrow$ Degraded substrate + Free HRP.

2) Free HRP + 2,2'-azino-di-(3-ethyl benzthiazoline-6-sulfonic)acid (ABTS) + hydrogen peroxide (H_2O_2) $\rightarrow$ Color production (414 nm).

The degradative enzyme, collagenase, if present in the unknown solution, cleaves the substrate, releasing HRP into solution. The HRP is then free to reduce its substrate (H_2O_2) which in turn oxidizes the chromagen (ABTS) to produce a color reaction. If no degradative enzyme is present HRP is not released, no reaction of HRP with its substrate occurs, and no color is produced. The concentration of original enzyme is quantified by spectrophotometrically measuring the amount of color produced after a known incubation time.

A preliminary report of a similar method utilizing HRP-elastin to measure elastase activity was presented in the 1980 Life Sciences Annual

Report by George Saunders and Zita Svitra of the Experimental Pathology Group.¹

A modification of the method of Nakane was used for conjugation of HRP to collagen.² The carbohydrate moieties of HRP were oxidized to aldehyde groups with $NaIO_4$. Subsequently the oxidized HRP was reacted at high pH with collagen. The Schiff base (HRP-HC=N-R-collagen) which was formed was then reduced with sodium cyanoborohydride to a more stable form (HRP-H-C-NH-R-collagen).

In order to immobilize the insoluble HRP-collagen substrate so that HRP released by collagenase could be monitored in the supernatant, a concentrated suspension of the conjugate was nonspecifically bound to polystyrene tubes. Figure 1 shows that 4 h was the optimum time for coating the tubes at room temperature and that some loss of activity occurred when the tubes were coated overnight. It was also found that the coating suspension could be reused numerous times, since only a very small amount of conjugate was bound after one use.

The assay was standardized using bacterial collagenase. Immediately after removing the HRP-collagen coating suspension and carefully rinsing the coated tubes, the following additions

Fig. 1. Collagenase activity as a function of time of coating polystyrene tubes. Tubes were coated for the indicated times and subsequently assayed for collagenase activity as described in the text. The supernatants were then removed and assayed for HRP activity. Results are expressed as absorbance read at 414 nm (proportional to collagenase activity).

were made: 1) 5 mM CaCl_2 ; 2) 30 $\mu\text{g/ml}$ bovine serum albumen (BSA); 3) 50 mM TRIS buffer, pH 7.5; and 4) 4 $\mu\text{g/ml}$ bacterial collagenase (Sigma Type IA) to start the reaction. The reaction mixture was then shaken at 37°C , and aliquots of the supernatant were removed and assayed for HRP activity at the desired times.³ Figure 2 shows that color production increased with time for at least 3 h when a HRP reaction time of 15 min at room temperature was used. Blanks with no collagenase gave negligible O.D. readings.

When an unknown solution, such as fluid lavaged from rat lungs, was to be tested for collagenase activity, the unknown solution was used in place of bacterial collagenase. It was also necessary to preincubate lavage fluid with H_2O_2 to inactivate endogenous peroxidases fluid which interfere with the HRP reaction.

The collagenase assay using HRP-collagen was compared to a similar assay using HRP-fluorescein as the substrate. Calf skin collagen was fluoresceinated by the method of Baici and coworkers.⁴ The fluorescein-collagen conjugate was bound to plastic tubes and the collagenase assay was carried out in the same manner as with the HRP-collagen conjugate except that free fluorescein instead of HRP in the supernatant was measured. The fluorescein assay was very reproducible. However, the spontaneous release of fluorescein in the absence of collagenase was

Fig. 2. Collagenase activity as a function of incubation time at 37°C . Tubes were coated for 4 h and assayed for collagenase activity. Supernatants were then removed and assayed for HRP activity.

very high (20-40% of the experimental sample containing collagenase). Using the HRP-collagen conjugate, a similar blank was only 2-10% of the experimental sample.

The assay using the fluorescein-collagen conjugate was also less sensitive than the assay with the HRP-collagen conjugate. When it is necessary to measure very small amounts of released HRP (i.e., very low collagenase activity), the HRP reaction time can be lengthened and the reaction temperature raised to enhance detection. On the other hand fluorescein fluorescence cannot be enhanced in a similar manner. It is also very convenient with unknown samples to be able to detect the color reaction by eye. With experience, one can pick the proper HRP reaction time merely by observation of the progression of color development.

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A Rapid, Sensitive Colorimetric Microplate Assay for Hydroxyproline

[M. S. Halleck]

Pulmonary fibrosis, a debilitating, often fatal, chronic disease is generally recognized as a derangement of collagen metabolism, often leading to focal scarring or diffuse increases in pulmonary collagen. These changes may be observable biochemically through measurements of the imino acid hydroxyproline, which occurs almost exclusively in collagen protein.¹ Thus, studies of the fibrogenicity of inhaled oil shale dusts require a rapid,

sensitive method for measuring hydroxyproline. The processing of a large number of samples from lungs of animals exposed to various oil shale byproducts, as well as the desire to measure small amounts of collagen in lavage fluids and tissue culture medium from cultured cells, requires a rapid, sensitive method for hydroxyproline determination to determine sequelae leading to fibrogenicity of energy-related products. Although a relatively sensitive, automated assay for hydroxyproline using a Technicon autoanalyzer and the colorimetric method of Stegemann², as modified by Grant³ and further modified by Pickrell *et al.*, for increased sensitivity has been described,⁴ the use of an autoanalyzer is not readily available to us. With the development of enzyme-linked immunoassays (EIA) at Los Alamos and the purchase of a multiple channel photometer for through-the-plate reading of microtiter plates (Titertek Multiscan, Flow Laboratories) the idea was developed to adapt the autoanalyzer colorimetric method to a microplate assay for hydroxyproline. The method turns out to be sensitive (detecting as low as 50 to 100 ng hydroxyproline/ml), rapid (1 h), and adaptable to a large number of samples at one time (there being 96 wells in a microtiter plate).

As in the original method by Stegeman,² hydroxyproline is oxidized by chloramine T, excess chloramine T is destroyed by perchloric acid, and the hydroxyproline chromogen is then reacted during heating with p-dimethylaminobenzaldehyde to form a pink color. The absorption is then measured at 550 nm. Reagents used are the same as the Grant procedure³ in about the same relative proportions. Aliquots of sample (125 μ l) are pipetted into wells of a flat-bottomed microtiter plate and 50 μ l of 12 mM chloramine T solution prepared according to Grant³ are added. Contents of the wells are mixed and allowed to react for 15 min. At this time excess chloramine T is destroyed by addition of 25 μ l of 20% perchloric acid. The contents of the wells are again mixed and after a period of no longer than 3 min 100 μ l 5% PDA (in n-propanol) are added to each well, with mixing. Plates are then sealed with clear tape and placed in an oven at 75°C to heat for 20 min. Following this heating period the plates are allowed to cool slowly at room temperature for approximately 20 min, after which

the absorbances of the contents of 8 wells (1 row) are read simultaneously at 550 nm on the Titertek Multiscan. Blanks are set up in the left-most row of the plate. All twelve rows of eight wells can be read in about 40 sec. Using a total of 300 μ l of solution per well, the length of the light path during optical measurement was calculated to be 0.906 cm.

A calibration curve for hydroxyproline standards measured by this assay is shown in Fig. 1. Absorbance is linearly related to concentration from 100 ng/ml to 4 μ g/ml. The relationship became nonlinear above this concentration. Reproducibility from day to day was fairly good. The method was optimized for the best color yield by adjusting the final chloramine T concentration, final volume in the well (which changes the path length), temperature of incubation, length of incubation, cooling time, and PDA concentration. Lower concentrations of chloramine T reduce the color yield, especially in the presence of other amino acids. Concentrations higher than 3.4 mM final (50 μ l 12 mM chloramine T in a total of 175 μ l) were also found to depress the color reaction. Increased incubation

Fig. 1. Standard curve showing absorbance at 550 nm vs concentration of hydroxyproline.
 $A_{550} = 0.278[\text{Hypro}] + 0.013$.

times or increased temperature also depressed the color yield. Contrary to the autoanalyzer method, in which a cooling coil is employed following the heating step, slow cooling at room temperature produced more color than rapid cooling on ice. Increased concentration of PDA also depressed the color reaction.

Interference by chromogens formed by other amino acids is presently being examined. An obvious possibility is the amino acid proline, similar to hydroxyproline and a common amino acid in collagen protein. Proline has been reported to react some 200 times more slowly than hydroxyproline in the above reactions,⁵ however. Indeed, when proline was added to blanks and hydroxyproline standards at 5 times the concentration of hydroxyproline, the color yield was not enhanced. Whether increased absorbances result from chromogens formed by amino acids other than proline and hydroxyproline remains to be determined. These do not appear to be a problem with the autoanalyzer method, however (J. A. Pickrell, personal communication).

It is expected that this method will prove to be useful for rapid analysis of hydroxyproline in lung and tissue culture samples. Replicate samples and standards assayed using this method gave virtually identical results to those obtained using the autoanalyzer at the Inhalation Toxicology Research Institute, Albuquerque, NM.

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Optimization of Culture Conditions for the Growth of Rat Bone Marrow and Alveolar Macrophages

[G. C. Saunders and A. Martinez]

A library of methodologies is being developed to answer questions regarding the toxicity of inhaled pollutants relevant to the oil shale industry. One measure of toxic exposure is to evaluate the clonogenicity of macrophages present in exposed animals compared to that in control animals of a similar age and genetic makeup. The Fisher 344 rat has been chosen as the experimental animal used in inhalation studies at Los Alamos. Data in the literature concerning the culture of rat macrophages are very limited;¹ we therefore proceeded to define and optimize requirements for their culture.

The limited rat macrophage culture data suggests that rat cells require growth factors obtained from the culture of rat fibroblasts, rat serum, either bovine or horse serum, plus a well-balanced tissue culture medium. Conditioned medium containing macrophage growth factors was obtained by treating the lungs from 9-day-old rats in one of two ways; they were either passed through a wire screen to obtain single cell suspensions or they were minced into small (1-2 mm²) pieces. Single cells or minced lungs were cultured in T 75 flasks (1 lung/flask) for 7 days in D-MEM with 10% fetal calf serum. The types of cell growth obtained in the two types of culture were markedly different. The minced preparation consisted mainly of fibroblast colonies (Fig. 1) with a few widely scattered round cell colonies (probably macrophages). The screened culture contained an almost confluent layer of adherent macrophages (Fig. 2); interspersed were a few fibroblast colonies. Medium from both types of culture was harvested, filtered, and used to evaluate its capability to support the growth of bone marrow macrophage colonies in agar. The medium used was D-MEM containing varying amounts of conditioned medium with 10% normal rat serum and 10% FCS in 0.75% agarose (Sea Plaque, FMC Corp.). Cultures containing conditioned medium from minced cultures exhibited two types of colonies, diffuse and spheroidal (see Figs. 3, 4), while cultures

Fig. 1. Photomicrograph of a typical fibroblast colony which developed in minced lung cultures. Notice that 1-micron fluorescent microspheres are not ingested by the cells. Compare to Fig. 2.

Fig. 2. Photomicrograph of macrophages obtained from screened lung culture. One-micron fluorescent microspheres added to the culture were readily ingested.

Fig. 3. Photomicrograph of a spherical colony obtained from rat bone marrow cells cultured in both screened and minced rat lung conditioned medium.

Fig. 4. Photomicrograph of a diffuse colony which is only obtained from rat bone marrow cells cultured in minced rat lung conditioned medium.

containing conditioned medium from the screened lung cultures contained only the spheroidal colonies. Colony growth required conditioned medium but the number of colonies per culture remained relatively constant in the range of 1-20% of added conditioned medium. Table I summarizes the bone marrow colony growth data.

On the basis of this and several similar experiments, the minced conditioned medium was selected as the standard conditioned medium. Other experiments using bone marrow and lung lavaged macrophages were set up to optimize the concentration of rat serum, fetal calf serum, and conditioned medium both in liquid and agar cultures. Also, various basic tissue culture media (D-MEM, RPMI 1640, RPMI 1634) were evaluated. Little difference was observed among the various basic media tested. The working medium developed and now used routinely for culturing either bone marrow or lavaged cells in liquid culture is presented in Table II and for agar colony culture is presented in Table III. Applications using these media are presented elsewhere in this annual report.²

TABLE I
COLONIES PER 10⁴ BONE MARROW CELLS

Percent Conditioned Media	<u>Minced Conditioned Medium^a</u>		<u>Screened Conditioned Medium</u>	
	<u>Diffuse Colonies</u>	<u>Spherical Colonies</u>	<u>Diffuse Colonies</u>	<u>Spherical Colonies</u>
None	0	0	0	0.25
1	10	11	0	9.5
5	13	11	0	11
10	12	13	0	9
20	8	12	0	8

^aMean of 4 cultures.

TABLE III

PROTOCOL FOR PREPARING MEDIUM FOR MACROPHAGE COLONIES IN AGAR

- A. To 100 ml basic medium (DMEM, RPMI 1640, RPMI 1634) add 1.5 gram Sea Plaque agarose and autoclave.
- B. To 35 ml of A add:
1. 7 ml fetal calf serum
 2. 7 ml rat serum
 3. 20 ml lung conditioned medium
 4. 0.7 ml sodium pyruvate (0.11 gm/liter)
 5. 0.7 ml glutamine (200 mM)
 6. 0.7 ml Penicillin, streptomycin fungizone^a
- One ml of the above is added to 35-mm culture dishes on day(-1). On the day of the experiment, the cells to be plated are mixed with 1 ml of the same mixture and then added to the culture dishes.
- C. After the agarose containing the cells has hardened add 1 ml of liquid medium (Table II).
- D. Incubate the cultures at 37°C in a humid atmosphere containing 10% CO₂. Bone marrow colonies are counted on day 7; lung cell colonies are counted on day 21.

TABLE II
MEDIUM FOR LIQUID MACROPHAGE CULTURES

<u>Ingredient</u>	<u>Amount (volume %)</u>
Lung Conditioned Medium	30
Normal Rat Serum	5
Fetal Calf Serum	10
Pyruvate (0.11 gm/liter)	1
L-Glutamine (200 mM)	1
Penicillin, Streptomycin, Fungizone ^a	1
Basic Medium ^b	52

^aPenicillin 10,000 units/ml; streptomycin 10,000 mcg/ml; Fungizone 25 mcg/ml.

^bRPMI 1634, RPMI 1640, or D-MEM.

^aPenicillin 10,000 units/ml; streptomycin 10,000 mcg/ml; Fungizone 25 mcg/ml.

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Differentiation of Mononuclear Phagocytes

[C. C. Stewart, E. Walker (University of New Mexico), and W. Walker (St. Jude Children's Research Hospital)]

The mononuclear phagocyte system is being used as a model system to understand normal cell differentiation because of the diversity of functions these cells express. This diversity can be thought to occur in several ways. First, expression of any function can represent a differentiated state through which all cells pass. Second, it could represent differentiation of progenitor cells into subpopulations which express a particular function; each functional subpopulation would sum together in the whole population to manifest the functional heterogeneity observed. Third, tissue-specific substances may induce or modulate functional expression. Finally, any combination of the three above processes may occur.

In order to evaluate which of the above possibilities are manifested, we have selected surface membrane markers as a sign to identify macrophages within a particular population. Since the legends to the markers can be fluoresceinated, we are able to identify and sort the subpopulations using flow cytometry.

Briefly, murine bone marrow cells are grown for 5-7 days as previously described.¹ The nonadherent and adherent cells are separated and analysed. While the experiments have not been completed, several results indicate the feasibility of the approach.

As shown in Fig. 1, culture derived nonadherent and adherent cells label with MAC I, a monoclonal antibody which labels all mononuclear phagocytes. The monoclonal antibody E-2, however, is able to distinguish virtually all progenitor cells (found in the nonadherent population) from the more mature macrophages. In contrast, the monoclonal antibody H-12 labels only the more mature, adherent cells. While the antigen on macrophages for which these two

Fig. 1. C₃H bone marrow marker positive populations determined by flow cytometry. Nonadherent and adherent cell populations were obtained, adjusted to 10⁶ cells/ml in PBS containing 2% fetal bovine serum, and stained with the appropriate reagent. MAC 1 is a monoclonal antibody, kindly supplied by Timothy Springer with specificity against all mononuclear phagocytes. E-2 and H-11 monoclonal antibodies were obtained using mouse spleen cells as immunogens and kindly supplied by Jeff Ledbetter and Leonard Herzenberg. RA31B6, kindly supplied by Robert Koffman, was obtained after rat immunizations with a B-cell tumor line. DNL-1.9 was obtained from Mike Loken, who used a B-cell tumor as the immunogen.

antibodies is specific is not known, it is clear the antibodies can be used for sorting progenitor cells from more mature cells.

Using the whole nonadherent cell population we grew colonies from them. We reasoned that if every cell in a colony were marker positive, then the cells would have cloned for that marker. In contrast, if only some cells in the colony were positive, then differentiation states through which all cells pass would be the most likely interpretation. The data obtained for 5 markers is shown in Fig. 2.

Nearly all colonies formed on day 4 from bone marrow have a variable proportion of C3 and Fc receptor positive cells. In contrast, the appear-

Fig. 2. Distribution of marker positive NMP in colonies. Bone marrow from C₃H (H-2K) or DBA (H-2d) mice was cultured in growth medium at 10⁵ cells/ml. Three ml were plated and incubated in two chamber lab-tek slides for four days. (Plastic tends to increase background adherence of SRBC.) Sheep RBC (SRBC) were coated with the appropriate monoclonal using 0.1% chromic chloride. In the figure we have plotted the percent of colonies (ordinate) as a function of the percent of cells in the colony which are positive for the marker (abscissa). In A, showing colonies and cells which label for the complement receptor C3b, there were no marker positive cells found in 6% of the colonies while 20% of the colonies had over 90% of their cells positive. These data were obtained in collaboration with Wm. S. Walker, D. M. Gandour, and P. B. Hester.

A. C3b receptor positive cells in colonies. B. Combined data of cells in colonies which have receptors for IgG2a and IgG2b. C. Ia 2 receptors on cells in colonies from C₃H (H-2k) mice. D. Ia 2 receptors on cells of colonies derived from the irrelevant haplotype, DBA/2 (H-2d). We interpret the 13 and 22% of colonies having 1-10% positive cells from the C₃H and DBA mice, respectively, as background.

ance of Ia appears to be on cells clonally derived. There are some colonies which have less than 10% positive cells in both the C₃H (relevant) and DBA/2 (irrelevant) haplotype for the monoclonal antibody used (Ia 2 or 17); we interpret this as a background. Only the relevant haplotype shows colonies containing 50 to 100% of positive cells. We found that 15% of nonadherent progenitor cells obtained 4 days after culture of bone marrow were Ia positive. This is similar to the percentage of Ia positive colonies. We recognize the importance of showing that Ia positive progenitor cells directly give rise to Ia positive colonies and experiments designed to do this are in progress. The colony forming ability of cells for this marker and its stability in time is of paramount importance because of the recent findings that Ia can be induced in macrophages by lymphokine-rich supernatants.^{2,3} While this may be

so, such a result is not mutually exclusive of the possibility that some, indeed perhaps the most important, macrophages have Ia all the time because they are the differentiated progeny of Ia positive progenitor cells. Two other laboratories using mass cultures of BMC have shown that Ia positive markers are present on them.^{4,5}

In looking at Fig. 2 some colonies contain only 50 to 90% Ia positive cells. We believe colonies which contain less than 90% positive cells are actually mixed colonies produced by an Ia positive and an Ia negative progenitor cell in close proximity or even aggregated. The evidence for this possibility is as follows. Ia positive cells within the mixed colonies are always in a group, they are never found randomly dispersed throughout the colony; the mixed colonies on day 4 have more cells in them than could have been produced by a single

progenitor cell, if that cell has the observed 16-h generation time; and finally, 5% of the initial cells in the bone marrow are in aggregates when they are plated.

Using the flow cytometer it will be possible to sort Ia positive progenitor cells which are not aggregated. When this is done we will be able to definitively prove whether Ia positive progenitor cells give rise to Ia positive clones (not colonies) whose progeny maintain the marker in a stable manner. While this would be an important finding, perhaps the most important finding will be whether these cells work as accessory cells in immunologic responses.

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Proliferation Kinetics of Mononuclear Phagocytes of the Bone Marrow

[C. C. Stewart and S. J. Stewart]

We have been studying the proliferation kinetics of bone marrow mononuclear phagocytes for several reasons. First, this cell system is a unique normal cell system which can be used to identify and unravel the mechanisms which control proliferation. Insights in this area may lead to fundamental understanding of neoplasia and how the body responds to infectious insults. Second, due to the diversity of function with this system, large

numbers of homogeneous populations of functional cells can be obtained for study. Finally, the senescence of cell division can be studied leading to a better understanding of the process of aging.

For this study, bone marrow cells were derived from C₃H mice and cultured in growth medium. The growth medium consisted of α MEM supplemented with 10% fetal bovine serum and 10% L-cell conditioned medium. The latter medium is a source of macrophage growth factor which is absolutely required for macrophage proliferation.¹

In Fig. 1, we have plotted the number of cells per culture as a function of time during the first week in culture. During the first 3 to 4 days of culture there is a dramatic decrease in the number of nonadherent cells recovered. Thereafter, they

Fig. 1. Growth of bone marrow cells. Bone marrow cells were derived from the femur of C₃H mice (yield 10⁴ cells/femur). Cells were adjusted to 10⁴ cells/ml growth medium (α MEM supplemented with 10% fetal (or newborn) bovine serum and 10% LCM) and 25 ml were plated in 75-cm² culture flasks. As a function of time cells were counted. Adherent cells were removed with the detergent getrimide as previously described.²

tend to stabilize in number. In contrast, adherent cells exponentially increase with a doubling time of about 24 h.

We removed the nonadherent cells daily and plated them in new dishes to evaluate the ability of these cells to form macrophage colonies. The results are shown in Fig. 2. The initial plating efficiency of bone marrow was 0.8% but by day 6, 60% of the nonadherent cells will form macrophage colonies. This increase is apparently the result of cell death by other nonadherent formed elements combined with a slow proliferative expansion of the nonadherent progenitor cell pool.

In order to confirm the latter point, non-adherent cells were serially subcultured every 3 or 4 days in growth medium. The results are shown in Fig. 3. Each subculture of nonadherent cells resulted in recovery of nonadherent cells about equal to the number initially plated. In addition, adherent cells were produced and these proliferated exponentially with doubling in about 24 h. Since the nonadherent cell yield has about the same as the number initially plated, we interpret this to mean that on the average, each nonadherent cell division resulted in the production of one nonadherent and one adherent cell. The adherent cells were not removed when the culture was rinsed.

These cells, however, cannot proliferate indefinitely and as shown in Fig. 3, by 4 wk no more nonadherent cells can be recovered from the culture and adherent cells have ceased to proliferate. Nevertheless, we calculate that each original progenitor cell was capable of undergoing about 27 divisions to produce about 10^8 progeny. This represents a tremendous proliferative potential and if all the progenitor cells from a single mouse femur were cultured, 1000 kg of macrophages could be obtained.

Fig. 2. Colony formation by cultured bone marrow nonadherent cells. Cells derived daily from cultures described in Fig. 1 were serially diluted 1/10 in growth medium and plated in 35-mm culture dishes. Cells were stained 7 days later and colonies counted.

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Fig. 3. Maintenance of nonadherent cell proliferation. Bone marrow cells from $C_{3}H/An$ mice were plated in 35-mm culture dishes at 3×10^5 cells/culture in 3 ml of growth medium. Every 3 or 4 days the nonadherent cells (X) were removed, pooled, and counted. These cells were then replated (3 ml) into new culture dishes. Fresh growth medium was added to the adherent cells (O, ●, △, ▲, □, ■, ◇) and they were counted a week later.

Sister Chromatid Exchange (SCE) Analysis in Bone Marrow of Mice Treated With Shale Oils and Shale Process Waters

[Julianne Meynel]

Analysis of SCE (symmetrical interchange between sister chromatids) and structural aberrations observed in metaphase chromosomes are two parameters commonly used to assay for genetic damage induced by environmental agents. SCE analysis is often considered to be the more sensitive test because many chemicals induce significantly increased frequencies of SCEs at doses below those required to induce increased frequencies of structural aberrations. Experimental evidence suggests, however, that despite some common features, structural aberrations and SCEs have

independent mechanisms of formation,¹ and the quantitative relationship between SCEs and structural aberrations depends upon the mechanism of action of the agent used.²

Previous cytogenetic analysis has shown that crude shale oil (PCSO) and hydrotreated shale oil (PCSO-HT) from an aboveground retort and crude shale oil (OCSO) from a modified vertical *in situ* retort induce increased frequencies of structural aberrations in murine bone marrow chromosomes (Table I). Process waters from aboveground (PHRR), modified vertical *in situ* (OCCD), and true *in situ* (GEOK) retorts also induced slightly elevated frequencies of chromosomal aberrations (Table II). The purpose of this study was to determine whether SCE would provide a more sensitive method of screening for DNA damage induced by these complex mixtures.

Female Swiss (CD-1) mice were treated with three intraperitoneal injections of either shale oil diluted in olive oil or process water diluted in saline given at 24-h intervals. Mice were killed 4 h after the last injection. Detection of SCE was based on bromodeoxyuridine tablet methodology and a modified fluorescence plus Giemsa staining procedure. Table I shows the results of the SCE analysis after exposure to shale oil. The PCSO sample induced dose-related increases in the frequency of SCE, but even at the 2-ml/kg dose this increase was only about 50% above the control level. At this dose, the frequency of structural aberrations was five times the control value. The PCSO-HT and OCSO samples demonstrated SCE frequencies within the control range at all doses tested. The known positive control treatment with 5 mg/kg cyclophosphamide was within the expected range for this agent.

Table II shows the results after treatment with the three process waters. No increase in frequency of SCEs was observed, although the doses tested induced about a two-fold increase of structural aberrations. Therefore, it appears that structural aberration analysis is more sensitive than SCE analysis for evaluation of the types of DNA damage induced in murine bone marrow by shale oils and process waters.

TABLE I
SCE ANALYSIS OF BONE MARROW OF FEMALE CD-1 MICE
AFTER INTRAPERITONEAL INJECTION OF SHALE OIL

<u>Treatment</u> ^a	<u>Mean Number of Structural Aberrations per cell $\pm$ s.d.</u>	<u>Mean Number of SCE per cell $\pm$ s.d.</u>
untreated	0.008 $\pm$ 0.006	6.84 $\pm$ 0.44
olive oil	0.009 $\pm$ 0.006	7.40 $\pm$ 0.35
0.5 ml/kg PCSO	0.023 $\pm$ 0.008	8.82 $\pm$ 0.42
1.0 ml/kg PCSO	0.026 $\pm$ 0.009	9.22 $\pm$ 0.59
2.0 ml/kg PCSO	0.045 $\pm$ 0.012	10.68 $\pm$ 0.32
0.5 ml/kg PCSO-HT	0.015 $\pm$ 0.007	6.57 $\pm$ 0.21
1.0 ml/kg PCSO-HT	0.017 $\pm$ 0.006	6.10 $\pm$ 0.17
2.0 ml/kg PCSO-HT	0.029 $\pm$ 0.011	6.84 $\pm$ 0.35
0.5 ml/kg OSCO	0.041 $\pm$ 0.014	6.36 $\pm$ 0.06
1.0 ml/kg OSCO	0.025 $\pm$ 0.009	6.46 $\pm$ 0.51
2.0 ml/kg OSCO	0.022 $\pm$ 0.007	6.82 $\pm$ 0.35
5.0 mg/kg CP		16.80 $\pm$ 0.97

^aPCSO, crude shale oil from aboveground retort; PCSO-HT, hydrotreated shale oil from aboveground retort; OSCO, crude shale oil from modified in situ retort; CP, cyclophosphamide.

TABLE II
SCE ANALYSIS OF BONE MARROW OF FEMALE CD-1 MICE AFTER
INTRAPERITONEAL INJECTION OF PROCESS WATERS

<u>Treatment^a</u>	<u>Mean Number of Structural Aberrations per cell $\pm$ s.d.</u>	<u>Mean Number of SCE per cell $\pm$ s.d.</u>
untreated	0.008 $\pm$ 0.006	6.73 $\pm$ 0.55
saline	0.007 $\pm$ 0.006	6.67 $\pm$ 0.47
1.0 ml/kg PHRR	0.015 $\pm$ 0.012	6.65 $\pm$ 0.57
2.0 ml/kg PHRR	0.014 $\pm$ 0.008	6.53 $\pm$ 0.39
4.0 ml/kg PHRR	0.020 $\pm$ 0.007	6.74 $\pm$ 0.44
1.0 ml/kg OCCD	0.013 $\pm$ 0.009	6.01 $\pm$ 0.65
2.0 ml/kg OCCD	0.015 $\pm$ 0.007	6.48 $\pm$ 0.25
4.0 ml/kg OCCD	0.018 $\pm$ 0.008	6.55 $\pm$ 0.68
1.0 ml/kg GEOK	0.011 $\pm$ 0.009	6.34 $\pm$ 0.19
2.0 ml/kg GEOK	0.015 $\pm$ 0.008	6.60 $\pm$ 0.38
4.0 ml/kg GEOK	0.020 $\pm$ 0.007	6.46 $\pm$ 0.66
5.0 mg/kg CP	-	15.57 $\pm$ 0.46

^aProcess water designations: PHRR, aboveground retort; OCCD, modified in situ retort; GEOK, true in situ retort; CP, cyclophosphamide.

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Sister Chromatid Exchange Rates in Geokinetics Oil Shale Workers

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

The development of alternative energy technologies will be accompanied by a certain degree of occupational risk to those working in processing plants. There exists a need for assessing genetic damage to workers exposed to a wide variety of genotoxic chemicals. A suitable test must be sensitive and reliable and use readily accessible human tissue or body fluid. The sister chromatid

exchange (SCE) assay, applied to human peripheral lymphocytes, fulfills these criteria.

Geokinetics, Inc. is currently developing a true in situ oil shale retorting process at their "Kamp Kerogen" site in Uintah County in northeast Utah. It is a unique process that does not involve any mining, in that the shale bed is explosively fractured, and retorting is carried out underground.

We have participated in a comprehensive occupational health survey of the Geokinetics oil shale workers and adult members of the families living at the site. The survey was conducted in conjunction with the Rocky Mountain Center for Occupational and Environmental Health of the University of Utah, and Health Division's Occupational Medical Group (H-2) and the Industrial Hygiene Group (H-5) of the Los Alamos National Laboratory. The objectives are to determine if environmental hazards exist in the workplace, determine the current health status of the group, and to establish a data base for prospective epidemiological studies. There are no "unexposed" people for controls in the area. The

families live "downwind" from the work site and the wives wash the clothes of the workers.

The medical examinations of the employees were carried out at the Geokinetics site in order to disrupt operations as little as possible. At this time blood was drawn from each individual into heparinized tubes for the SCE assay, placed in insulated boxes, and hand carried by air back to Los Alamos.

Lymphocyte cultures were initiated in triplicate on 15 individuals each day (for two days) within 10-13 h of the blood collection time. The

culture medium consisted of RPMI 1640 supplemented with 15% fetal calf serum or autologous serum, penicillin and streptomycin, and phytohemagglutinin M (Gibco). The thymidine analog, 5-bromodeoxyuridine, was added to a final concentration of 80 μ M (25 μ g/ml). Two hours prior to harvest, Colcemid (final concentration 0.08 μ g/ml) was added to the cultures and metaphase chromosome preparations were made at 66 h. Sister chromatids were differentiated using a fluorescent plus Giemsa staining technique¹ routinely used in our SCE screening laboratory.

TABLE I
SCE RATES IN GEOKINETICS OIL SHALE WORKERS AND FAMILIES

Individual	Sex	Age	Serum Used in Culture			
			Fetal Calf		Autologous	
			X SCE/cell ± Standard Error	Range n=25	X SCE/cell ± Standard Error	Range n=25
23	M	28	6.40 ± 0.40	3-10	6.80 ± 0.55	2-12
11	M	31	7.04 ± 0.51	3-15		
25	M	25	7.44 ± 0.47	3-13		
26	M	27	7.48 ± 0.53	2-13	7.88 ± 0.52	3-13
33	M	37	7.68 ± 0.53	4-14		
28	M	29	7.72 ± 0.49	4-14		
34	M	32	7.76 ± 0.50	4-13	6.32 ± 0.49	3-14
6	M	37	7.80 ± 0.39	4-11		
18	F	34	8.0 ± 0.42	3-15		
22	F	35	8.04 ± 0.45	4-16	11.20 ± 0.59	7-18
9	M	20	8.04 ± 0.53	4-14		
15	M	27	8.24 ± 0.58	4-14		
3	M	25	8.48 ± 0.68	4-18		
4	M	34	8.68 ± 0.72	3-16	8.16 ± 0.55	3-16
27	M	27	9.00 ± 0.55	5-14		
21	F	28	9.20 ± 0.77	3-20		
24	M	26	9.52 ± 0.53	5-17	10.92 ± 0.85	5-22
19	F	29	9.60 ± 0.70	5-19		
17	F	17	9.60 ± 0.67	5-16		
1	M	32	9.96 ± 0.88	3-20		
16	F	15	10.20 ± 0.61	5-15	7.52 ± 0.60	3-17
5	M	31	10.24 ± 0.72	2-18		
13	M	34	10.44 ± 0.74	5-19		
8	M	27	10.48 ± 0.84	4-19	10.36 ± 0.59	4-17
12	M	38	10.80 ± 0.69	6-19		
14	M	28	10.88 ± 0.86	4-20	7.60 ± 0.59	3-15
2	M	51	10.92 ± 0.75	5-19		
20	F	47	11.00 ± 0.88	3-24		
10	M	51	12.04 ± 0.85	6-20	10.0 ± 0.57	7-17

SCE rates were determined by counting the exchanges in second division chromosomes from metaphase cells that had 46 chromosomes. Initially 25 cells were scored per individual from cultures supplemented with 15% fetal calf serum. These results (Table I) were ranked in order from the lowest to the highest rates and a matched set of 10 slides was scored (25 cells) from the cultures supplemented with autologous serum. The SCE rates on all 29 individuals fell within normal ranges. SCE rates per individual in cultures using fetal calf serum were essentially the same as those cultured in autologous serum. These results will serve as baseline rates of SCE frequency for each individual in longitudinal studies. Results are available for 22 male workers ages 20 to 51 (1 culture did not grow) and 7 female family members, ages 15 to 47. Future samplings from these workers and their families will provide an opportunity to find out if the SCE rates change as a result of working and living in this unique environment.

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Comparison of Direct-Acting Cytogenetic and Mutagenic Effects of a Shale Oil Retort Process Water

[J. Meyne and D. J. Chen]

Process water from an aboveground retort contains direct-acting (without activation) mutagens which produce substantial mutagenic activity in 6-thioguanine-resistant (6-TG^R) mutants of human embryonic skin fibroblasts. Less severe effects are observed in Chinese hamster ovary (CHO) cell mutants. Since many mutagenic agents are also clastogenic, we have compared the cytogenetic and mutagenic effects of this process water under the same experimental conditions. Table I shows the results in CHO cells and Table II the results in human fibroblasts. The mutation enhancement index was derived by dividing the observed mutation frequency by the background mutation frequency. The CHO cells demonstrated significant, dose-related increases in the frequency of chromosomal breaks observed. The frequency of tetraploid cells (including endoreduplication) was increased in both cell types but dose-related only in human fibroblasts. Although there is no quantitative correlation between mutation frequency and the frequency of either chromosomal breaks or tetraploid cells in cultures treated with process water, this study provides additional support to the hypothesis that many mutagens also induce chromosomal changes.

TABLE I
CYTOGENETIC, MUTAGENIC, AND CYTOTOXIC EFFECTS OF AN
ABOVEGROUND RETORT PROCESS WATER IN CHO CELLS

<u>Process Water (pph)</u>	<u>Percent Cells With Breaks</u>	<u>Percent Tetraploid Cells</u>	<u>6-TG^R Mutation Enhancement Index</u>	<u>Surviving Fraction (Percent)</u>
0.0	0.5	0.2	1.00	100
0.1	2.5	5.5	1.44	90
0.3	6.0	12.5	3.49	74
0.5	13.0	11.0	4.63	44
0.8	26.0	13.5	9.58	25
1.0	39.5	13.0	7.18	12

TABLE II
CYTOGENETIC, MUTAGENIC, AND CYTOTOXIC EFFECTS OF AN ABOVEGROUND
RETORT PROCESS WATER IN HUMAN FIBROBLASTS

<u>Process Water (pph)</u>	<u>Percent Cells with Breaks</u>	<u>Percent Tetra- ploid Cells</u>	<u>6-TG^R Mutation Enhancement Index</u>	<u>Surviving Fraction (Percent)</u>
0.0	0.5	0.4	1.00	100
0.1	0.5	0.8	1.06	61
0.2	0.5	1.4	3.19	43
0.3	0.0	4.5	5.00	25
0.4	2.0	13.4	8.09	17
0.5	6.5	20.5	7.39	14

Effects of Prolonged Inhalation Exposure to Oil Shale Dust

[M. S. Halleck, G. C. Saunders, E. H. Tate, A. P. Stevenson, J. S. Wilson (LS-1), C. C. Stewart, W. D. Spall (LS-5), and A. Martinez]

The development of shale oil as an alternative energy source poses potential health hazards to those associated with the mining and milling of raw shales. Experiments in the Toxicology Group (LS-1) designed to assess the extent of pulmonary damage from inhalation exposure to highly respirable forms of oil shale dusts are currently in progress. These experiments have provided us the materials to develop assay systems to measure cellular and biochemical parameters on fluids lavaged from control and inhalation-exposed animals as well as biochemical measures of damage to lung tissues. This report describes preliminary results on a small sample of animals obtained after exposure for 16 months to a heavy aerosol dose of raw Paraho shale.

Two-month-old Fisher 344 rats were given nose-only exposure 6 h per day, 4 days per wk for 16 months to 150 mg/m^3 of Paraho raw shale in inhalation chambers at the Los Alamos oil shale exposure facility. The aerosol was generated by a Wright dust feed and was highly respirable; the mean mass aerodynamic diameter (MMAD) was $1.28 \mu\text{m}$ with a geometric standard deviation of 2.05 μm . Analysis by neutron activation indicated the lung burden of oil shale dust after 16 months to be 30 to 40 mg. We examined 3 oil-shale-treated animals from this group and compared them to 3 untreated colony control animals of the same age.

Following anesthetization with sodium pentobarbital and exsanguination by heart puncture, a

hemostat was placed on the bronchial connection to the left lung (to prevent entry of lavage fluid). Pulmonary lavage was performed by the hydrostatic method, a modification of Myrvick.¹ Lavage contents were analyzed for total cell yield, differential cell type, esterase activity, clonogenicity and tumoricidal activity of pulmonary macrophages, and elastolytic activity. Samples of bone marrow were also cultured for colony assays. Lung wet weights were measured for the unlavaged left lung only. Left lungs were analyzed for total hydroxyproline content (a measure of collagen content and fibrosis). Histological sections were prepared from the right lung following lavage.

Results of the cellular analysis of the lavage fluid are given in Table I. The total number of cells recovered from lungs of shale-exposed rats was more than an order of magnitude greater than the number recovered from control rats. Differential counts indicate a significant increase in the percentage of neutrophils in the lavages from shale-exposed rats, an indication of inflammation. The number of macrophages was also significantly increased in shale-exposed rats and most of these were loaded with phagocytized shale particles. Clonogenicity assays² to determine the proliferative capacity of cells recovered from the lavage provided the following results (Table I). Significantly greater numbers of cells grew in both liquid culture and on agar from cellular lavage content of controls than from shale-treated rats, although the same number of cells were initially plated. Plating efficiency of the macrophages lavaged from shale-treated lungs was also considerably less than that of the controls, thus, although there were significantly greater numbers of macrophages in

shale-treated lungs, they were apparently incapable of proliferation in vitro.

Evaluation of cells obtained from the bone marrow (Table II) demonstrated a slight decrease in the total number of bone marrow cells in shale-treated rats, although this was not statistically significant. Proliferative capacity of cells from controls appeared to be greater than the treated animals although there was considerable scatter in the data.

Tumoricidal activity of lavaged macrophages was assayed for both control and treated animals using a

"spot assay".³ Results indicated that if $> 4 \times 10^4$ macrophages were spotted, tumor cell growth was inhibited whether or not macrophages had been incubated in the presence of MAF (macrophage activation factor) prior to tumor cell addition. Tumor cell growth was inhibited in dishes containing macrophages from treated as well as from control animals, thus macrophages from shale-exposed lungs appear to have retained their tumoricidal activity. It remains to be determined, however, whether the shale particulates themselves contributed to the inhibition of target cell growth.

TABLE I
LAVAGE CONTENTS EVALUATION^a
(Number of Cells)

	<u>CONTROLS</u>		<u>TREATED</u>		<u>SIGNIFICANT DIFFERENCE</u>
Total Lavaged Cells	2.53 x 10 ⁶ ±	0.11 x 10 ⁶	3.03 x 10 ⁷ ±	0.66 x 10 ⁷	≤ 0.01
Percent Macrophages	92 ±	5.57	33.7 ±	3.06	≤ 0.01
Percent Esterase Positive Macrophages	94.5 ±	8.0	67.0 ±	12.9	
Percent Neutrophils	1.33 ±	1.15	61.3 ±	4.04	≤ 0.01
Percent Lymphocytes	6.67 ±	5.9	4.33 ±	5.86	None
Liquid Cultures ^b (cell number)	2.78 x 10 ⁵ ±	8.1 x 10 ⁴	4.83 x 10 ⁴ ±	2.7 x 10 ⁴	≤ 0.01
Agar Colonies ^c	7112 ±	880.1	317 ±	85.8	≤ 0.01
Macrophages Plating Efficiency (%)	77.7 ±	9.07	10 ±	3.41	≤ 0.01

^a Mean of 3 samples.

^b 25 000 cells plated, counted at 7 days.

^c 10 000 cells plated, counted at 21 days.

TABLE II
BONE MARROW EVALUATION^a

	<u>CONTROLS</u>	<u>TREATED</u>	<u>SIGNIFICANT DIFFERENCE</u>
Total BM Cells	$8.83 \times 10^7 \pm 3.18 \times 10^7$	$6.33 \times 10^7 \pm 2.08 \times 10^7$	None
BM Colonies (per 10^4 Cells)	41.67 ± 13.65	$17.17 \pm 8/25$	0.1
Liquid Culture ^b (Cell number)	1.34×10^5 $\pm 1.63 \times 10^4$	1.17×10^5 $\pm 4.22 \times 10^4$	None

^a Mean of 3 samples.

^b 25 000 cells plated, counted at 12 days.

Significant differences were, however, determined for elastolytic activity of the lavage fluid, measured by a method developed at Los Alamos utilizing peroxidase-labeled elastin (Table III). Lavage fluids from shale-treated lungs demonstrated higher elastolytic activity than controls. Whether these results represent increased macrophage or neutrophil secretion of elastase or cellular lysis remains to be determined.

Measurements of lung tissue indicated that wet weight of the shale-treated lungs was increased by more than 2.5 X the control values (Table IV), while collagen content (total hydroxyproline) was increased by more than 2 X controls (Table IV). On a per weight basis, however, collagen content does not appear to have increased with shale treatment. This was probably due to extensive hypercellularity (as seen in the lavage) and edema, due to inflammation, as well as increases in other proteins due to lipoproteinosis. Collagen content per lung was significantly increased, however, suggesting a fibrotic response in shale-treated lungs.

Microscopic examination of histological sections (Fig. 1) revealed many alveoli were filled with an eosinophilic protein-rich material. In addition, many alveoli contained dust-laden macrophages. There were also areas of alveolar septal thickening with some deposits of mature collagen fibers (demonstrated with trichrome).

TABLE III
ELASTOLYTIC ACTIVITY OF LAVAGE FLUID
(First Lavage)

	<u>Sample</u>	<u>Total Elastolytic Activity (ng)</u>
Control	1	24
	2	Not detectable
	3	Not detectable
Treated	1	69.5
	2	71.1
	3	65.9

TABLE IV
RESPONSE OF LUNG WET WEIGHT AND COLLAGEN
CONTENT TO OIL SHALE DUST INHALATION

	<u>LEFT LUNG WET WEIGHT (mg)</u>	<u>LEFT LUNG HYPRO CONTENT (mg)</u>
Control	393 ± 30.6	1.33 ± 0.18
Treated	1007 ± 110.1	2.89 ± 0.29
Increase Relative to Control	156%	117%

Fig. 1 Histological section from the lung of a shale-exposed rat demonstrating some alveoli filled with lipoproteinaceous material (P), thickened alveolar septae with collagen deposition (darkly staining) (C), and alveoli containing dust-laden macrophages (M).

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Rapid Staining Procedures for Analysis of Cellular DNA and Protein by Single and Dual Laser Flow Cytometry

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

Analyses of cellular DNA content by flow cytometry provides a rapid and convenient method for studying cell cycle kinetics and population ploidy levels. However, in heterogeneous cell populations, the kinetic patterns are a composite of all cell types and cell cycle progression profiles for subpopulations of interest are not easily derived. Furthermore, when malignant aneuploid cells become prominent in the flow-cytometry-distributions, cell-

cycle kinetic analysis by single parameter DNA content measurements is not possible.

Protein content of cells reflects in a general way the metabolic capacity of cells and, in most instances, is also a good descriptor for protoplasmic mass. Simultaneous flow-cytometry (FCM) analysis of cells stained for both DNA and protein can reveal subpopulations of different protein content located throughout the cell cycle or within aneuploid cell populations. Additional descriptive information such as the nuclear-to-cytoplasmic relationship³ and the DNA-to-protein ratios² for subpopulations can also be determined by flow cytometry and this approach can further enhance the analysis of heterogeneous cell populations.

In a recent report¹ we described a rapid and simplified method for staining hematopoietic cells with propidium iodide (PI) and fluorescein isothiocyanate (FITC) for subsequent analysis in a FCM system with a single excitation source. In contrast to our previous protocol,² all reagents were added sequentially to ethanol-fixed cells and, thus, no centrifugation steps were required. Staining time was reduced to 10 min as compared to 3 h by our previous method.² Analysis of the bone marrow samples from multiple myeloma patients showed mixed normal and aneuploid populations with a major portion of aneuploid cells having a significantly higher protein content. This approach permitted differential cell-cycle analysis of normal and aneuploid populations.

Our more current studies have focused on the development of two-color DNA-protein staining methods for use in the dual laser FCM system.⁴ With this system dyes with different excitation wavelengths such as mithramycin (MI) and rhodamine 640 (R 640) or substituted rhodamine isothiocyanate (X RITC) may be used for staining cellular DNA and protein, respectively. Alternatively the DNA reactive fluorochrome Hoechst 33342 (HO) may also be used in combination with R 640 or X RITC. One main advantage of using MI or HO is that these fluorochromes are highly specific for cellular DNA, thus RNase treatment is not required.

Cell staining is performed, in most cases, on ethanol-fixed cells; however, in one protocol, HO and R 640 were used to stain unfixed cells. In all cases the cells were stained and analyzed in the

combined stain reagents. MI-stained cells were excited at 457.9 nm, HO-stained cells at 350-365 nm (combined laser lines) and R 640- or XRITC-stained cells at 568 nm.

In the dual laser system artifacts arising from fluorescence emission overlap and energy transfer are avoided since the DNA stains, MI and HO, are both excited at significantly shorter wavelengths than either of the rhodamine protein dyes. Each dye of a particular two-color combination is preferentially excited by one of the appropriate laser beams and the beams are spatially separated so that stained cells are excited and analyzed sequentially. Complete details of the dual laser system are found in Reference 4.

The DNA and protein distributions (Fig. 1) for ethanol-fixed CHO cells stained with either MI-R 640 or MI-X RITC are both quite similar to distributions obtained in our previous studies using the PI-FITC staining procedure.² The protein distributions are typical of those obtained from a homogeneous cell population in which cell size increases somewhat linearly as a cell traverses from the G₁ to G₂+M phase. The coefficients of variation (CV's) of the G₁ peaks in the DNA profiles are about 3.5%. This value is virtually unchanged from CHO cells stained

Fig. 1. DNA and protein distribution profiles obtained by dual laser FCM analysis of CHO cells stained with a combination of MI and either R 640 (left) or X RITC (right). Ethanol-fixed CHO cells were stained for at least 10 min and then analyzed in a solution containing MI (100 μ g/ml) and either R 640 (1 μ g/ml) or X RITC (1 μ g/ml) in phosphate buffered saline containing 15 mM $MgCl_2$.

with MI alone indicating that very little rhodamine fluorescence enters the MI fluorescence analyzer, in which case a noticeable broadening in the DNA histogram would have been observed.

Similar observations to those above are apparent in the DNA and protein distributions shown in Fig. 2 for ethanol-fixed colon 26 cells stained with HO-R 640 or HO-X RITC. The CV's for the G_1 peaks typically ranged from 3.0 to 4.0%. Since these cells were maintained in tissue culture the protein distributions are similar to CHO cells and do not reflect the large variability in cell size that we have noted in cell population from the solid *in vivo* colon 26 tumor.

Viable staining of colon 26 cells was accomplished using HO and R 640. The CV for the G_1 peak (Fig. 3) is about 8%. This value is considerably larger than for the fixed cells in Fig. 2, but yet typical for these cells when stained viably with HO. The protein distribution, however, is quite similar to that for the R 640-protein distribution shown in Fig. 2. We are currently analyzing the plating efficiency of colon 26 cells following staining with HO-R 640, analysis, and cell sorting. We have observed that CHO cell populations stained only with HO (2 $\mu\text{g/ml}$), analyzed, and then sorted had an 85% plating efficiency, providing the UV laser power setting was not above 200 milliwatts.

COLON 26 CELLS (VIALE)

Fig. 3. DNA and protein distribution profiles obtained by dual laser FCM analysis of unfixed colon 26 cells stained with HO 33342 (2 $\mu\text{g/ml}$) and R 640 (5 $\mu\text{g/ml}$) for 90 min while maintained as monolayer cultures in medium at 37°C. Cells were trypsinized and analyzed at room temperature in culture medium containing HO 33342 and R 640 at the above concentrations.

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COLON 26 CELLS

Fig. 2. DNA and protein distribution profiles obtained by dual laser FCM analysis of ethanol-fixed colon 26 cells stained with a combination of HO 33342 (1 $\mu\text{g/ml}$) and either R 640 (1 $\mu\text{g/ml}$) (left) or X RITC (1 $\mu\text{g/ml}$) (right) in aqueous 0.15 M NaCl.

Flow Analysis of Karyotype Instability

[M. Bartholdi and L. S. Cram]

Karyotype instability is widely accepted as being associated with tumor cells. The role of this instability is unknown but may be related to tumorigenic potential by promoting continuous selection of cell variants, some of which are better able to avoid control mechanisms. Even in instances where tumor cells have been reported to have a normal karyotype it has been possible to demonstrate small DNA differences in individual chromosomes.¹ What constitutes a significant karyotype change is not known nor are answers to questions such as: What karyotypic changes correlate with tumorigenic potential?; Do some aberrations consistently appear?; Are some changes static and others dynamic?; At what frequency do aberrations appear? It is important to note that karyotypic changes alone, that is, in the absence of any in vitro transformation properties, have been shown to be present very early in a rodent cell line that spontaneously progressed from normal to tumorigenic.²

Investigations designed to evaluate the significance of karyotype instability have concluded that 1) some degree of non-random chromosome change is observable, and 2) karyotype changes occurring at low frequency and involving potentially small aberrations must be analyzed as they first appear in spontaneously developing tumors to be able to answer the question of the role of karyotype instability in tumorigenesis.¹ To do so has required the development of 1) a model system in which cells spontaneously progress from normal euploid cells to tumor producing cells, and 2) specialized flow cytometric instrumentation for quantitating several properties of individual chromosomes at very high analysis rates. The model system of spontaneous tumor development (whole Chinese hamster embryo cells, WCHE) has been described previously.³ The specialized flow-cytometric instrumentation designed to make high resolution and morphological measurements of isolated chromosomes is described below.

Isolated chromosomes are stained with a fluorescent probe, currently the intercalative dye propidium iodide. The dye is excited as the chromosomes flow singly through a laser beam. The total fluorescent emission detected is a measure of chromosome DNA content. The distribution of fluorescence along the chromosome measures chromosome morphology and stain localization. Chromosomes can be sorted on the basis of these measurements as they emerge in a fine stream of droplets that can be individually charged and deflected.

Performance of the high resolution chromosome analyzer has been evaluated and chromosome DNA content measurements of high precision can be achieved. The significant features of the instrument are high numerical aperture excitation and detection optics (currently used are microscope objectives) and a motor driven syringe for precise flow control.

Preliminary findings indicated that DNA content measurements of M3-1 Chinese hamster chromosomes were only intermittently of sufficiently high precision to completely resolve the hamster karyotype. A rigorous investigation of stability, precision, and linearity was undertaken. A first finding was that, despite stability of 1% over hours from fluorescent intensity measurements of 1.5- μ m diameter microspheres, fluorescence measurements from chromosomes could drift by as much as 10-20% within minutes. Recent reports showed that the intensity of fluorescence emission of propidium iodide intercalated into DNA depends strongly upon sodium ion concentration. Since the sample fluid containing chromosomes was 75 mM KCl and the sheath fluid, which surrounds the sample stream in flow, was 180 mM NaCl, it is likely that mixing of sample and sheath fluids occurred. Care in alignment of the flow nozzles minimized mixing, and matching sheath and sample fluids reduced the instability.

The dependence of the coefficient of variation (standard deviation/mean) with chromosome brightness (size) was found to be $1/\sqrt{N}$, indicating that photoelectron statistics were affecting the precision. Also, a light emitting diode flashed on the photomultiplier at equivalent gain settings generally produced a lower C.V. than that of the chromosomes, and microspheres produced an

Fig. 1. Chinese hamster chromosomes stained with propidium iodide; chromosome number 8 is identified as peak A. Chromosomes were mixed with 1.5- μ m microspheres, peak B, and simultaneously a light emitting diode flasher was shown on the photomultiplier resulting in peak C. The coefficient of variation of peak C (0.5%) is limited by photoelectron statistics. The CV of peaks A and B are 1.9% and 1.7%, respectively, showing that the errors due to photoelectron statistics have been reduced below the variation due to other factors.

intermediate C.V. as illustrated in Fig. 1. Evidently additional sources of error and instabilities are contributing variations of about the same magnitude as photoelectron statistics. One error found was nonlinearity of the integration of pulses as short as those produced by the small chromosomes. This has been corrected by developing a pretrigger for integration in which, after the dc trigger threshold is passed, the pulse is delayed 0.25 μ sec. Thus, integration starts at the point on the pulse 0.25 μ sec before the trigger, ensuring integration of the entire pulse over a wide range of pulse sizes.

In order to decrease the effect of photoelectron statistics below the level of other variations, the excitation intensity was increased. This was done in two ways. First, an argon ion laser with single line output powers of 1 to 5 watts was used. Second, the beam was focused to dimensions of 2 μ m by 27 μ m by a microscope objective, producing incident power densities of 1 MW/cm², more than 10 times greater than that in other flow cytometers. Tests showed that the ADC and photocathode responded linearly, but that a factor of two increase in laser power from 0.9 to

1.8 watts produced only a 10% increase in fluorescent intensity. Evidently, the rate of absorption is sufficient to produce a partially inverted population of fluorophores in the excited state which decays by stimulated emission. The striking finding is that the fluorescence emission intensity remains linearly proportional to fluorophore number, and thus chromosomes DNA content over a wide range of excitation powers. The major factor that contributes to high precision is now thought to be the insensitivity of fluorescence emission to excitation intensity. Thus, the effects of noise in the laser beam, and more important, the effects of variations in excitation intensity suffered by particles deviating slightly from the center of the sample stream, are significantly reduced. This last point obviates a major objection to this approach, that in using high numerical aperture optics, a sharply defined excitation intensity profile is produced and fluctuations of particles in the flow stream would limit precision.

To advance the state-of-the-art in analysis of human chromosomes, in detection of abnormal chromosomes, and in localization of antibody binding, recourse to scanning of chromosomes is indicated, as is operation of two lasers for sequential measurement of doubly stained chromosomes. The latter procedure is promising in light of the high precision data with propidium iodide, a probe not previously shown to yield high precision data. Operation with both ultraviolet and visible excitation would enable use of probes specific for AT and GC content of chromosomes.

To measure the distribution of fluorescence along the chromosome, an objective capable of focusing a laser beam of 2 to 3 watts in the UV (360 nm), to a spot size of 1 μ m, has been designed. The objective serves both as the illumination and collection optics with the fluorescent light directed toward the photomultiplier tube as illustrated in Fig. 2.

DNA content measurements of high precision, sufficient to resolve the Chinese hamster karyotype, can now be obtained reliably. Our experience is showing that the variation in instrument response can be less than that of the preparation protocol.

Fig. 2. Objective for high resolution chromosome analysis. The innermost ray tracings delineate the outer diameter of the laser beam, focused to a line across the chromosome whose fluorescence is collected by the same objective, as illustrated by the outer ray tracings. As a chromosome flows through the laser beam, it is scanned with a resolution of $1\ \mu\text{m}$.

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Flow-Cytometric Analysis of Cultured Bone Marrow Mononuclear Phagocytes

[C. C. Stewart and S. J. Stewart]

We have described a system to produce large numbers of both progenitor cells and mature cells of the mononuclear phagocyte system.¹ We have analyzed the autofluorescent profile of these two populations of culture derived mononuclear phagocytes using flow cytometry. Briefly, day 4 or day 7 cultures of bone marrow cells in macrophage growth medium, representing subconfluent and superconfluent cell

cultures, respectively, were rinsed to obtain nonadherent cells. The adherent cells were removed using sodium pyrophosphate. As shown in Fig. 1, when day 4 cells were analysed at the respective optimal gain settings, nonadherent cells were characterized as having a mean volume 4 times the nonadherent cells and a mean autofluorescence 16 times that of the nonadherent cells.

When day 7 cells were obtained the adherent cells showed the same characteristics as the day 4 adherent cells but, as shown in Fig. 2, the nonadherent cells were contaminated by adherent cells. As shown in the gated analyses, the cells with a low Coulter volume also had low autofluorescence while the cells with a high Coulter volume, most of which were off scale, had a very high autofluorescence profile.

These data show that mature macrophages can be distinguished from their progenitor cells on the basis of autofluorescence. They further illustrate a potential problem in studying membrane markers using fluorescent probes; namely, due to the high degree of autofluorescence of these cells, it is very possible that low density receptors will not be

Fig. 1. Autofluorescent profile of day 4 cultured bone marrow mononuclear phagocytes. C₃H mouse bone marrow cells were cultured 4 days in growth medium. After harvesting, the Coulter volume (CV) profile (A and C) and autofluorescent (AF) profile (B and D) of nonadherent and adherent cells were obtained. For nonadherent cells, the CV gain was 7 and for adherent cells it was 2. The AF gain was 8 and 2 for nonadherent and adherent cells, respectively.

Fig. 2. Autofluorescent profile of day 7 cultured bone marrow mononuclear phagocytes. See Fig. 1 caption for culture details. In A is shown the ungated Coulter volume profile and in B is shown their fluorescence distribution. In C, the first population cells were gated and their fluorescence distribution is shown in D while the fluorescence distribution of the gated larger cells (shown in E) is shown in F. Cells were analyzed at a CV gain of 4 and AF of 8.

resolved by fluorescent probes because the autofluorescence will be too high a proportion of total fluorescence.

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Flow-Cytometric Analysis of Human Lymphocyte Spheroids

[C. C. Stewart and J. A. Steinkamp]

When human peripheral blood mononuclear cells (MNC) are cultured at low concentration and density with phytohemagglutinin they were found to form discrete spheroids. Briefly, 2×10^5 MNC were cultured in 3.5 ml MEM containing 3% FBS and 6 μ g PHA/ml in 35-mm culture dishes. Each day replicate cultures were analysed. Free cells and cell clusters (spheroids) were suspended gently and fixed in 1% glutaraldehyde *in situ* for 1 h and then overnight in 70% ethanol. By carefully resuspending other cultures so as not to disaggregate spheroids, it was possible to separate the free cells from the spheroids and quantify the production of each population separately. The spheroids settled after 10 min to the bottom of a 15-ml conical centrifuge tube. The monodispersed cell-rich

supernatant was removed and the cells in it were counted as previously described.¹ The spheroids at the bottom, which had coalesced into a large mass were removed, vortexed to disperse the cells, and they too were counted. Both free cells and dispersed spheroids were then fixed in 70% ethanol. Cells still attached to the culture dish were removed by forceful pipetting in 5 ml saline, centrifuged, and the pelleted cells fixed in 70% ethanol. All preparations were stained with mithramycin (100 μ g/ml).

A special flow cell with a 500- μ m diameter orifice was used to allow spheroids to pass. The laser beam was shaped to produce a near planar beam of approximately 10 x 500 μ m at the intersection of the cell stream. Cells were diluted to about 10^5 particles per ml and the flow rate was adjusted to minimize coincidence.

During days 0-3, many cell clusters could not be resuspended as the lymphocytes were attached to adherent monocytes. By day 4 most spheroids were no longer adherent as monocytes were incorporated into them. On days 1-4, there were 6200 ± 1150 cell clusters found per 100,000 mononuclear cells plated. By day 7, there are approximately 2400 distinct spheroids per 10^5 cells.

As shown in Fig. 1, the number of cells in spheroids increase exponentially with a doubling time of 21 h from day 3 to day 6. Similarly, free cells accumulate in culture with the same doubling time from day 4 to day 6. No difference in the frequency of cycling cells was found between free cells and those in spheroids. The percentage of cells in S + G₂ on days 3, 4, 5, 6, and 7 was 47, 47, 35, 28, and 19%, respectively.

The size distribution of spheroids (Fig. 2) was determined using spheroids obtained after 7 days of culture. Diameters of 100 spheroids were measured using a microscope with an eyepiece micrometer. The mean spheroid size was 132 μ m in diameter. None were smaller than 30 μ m and 11% were larger than 335 μ m in diameter.

Time lapse movies (6 separate experiments) have allowed us to observe the fundamental kinetics of spheroid colony formation. These kinetics are shown in Fig. 3. During the first 24 h, lymphocytes accumulate over monocytes as has been reported by Werdelin *et al.*²⁻⁴ By 24 h, there are very few

Fig. 1. Distribution of cells in culture. Spheroids were separated from single cells by allowing them to settle 10 min. The single cells in the supernatant were removed and the spheroids resuspended and dispersed in saline. All cells, including those still adherent were counted as previously described.

free lymphocytes as most have either formed clusters of one to five lymphocytes per monocyte or have died. Thereafter, clusters increase in size by lymphocyte proliferation. By day 3 to 4 the monocyte attached to and holding the cell cluster firmly to the dish is incorporated into the spheroid. The spheroid now becomes free floating and shows active directed locomotion. Throughout this period there is an increasing frequency with which free cells can be seen leaving the spheroid. In the undisturbed culture, these cells form a monolayer under the spheroid. To our surprise spheroids can coalesce with one another to form larger spheroids. This always occurs as the

Fig. 2. Size distribution of spheroids. Spheroids were resuspended and fixed in 1% glutaraldehyde. Their diameters were measured using a micrometer eyepiece.

spheroids actively move about the culture coalescing with smaller ones.

The results show that when lymphocytes are cultured at a sufficiently low number per cm^2 , they will form discrete spheroid colonies. These colonies form by proliferation of individual lymphocytes and subsequent coalescence of small clusters into larger ones.

While we do not presently know the nature of these complex coalesced spheroids, we believe that this system may be useful in isolating individual colonies of lymphocytes. Such colonies, in the presence of Interleukin II, may be sufficiently expanded to study their functional potentialities.

FORMATION OF SPHEROID COLONIES

Fig. 3. Kinetics of spheroid formation. Cultures of human mononuclear cells were established ($25,000 \text{ cells/cm}^2$) in 35-mm culture dishes with 3rd growth medium containing $6 \mu\text{g/ml}$ PHA and time-lapsed movies were obtained over a 7-day observation period.

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Sequential Detection and Analysis of Multiple Electronic and Optical Measurements on Cells [J. A. Steinkamp]

Flow-cytometric experiments often require multiple electrical and optical measurements to determine cytological, biochemical, and functional properties of cells. Since many fluorescent stains have separated excitation spectra, two or more excitation sources operating at different wavelengths may be required.¹⁻⁶ In addition, electronic cell volume (Coulter principle) can be measured in conjunction with optical properties.⁷⁻⁹ Depending upon flow cell geometry, electrical and optically sensed signals normally occur sequentially. Therefore, electronics are required to sense, acquire, and temporarily store peak signal amplitudes which are separated in time for processing as frequency distribution histograms.^{7,10} This report illustrates the use of versatile electronics for coherently processing six sequentially occurring signals from electronic cell-size and optical sensors.¹⁰

Cells stained with fluorescent dyes, suspended in normal saline, are analyzed sequentially as they pass through the flow chamber (see Fig. 1). On entering the chamber, they pass through an electronic volume sensing orifice and then intersect three laser beams resulting in three fluorescent and light scatter signals. Measurable parameters include cell volume and surface area, three colors of fluorescence, and small-angle light scatter. Figure 2 shows the time sequence of electronic cell-size and optical signals as a cell stained with three fluorescent dyes traverses the flow chamber, first entering the volume sensing orifice, and then sequentially intersecting three laser beams spaced $250\text{-}300 \mu\text{m}$ apart. Electrical and optical signals are amplified, integrated, and fed directly to the signal processing electronics.

The electronics system can coherently process signals that arrive in sequential order, or it can be used in the simpler mode for single or simultaneous signal processing. The processor temporarily stores a maximum of six analog signals and two ratios in parallel using gated peak-sense

Fig. 1. Cutaway view of the flow chamber illustrating the cell volume sensing orifice (Coulter) and three-laser excitation with spatial positioning of beams. Lasers 1, 2, and 3 are Coherent models CR-10 (argon), CR-4 (argon), and CR-3000K (krypton), respectively.

and hold (GPSH) circuits to acquire crest values. GPSH signals are routed to the ADC's of the computer interface,¹¹ the outputs of which are input to an LSI-11 computer. Signals are stored in the LIST data acquisition mode on dual-density floppy diskettes and subsequently displayed as single-parameter, ratios, gated single-parameter, and two-parameter frequency distribution histograms.¹² Processor output signals are also routed to the cell separation electronics⁷ for cell-sorting experiments.

When single or simultaneously occurring signals are to be analyzed, sense gates are automatically triggered open by incoming signals for acquiring pulse height values. In the sequential analysis mode, individual trigger and sense delay switches are used to select trigger signals and sense gates for acquiring sequentially delayed signals. Front panel potentiometers and test points allow individual signal sense gates to be adjusted and monitored by oscilloscope for aligning incoming signals with respective sense gates. The delay between electronic cell-size and optical signals is easily set by dialing in the correct delay time and monitoring incoming signals and sense gates. A set of six input signal logic selector switches

Fig. 2. Diagram of electronic cell-size and optical signals as a function of time (passage of a cell stained with three fluorescent dyes through the flow chamber). The cell first enters the volume sensing orifice and then sequentially intersects three laser beams separated by 250-300 nm.

determine initial logic conditions prior to acquisition by the computer. Signals not satisfying initial conditions are rejected and the processor reset.

To evaluate sequential signal measurements, Colon-26 cells were stained vitally for DNA content, mitochondria, and total protein using Hoechst 33342,¹³ rhodamine 123,¹⁴ and rhodamine 640,¹⁵ respectively. Since the fluorescence excitation maxima are near 355, 495, and 570 nm wavelength for Hoechst 33342, rhodamine 123, and rhodamine 640, respectively, three-laser sequential excitation was required. The CR-10, CR-4, and CR-3000K (see Fig. 1) lasers were set to operate in the UV (near 350 nm), at 488 nm, and at 568 nm. Electronic cell-size followed by sequential laser excitation measurements were recorded by triggering processing on cell volume and surface area signals (see Fig. 2). The cell volume and surface area distributions (Fig. 3A) are similar to those previously reported.^{16,17} The DNA content distribution, recorded from UV-excited blue fluorescence measurements, is shown in Fig. 3B. The mitochondria distribution (Fig. 3C), as measured by exciting at 488 nm wavelength and recording green fluorescence signals, is broader than the total protein distribution (Fig. 3D) obtained by exciting at 568 nm and measuring red fluorescence signals. The protein and light scatter distributions (Fig.

3D) are nearly identical, illustrating correlation between protein content and cell size.¹⁶ This versatile system can be used for sequential multi-laser indirect fluorescence and other optical measurements at wavelengths ranging from the ultraviolet to infrared. We are now using the instrument experiments in immunology, tumor cell biology, and pulmonary toxicology.

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Infiltration of Host Defense Cells into a Foreign Substratum

[A. P. Stevenson, K. J. Bame, G. L. Travis, and P. M. Kraemer]

A simple system employing surgical sponges (Gelfoam[®]) as previously described¹ has been used to determine the host's response and the fate of tumor cells grown on sponges and then implanted subcutaneously into nude mice. Histological examination during such studies has shown that a host response develops and that this response includes infiltration of host macrophages and fibroblasts. Consequently, we began studies to characterize this host response using blank sponges.

Blank sponges (16 x 16 x 10 mm) were hydrated in growth medium (α MEM + 10% fetal calf serum) and then blotted on sterile gauze before implanting into nude mice as well as into standard hairy BALB/c mice. At 1, 2, 3, 7, or 10 days after implantation, the sponges were explanted and dissolved using trypsin, collagenase, and EDTA (TCE). A heavy infiltration of cells was found, followed by a drop in total cell number on days 2 and 3 (Fig. 1). On

Fig. 1. The infiltration of host cells. Total number of cells recovered per sponge as a function of time after implantation for normal nude mice is shown. Each data point represents the mean and standard deviation from 6-8 experiments. For the BALB/c mice each data point represents the average and the range from two experiments.

days 7 and 10 TCE is not sufficient to completely dissolve the matrix resulting from the consolidation of the sponge. Examination of this remaining matrix and the cells trapped within has shown that not all cells are recovered on these days. We are currently investigating alternative methods for dissolving the formed matrix so that a more accurate measure of cells within the sponge can be obtained.

Morphological examination of fixed and stained cells revealed changes in the types of cells recovered (Fig. 2). There was an initial rise in polymorphonuclear leukocytes (PMNs) to about 70% of all cells followed by a subsequent sharp drop. Macrophage/monocyte lineage cells rose from 20-25% on day 1 to about 80% on day 10. The percent lymphocytes remained between 5 and 10% throughout time. Unified cell types, probably fibroblasts and endothelial cells, increased slightly. For both strains the infiltration kinetics in the sponge are similar to those found in most exudates.

The colony forming ability of infiltrating macrophages as well as fibroblasts was also examined. In the absence of mouse L cell conditioned medium (as a source of macrophage growth

Fig. 2. Percent of macrophages, lymphocytes, polymorphonuclear leukocytes, and other cells as a function of time after implantation. Data are for nude mice but the data for the BALB/c mice were very similar.

factor)² only fibroblast colonies were observed, whereas in the presence of this conditioned medium, both macrophages and fibroblast colonies grew. (Macrophage colonies were identified by the phagocytosis of fluorescent beads 30 min prior to staining.) We observed an increasing percentage of fibroblast colonies for both strains (Fig. 3). The percentage of fibroblast colony forming units from sponges of nude mice was found to be about twice that from sponges of BALB/c mice (~5% for nude as compared to ~2.5% for BALB/c). The percentage of macrophage colony forming units also increases with time (Fig. 3), but we do not yet know whether the observed difference between the two strains is significant. It is clear, however, that colony forming macrophages infiltrate these sponges.

From the cell yield and the differential cell counts the absolute number of colony forming units increases through day 3. Whether the absolute number of cells continues to increase through day 10 will require complete dispersion of the matrix formed by days 7 and 10.

We also determined the percentage of cells found to be labeled by autoradiography when animals were injected with ³H-thymidine (given both i.p. as well as directly into the sponge) 30 min before the sponges were explanted. For the BALB/c mice 6% of

Fig. 3. Percent of fibroblast and macrophage colony forming units as a function of time after implantation. Each data point represents the mean and standard deviation from six replicate cultures.

all cells were labeled by day 10 and >80% were of the macrophage/monocyte lineage. For the nudes 3% of all cells were labeled by day 10, and >90% were macrophages.

Our results suggest that host defense cells not only infiltrate an implanted substratum but are also capable of proliferating, thereby increasing the potential for the involvement of these cells in the host defense mechanism. We are currently investigating whether these macrophages can be activated to tumoricidal activity *in vitro*.

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On the Emergence of Permanent Cell Lines from Cultured Diploid Cell Strains

[P. M. Kraemer and G. L. Travis]

Permanent, aneuploid cell lines often emerge in monolayer cell cultures initiated with normal, diploid rodent cells. It is well documented that such cell lines frequently become tumorigenic spontaneously with passage in culture.¹ Tumorigenicity, as well as altered *in vitro* characteristics, can also be induced in such cell lines by treatment with chemicals, viruses, or radiation. However, cultured cell strains that do not generate permanent cell lines, such as human diploid cells, are relatively resistant to induced malignant transformation.² Hence, the emergence of permanent cell lines from diploid cell strains is currently viewed by some workers as a preneoplastic event.

Both the cell line emergence phenomenon, as well as the further progression to frank tumorigenicity appear to be quite variable. Accelerated or delayed changes have been related to the method of subcultivation,³ to serum components,⁴ or to endogenous Type C viruses.⁵ In general, however, the determinants of these phenomena are obscure. The present report describes attempts to determine whether this variability would be eliminated in replicate cultures of a given normal diploid cell population. For this purpose, a single, whole, 18-day-old, Chinese hamster fetus was diced, trypsinized, and the resulting cell suspension divided into 10 primary cultures. For 20 wk thereafter, all 10 lineages (designated A through I) were subcultivated each wk, using 10^5 cells to initiate the next passage. In general, all cultures reached confluency each wk and the number of cells/flask (of 75 cm² area) at the time of subcultivation reflected the saturation density. In addition, the average number of doublings/day was calculated from the weekly cell numbers.

Results for one lineage, lineage G, are given in Fig. 1. These data illustrate several features common to most Chinese hamster lineages. First, the

Fig. 1. Saturation density [cells/flask (●)] and growth rate [doublings/day (▲)] estimates for the "G" lineage of Chinese hamster fetal cells, between passages 1 and 20.

saturation density (cells per flask $\times 10^{-6}$) remained quite low ($< 5 \times 10^4/\text{cm}^2$) for a number of passages. In Fig. 1, for instance, it remained low for 15 passages, which represented 55.3 cumulative doublings. Over the same period, the net rate of cell growth was quite variable but showed a characteristic "crisis" phase at passages 5 and 6. The "crisis" phase represented the period of maximal cell loss of the normal diploid pedigrees, which are subject to cellular senescence.⁶ The emergence of the abnormal permanent lineages was signaled by the post-crisis increase in growth rate (to a higher level than precrisis), and the increase in saturation density.

Data similar to that illustrated in Fig. 1 was prepared for all 10 lineages and analyzed for variability. The overall growth rate data (Fig. 2) showed the expected increase in doublings/day for the later passages. However, the crisis phase, which was clearly discernible in only five of the ten individual lineages, was also not clearly discernible in the aggregate. In addition, the variability of the growth rate data seemed similar over the entire experiment.

By contrast, saturation density (Fig. 3) appeared to be more distinctive. The earlier passages consistently reached less than 4×10^6

Fig. 2. Doublings/day ($\bar{x} \pm$ standard deviation) vs passage level for 10 Chinese hamster cell lineages from a single fetus.

Fig. 3. Saturation density (cells/flask) estimates ($\bar{x} \pm$ standard deviation) vs passage level for the same 10 lineages of Fig. 2.

Fig. 4. Passage level of the different lineages from a common fetus at which the saturation density exceeded 4×10^6 cells/flask.

cells/flask. After passage 6 (cumulative doublings, 22.3 ± 0.8) this parameter increased steadily, but the variability of it, between the 10 lineages, increased drastically. This reflects the fact that the passage level at which the different lineages began to grow to an abnormal density varied remarkably. Figure 4 illustrates this construction of the data, using a saturation density of 5.3×10^4 cells/cm² (4×10^6 /flask) as a convenient level dividing normal from abnormal. It permits the conclusion that under the conditions of maximal homogeneity of the initial normal cell populations, the emergence of abnormal cell lines occurred over a wide range of passage levels. Lineage A, at the 6th passage, had doubled 24 times *in vitro*, while lineage H, at the 18th passage, had doubled 74 times.

This experiment did not critically address the question of the relative contribution of pre-existent endogenous factors (within cells of the fetus) and the cell culture environment in the etiology of spontaneous malignant progression. It illustrated, however, that the phenomena is intrinsically stochastic in some critical way.

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Potassium Ion Influx Measurements on Cultured Chinese Hamster Cells Exposed to 60-Hertz Fields

[A. P. Stevenson and R. A. Tobey (LS-1)]

Studies of the effects of 60-Hz electromagnetic fields (ELF) on the growth and survival of cultured Chinese hamster (line CHO) cells showed that cell growth over prolonged time periods is not affected by fields up to 2.85 V/m.¹ These findings led to the search for a more sensitive parameter for measuring effects of such fields on cellular processes. The study of potassium ion influx was considered to be of particular importance in view of both the role of potassium ions in protein synthesis, a process necessary for cell growth and survival, and the fact that the pumps responsible for transport of this ion into the cell are electrogenic, and therefore, perhaps sensitive to the effects of impinging fields.^{2,3} Additionally, measurement of K^+ influx in cultures of CHO cells exposed to hyperthermia (42°C) had demonstrated previously that changes in transport could be measured immediately (as early as one minute after treatment).⁴ Therefore, it was decided to investigate in CHO cells the effects of 60-Hz fields on K^+ influx.

The magnetic induction exposure chamber used in these studies is capable of yielding fields up to 2.85 V/m and has been previously described.⁵ Briefly, the exposure vessel consists of a glass toroid that contains cells and growth medium and is inserted through a transformer core. Thus, the conducting nutrient medium forms a shorted turn on the transformer core, and electric fields can be generated by magnetic induction. A primary winding on the core is excited by a variable transformer to produce varying electric fields. The vessel is shaken in a circular configuration to keep cells

uniformly dispersed, and the chamber holding the vessel is maintained at 37°C. Sham control experiments were performed in two ways: 1) with zero field applied and with a normal toroid inserted through the transformer; and 2) with a 2.85-V/m field (field strength obtained in the conductive culture medium) applied but with a glass septum in the toroid to interrupt the induced current flow and so eliminate the electric field.

K^+ influx was determined from the kinetics of ^{42}K uptake. CHO cells were grown in suspension culture with zwitterion buffer added to maintain pH during shaking of the culture vessel in the exposure chamber. For each influx determination, 500 ml of cell suspension (normally 3.0 to 3.7×10^5 cells/ml) were centrifuged and resuspended in the toroid in 55 ml of complete nutrient medium containing ^{42}KCl as a trace amount of total KCl . At frequent intervals, duplicate 1-ml aliquots were removed, and the cells were washed once with serum-containing medium before the final cell pellet was counted in a well-scintillation counter.

It had been determined previously that CHO cells exhibit two components of influx, A_{12} and A_{23} , as evidenced by the fact that log plots of uptake vs time do not yield straight lines but show a change in slope at ~ 5 min (see Fig. 1a). An early ^{42}K uptake experiment suggested a trend toward significant reduction in the slow component of K^+ influx (A_{23}) for cells exposed to 60-Hz fields of 2.85 V/m. However, subsequent experiments have shown that if one compares the plots of $-\ln(-S/\bar{S}_\infty)$ vs time (where $S/\bar{S}_\infty$ reflects the degree to which the distribution of ^{42}K has progressed toward equilibrium), the observed differences between uptake by field exposed and control cells are small and sometimes appear to resemble the differences due to experimental error.

As an example of the experiment-to-experiment variability encountered in these studies, Figs. 1a and 1b provide data obtained in two paired ($\pm$ field) experiments; in Fig. 1a, there is an obvious difference in flux rate between the field-exposed and control culture, yet an identical experiment produced the results in Fig. 1b in which the difference in flux rate is greatly reduced. Note that the difference observed in Fig. 1b is not much greater than the difference between measurements on

Fig. 1. Effect of a high-strength electric field (2.85 V/m field in the conductive culture medium) on ^{42}K uptake by exponentially growing CHO cells. For three experiments (A, B, and D) plots of $-\ln(1 - \bar{S}/\bar{S}_0)$ vs time (where $\bar{S}_0$ equals the specific activity of isotope in the cells at time t and $\bar{S}$ equals the specific activity at equilibrium) are shown for control cells (o) and for exposed cells (Δ). For one of these experiments (D), the results are also shown for sham exposed cells ($\square$) (i.e., with a 2.85 V/m field applied but with a glass septum in the toroid to eliminate the electric field.) For one experiment (C) plots are shown for duplicate (control) cell cultures (o and Δ).

duplicate (control) cultures (Fig. 1c). Similar small differences are observed when one attempts to distinguish field effects from purely magnetic effects utilizing a septum-containing toroid (Fig. 1d). (Additionally, measurements on cells in the septum-containing toroid showed considerable sampling variability, possibly due to incomplete mixing between samples taken every two min.)

Comparison of the calculated fluxes for a series of experiments (Table I) revealed the difficulty in quantitating such small differences for each paired experiment. When the fluxes were averaged for all experiments, the field-exposed cultures showed a 4% reduction in the slow component of uptake and a 7% reduction in the fast component (Table I); however, these differences fall within the calculated standard deviations. In spite of the experiment-to-experiment variability evident in the calculated fluxes as well as in the log plots, it was observed in all but one experiment that the plotted data points for the exposed cultures were below those for control cultures. This difference varied somewhat from experiment to experiment but generally remained extremely small and so is of questionable significance.

However, given the possibility that small magnitude field-induced perturbations in ion flux rate may occur, the important consideration is the long-term effect of putative perturbations on internal potassium concentration. Therefore, the level of intracellular potassium was monitored over a prolonged period (multiple generations) in order to increase the probability of detecting a gradual change in internal concentration. It was expected that if the level remained unchanged throughout the entire period, it would imply either that the field effect was negligible or that the induced perturbation could be offset by a compensatory cellular mechanism.

Figure 2 illustrates results obtained when the intracellular concentration of potassium was determined directly at various intervals via atomic absorption analysis on control cells and on exposed cells maintained in a 2.85-V/m field over a period of 96 h (approximately six culture doubling times). These results demonstrate that there is no apparent difference in intracellular $[\text{K}^+]$ between the cultures over the entire course of the experiment.

TABLE I

VALUES OF K^+ INFLUX: MEASUREMENT ON EXPOSED CELLS COMPARED TO MEASUREMENT ON CONTROL CELLS

Experiment Number	Field-Exposed (2.85 V/m)		Septum Control		Control	
	A_{12}^a	A_{23}^a	A_{12}^a	A_{23}^a	A_{12}^a	A_{23}^a
91	9.40	2.76			10	3.82
92	8.01	3.38			8.56	2.83
94	5.71	1.79			-----	-----
95					5.71	3.91
96	5.87	1.79			5.35	2.40
97			9.36	3.76	6.12	3.85
98					7.42	2.10
					7.45	2.33
99	6.73	3.62	6.33	3.47	7.44	3.87
102	6.54	2.93	6.63	1.34	7	3.08
103	4.62	3.76	-----	-----	5.82	2.55
Average	6.70 ± 1.58	2.86 ± 0.81			6.99 ± 1.44	3.07 ± 0.73

^aExpressed as femtomoles/cell/min.

Fig. 2. Effect of a high-strength field (2.85 V/m) on intracellular potassium concentration. Intracellular $[K^+]$ (expressed as mM/l of packed cells) was determined for control cells (solid) and for exposed cells (hatched) maintained in a 2.85-V/m field over a period of 96 h.

Additional studies (data not shown) revealed that the internal potassium level also remained unchanged at early stages of exposure to the field (e.g., during the first 20 min in contact with the field, the period studied in the influx measurements).

The major conclusion to be drawn from these studies is that electric fields of magnitude greatly in excess of those encountered by the general population have little or no effect on the rate of transport of potassium ions. Supporting this conclusion are the observations that a) the intracellular concentration of K^+ is maintained during prolonged periods of exposure to high-strength fields, and b) the growth rate and survival of field-exposed cells is comparable to values obtained in control (non-exposed) cells, suggesting that K^+ -dependent processes such as protein synthesis are not greatly affected by fields as great as 2.85 V/m.

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INTRODUCTION

In Group LS-5, there are two areas of programmatic research—the stable isotopes of carbon, oxygen, and nitrogen (ICONS) program and the analytical chemistry of oil shale program. The major activity in Group LS-5 is the ICONS program, which has as its objective the facilitation of the use of naturally occurring stable isotopes in the physical and life sciences. Stable isotopes can be used as nonradioactive tracers to monitor biochemical, agricultural, environmental, metabolic, and chemical processes. Such uses of stable isotopes require compounds labeled with stable isotopes. The synthesis of compounds labeled with stable isotopes is conducted by Group LS-5. Both chemical syntheses and biochemical syntheses are used to prepare labeled compounds. Labeled compounds are made available to outside investigators through the National Stable Isotopes Resource (SIR) which is sponsored by the National Institutes of Health. Investigators may apply to the SIR for

labeled compounds, analytical services, training, or cooperative research. Core research within Group LS-5 is concerned with the development of methodology for preparing compounds labeled with stable isotopes. In recent years, our research has focused on isotopically labeled amino acids with an emphasis on methods that afford the naturally occurring L-stereoisomer.

The analytical chemistry of oil shale is the second programmatic activity in Group LS-5. This work has two objectives. The first objective is to provide analytical data on the materials being studied in the Los Alamos program to assess the health effects of shale oil production. The second objective is to develop new analytical methods as required to address the questions arising from the health effects studies. Because of the diversity and complexity of samples in the oil shale program, this work relies heavily on chromatographic analyses, particularly the combined techniques of gas chromatography-mass spectrometry.

Organic Synthesis With Stable Isotopes

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

As in previous years, we have conducted various routine preparations. These compounds have served as starting materials for other syntheses and also for preliminary studies in our own work. These preparations have included methanol- ^{13}C , acetic-1- ^{13}C acid, bromoacetic-1- ^{13}C acid, glycine-1- ^{13}C , acetic-2- ^{13}C acid, bromoacetic-2- ^{13}C acid, glycine-2- ^{13}C , and acetic- $^{13}\text{C}_2$ acid.

In our last report, we described a synthesis of ornithine-2- ^{13}C which afforded a mixture of 70% L-isomer and 30% D-isomer because of poor stereoselectivity in the chymotrypsin ester cleavage of α,δ -diacetyl-D,L-ornithine ethyl ester. In order to recover optically pure material from this preparation, we chose to conduct a second resolution with acylase, which requires α,δ -diacetylornithine as a substrate. Investigations into the acetylation of ornithine showed that formation of the α -acetyl- δ -lactam was a significant ($\sim 30\%$) side reaction that could not be circumvented. However, pure diacetylornithine could be isolated from the reaction. The enzymatic resolution of α,δ -diacetylornithine-2- ^{13}C (still an unequal mixture of D and L isomers) with acylase gave δ -acetyl-L-ornithine-2- ^{13}C . Hydrolysis of the remaining acetyl group afforded optically pure L-ornithine-2- ^{13}C .

Our initial plan for synthesizing L-arginine-1- ^{13}C was to prepare L-ornithine-1- ^{13}C using the methods developed for L-ornithine-2- ^{13}C and then convert ornithine into arginine. However, in light of our experience with chymotrypsin and acylase, we felt it would be preferable to modify the ornithine synthesis to allow use of the latter enzyme. We felt that this could be accomplished by conducting the synthesis with the methyl ester instead of the ethyl ester. Since iodotrimethylsilane¹⁻⁴ has been reported to be a reagent that cleaves methyl esters, we planned to obtain the acylase substrate (α,δ -diacetyl-D,L-ornithine) by cleavage of the methyl ester. Thus, glycine-1- ^{13}C

was converted to the Schiff's base methyl ester 1 by esterification and condensation with benzophenone.

The Michael reaction of 1 and acrylonitrile gave 2.

Hydrolysis of the diphenylmethylidene group, hydrogenation of the cyano group, and acetylation all proceeded smoothly to give α,δ -diacetyl-D,L-ornithine-1- ^{13}C methyl ester (3). Despite extensive

experimentation, the methyl ester in 3 could not be cleaved with iodotrimethylsilane or derived reagents. Rather than attempting to use other chemical reagents to cleave the ester group in 3, we returned to the enzymatic hydrolysis with chymotrypsin. This procedure gave α,δ -diacetylornithine-1- ^{13}C (4), which was 92% L-isomer and 8% D-isomer,

a result similar to our preliminary studies in the ornithine-2- ^{13}C synthesis. The diacetyl compound 4 was then treated with acylase to remove the α -acetyl group from the L-isomer. Hydrolysis of the remaining acetyl group gave L-ornithine-1- ^{13}C (5).

Conversion of 5 to L-arginine-1- ^{13}C (6) was effected by treating the copper complex of 5 with O-methylisourea tosylate.

Because of the two chiral centers in the 2-amino-3-methylpentanoic acid (7) skeleton, syntheses from achiral precursors afford a mixture

of four stereoisomers—i.e., D,L-isoleucine and D,L-alloisoleucine. To our knowledge, there is not a truly satisfactory method for separating isoleucine and alloisoleucine. During the past year, we learned of the details of the method of LeMaster and Richards⁵ for separating the amino acids of protein hydrolysates and have modified their method for our isoleucine-alloisoleucine separation problems. We have been able to separate 5 g of a mixture of D,L-isoleucine and D,L-alloisoleucine on a Dowex 50 column (200–400 mesh, 2.2 x 85 cm, pyrazolium form). Elution of the column with pyrazolium chloride (0.15 M, pH 2.30, 2 ml/min, 25-ml fractions) gave D,L-alloisoleucine (fractions 125–150) and D,L-isoleucine (fractions 152–170). We feel that slightly higher loading of the column is possible.

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Biochemical Synthesis With Stable Isotopes

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

We are continuing to utilize the algal systems described in previous reports as sources of both labeled organisms and labeled compounds. The marine alga *Agmenellum quadruplicatum* is an excellent organism for producing glucose-¹³C₆ (and also glucose-¹²C₆) since the organism can be induced to accumulate high levels of cyanophycean starch when grown under iron- and nitrogen-limited conditions. During the past year, 234 g of [¹³C] *Agmenellum* and 53 g of D-glucose-¹³C₆ were prepared.

We have previously noted that protein hydrolysates from multiple labeled organisms, together with density centrifugation analytical techniques, are being used by several investigators to study biosynthetic processes. We have employed *Chlorella pyrenoidosa* for multiple labeling experiments. Labeling of the alga with carbon-13 and nitrogen-15 can be accomplished readily by growing the organism on carbon-¹³C dioxide and potassium nitrate-¹⁵N. We have produced 28 g of [¹³C, ¹⁵N] *Chlorella* during the past year. Additional density labeling of the alga with deuterium requires adaptation of the organism to growth on D₂O by culturing in H₂O–D₂O mixtures in which the D₂O content is gradually increased to 99%. After adaptation, triple labeled organisms are obtained by growth on carbon-¹³C dioxide and potassium nitrate-¹⁵N in the D₂O medium. As would be expected, growth rates in D₂O are significantly slower than rates in H₂O. We are preparing an additional 80 g of [²H, ¹³C, ¹⁵N] *Chlorella*.

There has also been some interest in labeled nucleic acids. *Escherichia coli* are a convenient source of nucleic acids since they contain approximately 2% DNA and 2% RNA. [¹³C] *E. coli* have been obtained by growth of the organism on 20 mol % ¹³C acetic-¹³C₂ acid in a bench-top, 14-l fermentor. In five experiments, 73 g of labeled cells were obtained. On this scale, we did not consider it practical to recover the carbon-13 lost because of metabolism of acetate by the *E. coli*. We have also prepared [¹⁵N] *E. coli*. In these experiments, the bacteria were grown using glucose as a carbon source and ammonium-¹⁵N sulfate as a nitrogen source. We had anticipated that organisms grown on glucose would reach culture densities compatible with

bench-top fermentors; however, this was not realized. After five experiments in the 14-1 fermentor giving 103 g of [^{15}N]E. coli., we switched to a 250-1 fermentor and obtained 420 g of [^{15}N]E. coli. in one experiment.

Another bacterium, *Lactobacillus casei*, has been employed to produce L-(+)-lactic- $^{13}\text{C}_3$ acid from D-glucose- $^{13}\text{C}_6$. This biochemical synthesis afforded 29 g of L-(+)-lactic- $^{13}\text{C}_3$ acid which is isolated and purified as a zinc salt.

Asymmetric Syntheses of Amino Acids

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

In prior reports we have discussed the rationale and background for our work on asymmetric syntheses of amino acids, i.e., reactions that will afford the naturally occurring L-isomer of α -amino acids as the major, if not exclusive, product. We are investigating two approaches to this problem -- chiral hydrogenation reactions and chiral alkylation reactions.

We have previously described the synthesis of (-)-1,2-bis(*o*-anisylphenylphosphino)ethane and its subsequent conversion to the rhodium(I)-cyclo-octadiene-bisphosphine tetrafluoroborate catalyst employed by Vineyard *et. al.*¹ in chiral hydrogenation reactions. During the past year, we have used this catalyst in a synthesis of L-tryptophan-1- ^{13}C . This synthesis requires the unsaturated acid 8 as a precursor. In order to prepare 8 via the azlactone (to give the necessary

Z-stereochemistry about the double bond), a condensation between N-acetylindole-3 carboxaldehyde (9) and an N-acylglycine-1- ^{13}C (10)

is required. The condensation of 9 and N-acetylglycine (10, $\text{R} = \text{CH}_3$) using acetic anhydride without a solvent gave the azlactone, but in low yield and contaminated with a side product shown to be the diacetate of the aldehyde 9. The condensation of 9 and N-benzoylglycine (10, $\text{R} = \text{C}_6\text{H}_5$) using lead acetate in tetrahydrofuran gave the azlactone in good yield. The azlactone ring was opened (sodium ethoxide) and the unsaturated ester was hydrogenated with the chiral rhodium catalyst; however, the benzoyl group could not be removed without significant racemization of the tryptophan. All of these problems were finally overcome when suitable conditions (acetic anhydride in dimethylformamide) were found for condensing 9 and N-acetylglycine-1- ^{13}C (10, $\text{R} = \text{CH}_3$). Ring opening of the azlactone (water in dioxane), hydrogenation, and hydrolysis (2 N hydrochloric acid, reflux 2.5 h) afforded L-tryptophan-1- ^{13}C of high optical purity (> 93%). One recrystallization gave pure L-isomer.

Our second approach to synthesizing amino acids is the alkylation of a chiral glycine-iminoester such as 11, which is in turn prepared from a

hydroxyketone such as 12 and a suitable derivative of glycine.

In the last report, we described the conversion of 12 into the ester 13.

We had also removed the benzyloxycarbonyl group with iodotrimethylsilane to give 14. Attempts to cyclize 14 to 11 in aqueous solution at pH 5,

conditions under which we felt Schiff's base formation should occur,² were unsuccessful. Attempts to cyclize 14 with boron trifluoride etherate in benzene were also unsuccessful with a mixture of products being obtained. It was ultimately found that 14 was a mixture of hydrochloride and hydroiodide and that the deprotection of 13 with iodotrimethylsilane was unsatisfactory. We have since found two methods that can be used to deprotect 13 and give pure products — hydrogen bromide in acetic acid or trifluoroacetic acid. We are continuing to study the cyclization of 14 to 11. We have completed the synthesis of 15, a fourth hydroxyketone reagent.

The product was obtained as a mixture of epimeric alcohols and we are currently deciding whether or not to continue work with this reagent.

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National Stable Isotopes Resource

[T. W. Whaley]

A summary of the investigators who were provided with labeled compounds during the past year is given below.

1. G. J. Blomquist -- Department of Biochemistry, University of Nevada Medical School -- Biosynthesis of the Housefly Sex Pheromone: a Carbon-13 NMR Approach -- 1.9 g D,L-Valine-¹³C₃.
2. R. M. Cowett -- Department of Pediatrics, Women and Infants Hospital of Rhode Island -- Glucose Kinetics in the Pregnant Woman and Her Newborn -- 6.7g Zinc L-Lactate-¹³C₃.
3. H. G. Floss -- Department of Medicinal Chemistry and Pharmacognosy, Purdue University -- Biosynthesis of the Novel Macrolide Antibiotic Chlorothricin -- 1.0 g D-Ribose-1-¹³C.
4. E. W. Gertz -- Veterans Administration Medical Center, San Francisco -- Myocardial Carbohydrate Metabolism: Uptake and Oxidation of Glucose and Lactate -- 13.4 g Zinc L-Lactate-¹³C₃.
5. F. R. N. Gurd -- Department of Chemistry, Indiana University -- Specific Enrichment of Myoglobins for Carbon-13 NMR -- 1.6 g L-Alanine-¹³C₃, 2.4 g L-Aspartic-¹³C₄ acid, 1.2 g L-Glutamic-¹³C₅ acid, 0.01 g L-Isoleucine-¹³C₆, 0.12 g L-Valine-¹³C₅.
6. N. B. Hecht -- Department of Biology, Tufts University -- RNA Synthesis in Haploid Cells -- 10.0 g [¹³C, ¹⁵N] *Chlorella pyrenoidosa*.
7. V. J. Hurby -- Department of Chemistry, University of Arizona -- Studies of Peptide Hormone-Binding Protein Interactions Using NMR of Specifically Labeled Hormones Containing [3-¹³C]cystine -- 0.6 D,L-Cystine-3,3-¹³C₂.
8. M. D. Lane -- Department of Physiological Chemistry, The Johns Hopkins University School of Medicine -- Regulation of Insulin Receptor Level in Animal Cells -- 120 g [²H, ¹³C, ¹⁵N] *Chlorella pyrenoidosa*.
9. G. C. Levy -- Department of Chemistry, Syracuse University -- ¹⁵N and other Nuclei NMR Spin Labeling Studies of Nucleotide-Metal Ion Binding -- 420 g [¹⁵N]*Escherichia coli*.
10. J. L. Markley -- Department of Chemistry, Purdue University -- Subzero NMR Studies of Enzyme Catalysis-1.0 g L-Arginine-1-¹³C, 1.0 g L-Tryptophan-1-¹³C.

11. A. S. Mildvan -- Institute for Cancer Research -- Conformations of Enzyme-Bound Nucleotide, Deoxynucleotide and Pentose Phosphate Substrates -- 3.0 g [^{13}C] *Escherichia coli*.
12. K. L. Rinehart, Jr. -- Department of Chemistry, University of Illinois -- Incorporation of D-[U- ^{13}C]Glucose Diluted with D-[U- ^{12}C]Glucose into C₇N Units of Geldanamycin and Pactamycin--36.0 g D-Glucose- $^{12}\text{C}_6$.
13. J. H. Siegel -- Department of Surgery, Buffalo General Hospital -- The Role of Occult Myocardial Failure on Human Shock: I-Myocardial Preservation in Cardiac Surgery -- 9.0 g D-Glucose- $^{13}\text{C}_6$.
14. J. L. Sudmeier -- Department of Chemistry, University of California, Riverside -- FTNMR Studies of Biosynthetically ^{15}N -Labeled Zinc Metalloenzymes -- 10.0g D,L-Histidine-1- ^{15}N .
15. M. Walser -- The Johns Hopkins School of Medicine -- Quantitation of Polyamine Turnover Rates in Normal Subjects and in Patients With Gyrate Atrophy -- 2.1 g L-Ornithine-1- ^{13}C .

Analytical Chemistry of Oil Shale

[W. D. Spall]

A. Solvent Extraction Studies of Oil Shale Materials

Procedures for the analysis of extracts of crude oils and solids have been developed in order to determine the compounds responsible for observed biological activity of the material. The cellular mutagenesis or carcinogenesis tests commonly employ dimethyl sulfoxide (DMSO) as the solvent for the test materials, which renders the material soluble in the test media. The quantity of material extracted into DMSO is determined by the isotope dilution GCMS technique we developed in 1979. For shale and petroleum oils, from 3 to 50 weight percent of the oil may be extracted. In order to determine the degree of extraction of various compounds and to compare the DMSO extraction from oil to oil, we selected 67 specific compounds, representing 27 classes of chemicals, and followed these compounds through the extraction procedure for three different oils. The following conclusions can be drawn from the study. Partition coefficients for each compound are dependent on the composition of the original oil. Polycyclic aromatic

hydrocarbons (PAH's), alkylated PAH's, polar-aromatic and polar-alkyl compounds are extracted well by DMSO. Paraffinic, olefinic, cyclic, and low-polarity hydrocarbons, including long-chain monofunctional compounds, are poorly extracted. Water present in the oil greatly changes the partitioning of many compounds. This study shows that the variability of the chemical composition of extracts can present problems in the use of biological tests to assess the potential biological hazard of crude oils, fractional oils, and extracts from energy-related materials.

Mouse skin painting studies have been carried out using a solvent carrier, generally 70% acetone-30% cyclohexane. In a preliminary study to determine the solubility of various oils in other solvents, we determined the solubility of 5 oils in 11 solvents. The results are given in Table I.

TABLE I
SOLUBILITY OF CRUDE OILS IN VARIOUS SOLVENTS

Solvent	Weight Percent Dissolved				
	OXY ^a	601 ^b	011/602 ^c	KCO ^d	LCO ^e
70% Acetone, 30% Cyclohexane	91.1	102.0	94.0	76.6	74.1
Cyclohexane	91.7	85.9	85.7	76.4	75.2
Acetone	81.0	83.6	66.5	50.6	54.3
Methanol	40.8	38.2	16.7	9.9	13.3
Ethanol	75.8	81.6	46.4	30.5	37.7
Propyl Alcohol	-	83.2	74.8	46.5	52.2
Tetrahydrofuran	107.7	88.2	78.8	75.8	75.6
Toluene	95.4	93.8	75.6	77.1	75.5
Xylenes	103.0	98.5	79.3	80.0	75.3
Hexane	87.2	90.4	84.1	70.5	72.8
DMSO	17.0	44.9	16.2	5.0	13.4

^a Occidental crude shale oil from room #6 retort.

^b Paraho composite crude shale oil, ORNL repository #4601.

^c Paraho composite hydrotreated shale oil, ORNL repository #4602.

^d API standard petroleum crude oil from Kuwait.

^e API standard petroleum crude oil from Louisiana.

Oil shale mining and processing can produce large amounts of dust which presents a potential worker hazard. The analysis of organic compounds in solids requires extraction before testing. A series of 15 solvents were used to determine the amount of extractable material from a sample of Anvil Points raw shale. The shale was sieved to pass a 270-mesh screen and be retained on a 300-mesh screen before extraction. The values obtained for amount of extractable material are shown in Table II. Tetrahydrofuran (THF) is the best solvent for Anvil Points raw shale. It should be noted that some inorganic material was probably simultaneously extracted, so that the values reported are high. If the Fisher assay for this shale represents the total extractable kerogen, this shale was 9.22 weight percent kerogen. The THF extracted 5.41 weight percent of the shale or 58% of the total kerogen. In future studies of airborne particulate matter, THF will be used to extract the particles before analysis.

TABLE II
EXTRACTABLE MATERIAL FROM ANVIL POINTS
RAW SHALE BY VARIOUS SOLVENTS

<u>Solvent</u>	<u>ppt Extracted^a</u>
Cyclohexane	14.40
Carbon Disulfide	42.64
Tetrahydrofuran	54.07
Ethyl Acetate	13.16
Methylene Chloride	17.96
Ethanol	13.92
Benzene	10.78
Chloroform	16.84
Methanol	16.18
Toluene	17.28
Hexane	11.31
Acetonitrile	9.73
Acetone	20.46
Dimethoxymethane	18.59
Carbon Tetrachloride	17.34

^aConcentration expressed as parts per thousand.

B. Evaluation of Air and Dust Samples Derived from Oil Shale

Two different types of air samples were investigated — vapor phase organic materials collected with various sampling tubes and respirable-sized dust particles generated in the inhalation facility (Industrial Hygiene Group, H-5). The vapor phase organic samples were collected from four oil shale pilot facilities. Additional samples were obtained from a sampling trip by Earth and Space Sciences Group personnel to the region of tract C-a and are treated as background samples. The primary goal of the analysis was to identify as many compounds as possible in each sample. The approximate concentrations of the materials could then be estimated from the GCMS peak heights. Comparison of results from facility to facility could point out specific compounds which may be indicative of the process at that facility. Furthermore, knowledge of compounds present in samples taken near the retorts can be used to identify monitor compounds whose presence would signify a fugitive leak source at the facility.

Nearly 400 compounds were identified in the samples, and some 50 target compounds have been identified as monitor compounds. Background compounds are predominately paraffinic with dimethylcyclopentane isomers, hexane, ethane, and propane the predominant species. Isoprene and ethylene were the most predominant olefinic compounds, while toluene and dimethyl benzenes were the most prevalent aromatic species. The samples from near the retorts are characterized by high alkane and olefin contents; up to 80% of all material collected are of these classes. There was some concentration variation from facility to facility, but the major components were the same for all facilities. The principal variation between facilities occurs in the aromatic compounds, but the low concentrations of these species make a good comparison difficult. It appears that polycyclic aromatic species of 3 or more rings, and in particular, alkylated PAH compounds, may be the best indicator compounds for retort emissions. All sample collections were made using charcoal tubes, and only a few samples have been collected using other adsorbent materials. Further study will be required to decide which adsorbent is best for the

vapors, and additional samples must be taken from each facility to determine the local concentrations on species fluctuations characteristic of the retort.

Respirable-sized dust particles of Anvil Points raw shale were generated by a Wright dust feed and collected by an Andersen cascade sampler. Sequential samples were collected and grouped by particle size. Each fraction was assayed by several procedures, including neutron activation analysis for metallic species, x-ray fluorescence for mineral species, extraction with tetrahydrofuran for organics, and GCMS analysis of the extracts to determine specific compounds present. The following conclusions may be drawn from the results. Mineral species vary with particle size, with the clay-like minerals tending to be associated with the smaller particles. Free quartz (d-silica) levels vary by as much as a factor of 2 with particle size, and the variation is irregular. Elemental variations follow mineralogical variations. The amount of extractable organic material per gram of particles increases as the particle size decreases. In general, the amounts of all specific organic compounds tested increases as the particle size decreases, but rate of increase for each species is different.

C. Fractionation and Analysis of Oil Shale Retort Water

A more detailed investigation of retort water obtained from the Paraho process has been undertaken. Previous studies of the water showed it to possess the greatest mutagenic activity in the Ames test and greatest organic content of any of the waters tested. In an attempt to determine the chemical species responsible for the observed mutagenic activity, the water was fractionated and each fraction was tested and fractionated further as required. This work is still in progress, so this report should be considered preliminary.

Two different fractionation schemes were utilized--a solvent extraction pH adjustment scheme derived from the EPA priority pollutant procedure and a column extraction scheme using a C_{18} reverse phase HPLC resin.

The EPA scheme gives five fractions of the water plus the initial water sample. The pH was adjusted to 13 using concentrated NaOH, and the black tar and gelatinous mixed precipitate filtered.

This fraction of base tars and insoluble metal hydroxides has been called the base precipitate. The water at pH 13 was extracted 3 times with an equal volume of methylene chloride to afford the base-neutral fraction. The pH was then adjusted to 4 and the acid precipitate fraction was separated. Extraction with methylene chloride gave the acid fraction, and a residual water sample. The results of the extraction are shown in Table III. For Ames testing fractions were dissolved in 50 ml H_2O and tested. The two precipitates were not soluble in water, nor could they be easily solubilized for testing.

It has been reported by workers at Pacific Northwest Laboratory that it is possible to remove nearly all (95%) of the Ames activity from oil shale product waters by repeatedly passing the water through C_{18} packing. The compounds removed by the packing may be recovered by elution with methanol. We have applied the scheme to Paraho water by passing 60 ml of the water through a 27-ml bed of C_{18} HPLC packing, 27-53 micron diameter. The column was rinsed with 30 ml deionized water after each pass, then flushed with 100 ml of methanol. The water wash was evaporated and the solid added to the column eluant. After nine passes, the mass recovery from the column as measured by evaporation of the methanol was only 12 mg, and after 12 passes, nothing could be eluted from the column with methanol. The 60 ml of initial water yielded 2.097 g of solid for a mass recovery of 17.5%. The remaining water was found to contain 170.2 mg/ml dissolved solids. This gave an overall mass recovery of 100.0%. The methanol fraction was taken up in 60 ml deionized water and all fractions were tested in the Ames system. The results are shown in Table IV. There are two explanations for the observed data; first, the compounds responsible for most of the Ames activity had been irreversibly trapped by the C_{18} column. The mass balance would indicate that, if this is so, concentrations of these compounds are extremely low. The second possibility is that the chromatography has caused chemical changes in compounds which caused these compounds to lose their mutagenic potential. At this time, we cannot say which of these possibilities is true.

TABLE III
RESULTS OF THE EPA A/B/N FRACTION OF PARAHO PRODUCT WATER

<u>Sample</u>	<u>Mass (mg/ml)</u>	<u>Percent Mass</u>	<u>Specific Ames-Activity rev/μg</u>	<u>Percent Activity</u>
Initial water	205	100.0	0.497	100.0
Base ppt	90.4	44.1		
Base-neutral	0.612	0.29	88.3	53.0
Acid ppt	63.3	30.9		
Acid	0.52	0.25	1.68	0.86
Residual water	40.0	19.5	0.004	0.16
Recovery	95%			54%

TABLE IV
RESULTS OF THE C18 COLUMN FRACTIONATION OF PARAHO PRODUCT WATER

<u>Sample</u>	<u>Mass (mg/ml)</u>	<u>Ames Specific Activity rev/μg</u>	<u>Percent Activity</u>
Initial water	205	0.497	100.0
Methanol fraction	34.95	0.55	19.0
Aqueous fraction	170.2	0.06	10.0
Recombined fractions	205.2	0.104	20.9

Due to this uncertainty, we have decided to concentrate on the base-neutral fraction of the EPA procedure. The initial results of this study are

reported by J. W. Nichols and G. F. Strniste - Mutagenicity of Various Oil Shale Retort Process Waters in the Ames/Salmonella Bioassay, in this report.

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INTRODUCTION

The Environmental Science Group is a multi-disciplinary organization that conducts research relevant to the identification and resolution of environmental issues associated with emerging energy technologies, nuclear waste management, and the Los Alamos National Environmental Research Park (LA/NERP). A primary focus of the group's activities is upon the requirements for information about air, water, and land disturbances resulting from energy developments.

Current research activities include pollutant source characterization, identification of mobile contaminants, transport of pollutants in the environment, assessment of the effects of environmental pollutants, and the identification or development of methods to control environmental contaminants. Specific research programs in support of developing energy technologies include environmental assessment of geothermal systems, conditioning and stabilization of uranium mill tailings, deer population dynamics near prototype oil shale development areas, disposal and control of coal fly ash/scrubber sludges, and transport of contaminants by air- and water-borne sediments. In the area of nuclear waste management, projects include field experiments on erosion control, biobarriers, water

management and advanced engineering concepts, unsaturated transport modeling, alternatives to shallow-land burial, and the attenuation of radio-nuclides in liquid wastes by soil. LA/NERP projects include a survey of endangered plant species, raindrop transport of contaminated soil, animal population dynamics, elk nutrition/vegetation dynamics, ponderosa pine regeneration, and the assessment of nitrogen stress in the environment.

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

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

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

Development of a Simplified Model to Predict Runoff,
Sediment Yield, and Contaminant Transport in
Mortandad Canyon

[L. J. Lane, T. E. Hakonson, and G. C. White]

Contaminants associated with large volume wastes may be transported from waste disposal sites with eroding soils. Soil erosion and subsequent sediment transport are of particular concern in the semiarid western United States where much of the annual precipitation occurs during intense summer thunderstorms. Runoff from such storms can result in accelerated loss of soil and associated contaminants from a disposal site. Once the contaminants enter a stream channel system, the stream bed sediments are often the major repository of radioactive and stable elements released to the environment. Subsequent storms produce runoff which can result in offsite transport of the sediment and contaminants in the channel system.

Differential erosion, transportation, and deposition result in sediment particle sorting. As these processes are selective, as a function of particle characteristics, the result is that transported sediment is usually enriched in the finer particles. Because of physiochemical processes, again as a function of particle characteristics, contaminants can be more strongly associated with the smaller sediment particles. The combined processes of particle sorting during erosion, transportation, and deposition and the differential association of contaminants by sediment particle size produce complex relations between runoff, sediment transport, and associated contaminant transport. Because knowledge of contaminant transport is important in designing monitoring systems, in estimating contaminant inventories, and in contaminant-risk assessments, there is a need to determine the influence of particle sorting on contaminant transport rates.

Predicting the transport of contaminants associated with sediment particles is important in the management of chemicals associated with agriculture¹ and energy wastes.² Although the environmental problems and circumstances pertaining to agriculture and to energy wastes appear to differ, the transport of contaminants in association with sediment particles is common in both areas. Recent advances in predicting runoff, sediment

transport, and chemical transport in agricultural systems^{1,3,4} have been adapted to predicting the transport of contaminants associated with energy wastes.⁵

We believe that models or procedures used to predict particle transport should be conceptually and operationally simple. The procedures should not be oversimplified, but they should require a minimum amount of calibration data, they should be able to make predictions without extensive parameter optimization, and the information they provide should be useful in decision making.

Toward this end, we have developed a procedure to predict runoff from upland areas using precipitation, soils, and vegetation data. The runoff is then routed through stream channel systems to compute sediment transport by particle size classes. The runoff and sediment particle transport data are then used to compute contaminant transport rates and amounts. The hydrologic model was developed using data from 65 experimental watersheds operated by the U. S. Department of Agriculture (USDA) and from 16 gaging stations operated by the U. S. Geological Survey (USGS). Data from these 81 experimental watersheds in 12 areas of the United States represent several hundred runoff events. Sediment data from 8 USDA and USGS watersheds were used to develop the sediment transport equations.⁵ Based on analysis of these data, we concluded that the hydrologic model and sediment transport equations were sufficiently tested and accurate enough to use in predicting particle-contaminant transport at Los Alamos.

This report describes a method to predict sediment transport, by particle size classes, in alluvial streams with noncohesive sediments. Based on a knowledge of contaminant concentrations in the bed sediments, procedures are developed to predict the transport rate of contaminants traveling in association with sediment particles. Runoff, sediment, and contaminant rates are integrated over a given period of runoff (the runoff hydrograph) to estimate water, sediment, and contaminant yields. The results of this routing procedure were compared with empirical methods, such as loading functions and enrichment ratios, that are commonly used to predict contaminant yields. The routing method includes the influence of particle sorting and thus

represents an improvement over the loading function-enrichment ratio approach.

An example application is for plutonium transport in an effluent-receiving canyon at the Los Alamos National Laboratory. Plutonium concentrations in the bed sediments of the alluvial stream channel were found to vary by an order of magnitude as a function of particle size.⁶ Errors in computed plutonium transport rates as a result of ignoring sediment particle sorting ranged from less than 10% for large runoff events to near 100% for very small runoff events. For flood events smaller than the average annual flood, predicted plutonium yields ignoring particle sorting differed by over a factor of two from yields computed using the routing procedure. Moreover, the routing procedure accurately predicted measured plutonium transport rates during a flood event. This illustrated the importance of particle sorting on plutonium transport in Mortandad Canyon at Los Alamos.

A second example application involved developing frequency distributions for runoff, sediment yield, and contaminant yield. Procedures were developed to predict the distance a contaminant will travel as a function of storm size and to determine the probability distribution for travel distances. This application for contaminant transport in ephemeral streams illustrated the importance of accurately predicting transport and deposition of sediments by particle size classes.

The methodology presented is general and applicable to a variety of sediment-contaminant transport problems independent of the specific models used to compute runoff and sediment transport. However, the models used to compute runoff rates and amounts and sediment transport rates should incorporate differential sediment particle transport as sediment particle sorting is shown to be highly significant in the example applications.

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Assay for Bioavailability of Uranium Mill Tailings Contaminants

[D. R. Dreesen, E. J. Cokal, and M. A. Maez]

A plant uptake assay technique has been under development at Los Alamos for the past several years.¹ The aim of this research is to develop a method to rapidly determine bioavailable contaminants in various types of solid wastes using a variety of plant species. Details of the assay technique have been described in an earlier report.¹

The first experiment evaluated uptake of contaminants by one species, four-wing saltbush (Atriplex canescens), grown from seed for one month in the vermiculite potting mixture. After this initial growth period, the root mass of the shrub was placed in contact with one of the following substrate treatments: control-vermiculite potting mixture (VERM), 4 replicates; alkaline tailings, Durango, CO (DGO) site, 3 replicates; acid tailings, Shiprock, NM (SHIP), and Salt Lake City, UT (SLC) site, 3 replicates each. The aboveground plant parts were harvested after one month's growth in the various treatments, then washed and air-dried. Roots from the upper vermiculite layer of each treatment were also harvested, washed in an ultrasonic bath, and air-dried. These plant materials were ground in a Wiley mill to less than 20 mesh and submitted for neutron activation analysis. The results of these analyses are summarized in Table I, which shows the ratios of the

TABLE I
ELEMENTAL ENRICHMENT IN PLANTS: CONCENTRATION
RATIOS (CR) OF ROOTS AND SHOOTS GROWN IN
TAILINGS VERSUS CONTROLS

Tailings	Plant Parts	CR < 1	1 < CR < 5	CR > 5
SHIP	Shoots	Br	U, Co, Al	V, Se, Eu
	Roots	Ca, As	U, Cl, Cu, Ba, Br	Se
SLC	Shoots	-----	Al, Na, Mn, Zn	U, Co, Cl, Sm
	Roots	Ca, Br	V, Co, Na, Cu, Ba, Ga, Hf, Ta	U, Cl, La, Ce, Lu, Sm
DGO	Shoots	Mn, Zn	----	V
	Roots	Cl, Sm, Na, Mn, Zn, Ca, As, Br	Ba	----

mean concentrations for shoots and the composite concentration for roots grown in tailings versus plants grown in the control treatment. The elemental concentrations in the roots have been standardized using the concentrations of immobile elements (i.e., Ti, Sc, ...) to reduce the variance among treatments due to inconsistent removal of vermiculite from the roots during washing.

The plants grown in acidic tailings (SHIP and SLC) have enriched levels of the acid mobile metals U, Co, and Al in aboveground plant parts. Shoots from the SHIP treatment show V and Se enrichment; both of these elements are highly enriched in these tailings. The shoots from the SLC treatment have enriched Na and Cl levels as well as elevated concentrations of acid mobile metals, Mn and Zn. The only element significantly enriched in shoots from plants grown in the alkaline DGO tailings is vanadium; Mn and Zn are depleted versus the control. The rare earth elements, Eu and Sm, are enriched in the SHIP and SLC shoots, respectively. Barium is present in elevated levels in roots grown in all the tailings materials. The roots from the various tailings treatment seem to be depleted in Ca, As, and Br. Many of the elements elevated in the aboveground portion of plants grown in acid tailings are also enriched in the roots (e.g., U, Se, Cl, Na, and Sm).

The second experiment investigated uptake by a variety of species—a grass, barley (*Hordeum vulgare*); two legumes, yellow clover (*Medicago*

scoparia); and a native shrub, four-wing saltbush (*Atriplex canescens*). These plants were grown from seed for one month before they were placed on tailings diluted 90% by coarse sand. Tailings from Ambrosia Lake, NM (AML) were assayed in addition to SLC, SHIP, and DGO tailings and a clay soil from the Bluewater area (BLUE) of the Grants mineral belt, NM. No replicates were used in this complete block design except 4 replicates of barley grown in AML tailings; the remainder of experimental protocol was as previously described for the first experiment.

The aboveground plant parts from the second experiment were dissolved and the solutions were analyzed by atomic absorption spectrophotometry, inductively coupled plasma emission spectrometry, fluorimetry (for U), and ion chromatography. The results for 4 elements (Mo, Se, Co, and U) are shown in Table II. Molybdenum appears to be easily assimilated by all these species; however, the plant concentrations do not reflect the Mo levels in the tailings or in the water extracts of tailings. Barley seems to take up more Mo than yellow clover, which shows greater uptake than the other species. Four replicates of barley grown in the AML tailings show a coefficient of variation for within treatment Mo concentrations of 16%. Selenium uptake and translocation is most noticeable for the legumes; appreciable Se is present in plants from AML and SHIP, which have high extractable or total Se contents, respectively. The coefficient of variation for Se in the four barley replicates is 28%. Some uptake of Co is apparent for the acid tailings, SHIP and SLC, which have appreciable extractable Co. Barley does not appear to assimilate and translocate cobalt. Uptake of uranium seems to be observable for some tailings, in particular AML; barley and yellow clover appear to take up more U than the other species.

Transition metals such as Ni, Zn, and Cd are assimilated in a manner similar to that of Co, i.e., more uptake from acid tailings. Levels of nutrients such as B, Mn, Zn, and S (as SO_4^{2-}) appear to be more controlled by the plant species with little differences in concentrations due to treatment. Appreciable chloride levels were found for all species when grown in the SLC tailings.

The results of these plant uptake assay experiments have indicated that appreciable

TABLE II
CONCENTRATIONS OF MO, SE, CO, AND U IN ABOVEGROUND PORTIONS OF PLANTS GROWN IN TAILINGS AND CONTROLS

Element	Treatment	Mesa ^a ($\mu\text{g/g}$)	Meof ^a ($\mu\text{g/g}$)	Hovu ^a ($\mu\text{g/g}$)	Atca ^a ($\mu\text{g/g}$)	Kosc ^a ($\mu\text{g/g}$)	Hovu x	(n=4) s.d.	Soil or Tailings Conc. ($\mu\text{g/g}$)	Water Extract Conc. (mg/l)
Mo	VERM ^b	<0.3	0.9	1.8	-	1.1			-	-
	BLUE ^b	1.4	1.5	1.2	1.5	1.5			0.3	0.18
	AML ^b	3.9	8.0	9.4	5.1	5.8	8.6	1.4	65	7.7
	SLC ^b	4.1	4.8	8.0	3.4	2.6			800	5.4
	SHIP ^b	2.9	3.9	6.0	2.6	3.3			96	0.1
	DGO ^b	2.7	4.8	2.4	1.8	1.7			11	0.7
	MAX. ^c	2.9	5.3	7.8	3.4	3.9				
	BLUE									
Se	VERM	<0.5	<0.5	<1	-	<1			-	-
	BLUE	<0.5	<0.5	<1	-	<1			1.2	0.02
	AML	5.4	2.4	2	<0.5	<1	2.5	0.7	33	2.7
	SLC	1.1	0.5	4	<0.5	<1			56	<0.1
	SHIP	3.9	6.0	3	1.6	4			200	0.2
	DGO	<0.5	<0.5	2	<0.5	<1			29	0.1
	MAX.	>11	>12	>4	-	>4				
	BLUE									
Co	VERM	0.6	0.6	<0.2	-	0.5			-	-
	BLUE	0.8	0.7	<0.3	-	0.3			9.0	-
	AML	1.1	0.3	<0.3	-	0.6	0.2	0.1	44	-
	SLC	1.4	1.8	<0.3	0.9	0.6			26	1.4
	SHIP	1.7	0.7	<0.3	0.6	1.4			5.3	0.2
	DGO	0.7	0.3	<0.3	0.7	0.5			110	<0.1
	MAX.	2.1	2.6	-	-	4.7				
	BLUE									
U	VERM	0.02	0.02	0.06	-	0.08			-	-
	BLUE	0.01	0.02	0.04	<0.03	-			2.4	0.01
	AML	0.06	0.28	0.17	0.13	0.30	0.16	0.14	176	3.7
	SLC	0.04	0.03	0.13	-	-			180	8.0
	SHIP	0.03	0.02	0.09	-	0.03			49	0.7
	DGO	0.03	0.16	0.14	0.03	0.03			470	1.1
	MAX.	6	14	4	-	-				
	BLUE									

^aMesa = *Medicago sativa*, alfalfa
^aMeof = *Melilotus officinalis*, yellow clover
^aHovu = *Hordeum vulgare*, barley
^aAtca = *Atriplex canescens*, four-wing saltbush
^aKosc = *Kochia scoparia*

^bVERM - vermiculite potting mixture
^bBLUE - Bluewater, NM soil
^bAML - Ambrosia Lake, NM tailings
^bSLC - Salt Lake City, UT tailings
^bSHIP - Shiprock, NM tailings
^bDGO - Durango, CO tailings

^cMaximum plant conc.
in tailings/plant conc.
BLUE

concentration ratios for bioavailable contaminants are obtained for plants grown in tailings for one month compared with controls. The apparent differences in root and shoot content for many elements illustrate the need for further refinement in experimental methods to better determine if some contaminants are fixed in the roots. The results of the most recent assays indicate a very complex relationship between contaminant uptake and plant species, tailings type, and the element of interest. It is clear that knowledge of total or extractable elemental contents cannot be directly related to uptake. In addition, the assimilation and translocation of contaminants by one species can not necessarily be extrapolated to other species.

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Subsurface Transport of Contaminants from Energy Process Waste Leachates

[L. E. Wangen and E. A. Stallings]

A number of inorganic components were present in leachates from energy processing wastes at concentrations high enough to contaminate water supplies if the leachates are added in sufficient quantities. Included in these wastes are uranium mill tailings¹, coal ashes and flue gas desulfurizations sludges², coal cleaning wastes,³ and spent oil shales.⁴ Ground water quality

degradation by such leachates is likely to be restricted to the vicinity of the disposal site but to continue for a long time. Contamination of ground water, where it has occurred as a result of improper waste disposal, has proven to be practically impossible to control. Thus, present wisdom advocates prevention rather than treatment where ground water contamination is a possibility. In general, the severity of contamination from a given waste will be site specific, i.e., dependent on local land use, geohydrology, climate, water quality, and underlying conditions of soil and rock. These factors may aggravate or mitigate the contamination potential posed by any given waste and should be understood in order to properly manage wastes. Studies have shown that natural soils have varying capacities for attenuating contaminants and that different contaminant species are attenuated to different degrees by the same soil.⁵⁻⁸ Such information demonstrates the possibility of using natural mechanisms to minimize the chance of environmental degradation resulting from waste disposal by use of informed management and engineering practices. Thus, it is desirable to obtain knowledge regarding the effectiveness of soils at reducing the migration of contaminants from energy processing wastes. In FY 1980, Los Alamos initiated experiments designed to quantify the migration of inorganics through soils using column methods.

Trace contaminants often present in leachates from energy process wastes at higher than acceptable concentrations include As, B, Cl, F, Mn, Mo, Se, NO₃, Ni, and Cd. Major matrix components include SO₄²⁻, Ca, and Mg. Species to be studied as to migration rates through soil columns are selected from these. At present the transport of As, B, Ca, Cd₂, Cl, Mo, Ni, NO₃, Se, and SO₄²⁻ are being studied. Speciation assumed for these are AsO₄³⁻, B(OH)₃, Cd²⁺, and SeO₄²⁻. A mixture containing the nitrate salts of Ni and Cd at ~ 10 mg/l was used to develop breakthrough profiles in foil columns. Ionic strengths and densities of these solutions are also adjusted with 0.1 M KNO₃.

Effluent breakthrough curves for these species are being developed for 4 real subsoils and 2 important soil mineral components. The mineral components are kaolin and goethite. The 4 real soils are a calcareous and acidic subsoil from

Illinois, a dunal sand from Indiana, and a high oxide sandy subsoil from Georgia. Soil components and the first two real subsoils have been diluted with a fine- to medium-grained sand by a coating procedure to produce a soil/sand mixture with sufficient hydraulic conductivity for performing leachate migration experiments. Batches of each material are homogenized and all experiments on a given material are performed on samples from this batch.

The soil columns used in these experiments are constructed of lucite with dimensions of ~ 10 cm long by 5 cm I.D. These are packed with soil using recommended procedures^{9,10} for uniformity of packing to a depth of 10 cm under CO₂ atmosphere. Packed columns are filled with a solution containing 0.1M KNO₃ and washed for several pore volumes. After removal of Cl ion from the column, the dispersivity of each column is determined using either a KCl solution or tritiated water. Dispersivity and leachate attenuation experiments are performed using downward flow at a flow rate of ~ 5 ml/hr. Chemical analyses on solutions is by DC arc plasma atomic emission for As, B, Ca, Cd, Mo, Ni, and Se. Hydrogen ion activity is monitored throughout while Eh and carbonate content are periodically checked.

Experimental soil column breakthrough curves have been developed for As, B, Cd, Mo, Ni, and Se in kaolinite and a dunal sand from Michigan City, Indiana. In addition such curves have been developed for the anionic species of B, Mo, and Se in goethite. Arsenic, assumed present as the +5 oxyanion, has not come through the goethite column after 9 months. These experiments have been performed in duplicate and the agreement has been satisfactory.

Cd and Ni are not highly adsorbed by either kaolinite or dunal sand. The quantities of Cd and Ni adsorbed by Kaolinite are 0.040 and 0.078 millequivalents per 100 grams, respectively, as compared to a kaolinite cation exchange capacity of 5.8 meq/100g, respectively. The cation exchange of the dunal sand is ~ 0.25 meq/100g.

Breakthrough curves for Cl⁻, Cd²⁺, and Ni²⁺ in the dunal sand from Michigan City, Indiana are shown in Fig. 1. We define the breakthrough point as the eluent volume corresponding to a C/C⁰ value of 0.5. C is the eluent concentration and C⁰ is the influent

Fig. 1. Breakthrough curves for Cl, Ni, and Cd in a dunal sand from Indiana.

concentration. Chloride behaves like a conservative tracer in this material and consequently has a $C/C^0 = 0.5$ at 1 pore volume. Of the cations, Ni^{2+} is less retained ($C/C^0 = 0.5$ at 4.85 pore volumes) than Cd^{2+} ($C/C^0 = 0.5$ at 7.45 pore volumes). After subtracting one pore volume from these to account for the pore water originally in the soil bed we calculated the above masses of each species retained or sorbed by the soil bed.

There is a bump in the initial part of the Cd^{2+} curve in Fig. 1. This may be caused by a decrease in pH of column eluent that occurred at about this time. If cation adsorption is pH sensitive in this material, some desorption, thus a somewhat higher concentration in solution, could have occurred as a result of decreased pH in the soil bed.

Breakthrough curves for Cl^- , H_3BO_3 , SeO_4^{2-} , and $H_2AsO_4^-/HASO_4^{2-}$ are shown in Fig. 2 for the same Michigan City dunal sand. Breakthrough ($C/C^0 = 0.5$) occurs at 1.0, 1.1, 1.2, 1.5, and 7.5 pore volumes for Cl^- , B, Se, Mo, and As, respectively. Thus, except for As, there is little adsorption of these species by the sand. The breakthrough pattern of Se is unusual in the presence of two somewhat S-shaped curves following one another. This was observed in two separate columns containing the dunal sand and could result from two different species of Se, e.g., Se(IV) and Se(VI). This possibility is being investigated. The breakthrough curve for As is also different in that it begins to level off at C/C^0 between 0.6 and 0.7. Although not shown in Fig. 2, arsenic C/C^0 values do continue to slowly increase with increasing pore volume, attaining a value of 0.83 at 17 pore volumes at which time the experiment was terminated. A slowly rising breakthrough curve

Fig. 2. Breakthrough curves for Cl, B, Mo, Se, and As in a dunal sand from Indiana.

such as that shown is indicative of a kinetic controlled reaction between $As(V)$ and reaction sites associated with the soil. Slow reactions have been noted between phosphate and iron oxides and it is possible, given similarities in chemical behavior, that such reactions could also be operative for arsenate.

Calculated adsorption, in mmoles per 100 g, for these species on kaolinite and dunal sand, respectively, are As (0.25, 0.019), B (0.032, 0.0021), Mo (0.047, 0.0010), and Se (0.028, 0.00054). In contrast, approximate adsorption values for these elements on goethite (iron oxide) are As (> 26.0), B (1.88), Mo (6.52), and Se (1.46) mmoles/100g. Thus, the iron oxide adsorbs vastly larger quantities of these anions than do kaolinite or the dunal sand.

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Predicting Brine Spillage from Hydrothermal Energy Electrical Development

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

A major concern in the development of hydrothermal energy, pinpointed by the workshops held at the Los Alamos National Laboratory in 1980, is the accidental discharge of brines into the environment.¹ Such discharges were postulated to subject the exposed ecosystems to hot saline water which could also contain toxic elements. Concern was expressed that these releases might adversely affect soils, plants, and nearby water systems. As part of a multilaboratory effort to address environmental issues attendant to developing hydrothermal energy, Los Alamos has undertaken the task of evaluating the effects that might result from the acute conditions created by a brine spill. This report briefly describes one of the necessary first efforts needed to describe and define spill effects,

i.e., to provide a prediction of the kinds and sizes of spills and how often they occur.

Spills associated with hydrothermal, or water-dominated, geothermal energy development are likely to be similar to those experienced during the extraction of energy from a steam-dominated energy source. The main difference will be the larger amounts of liquid that must be moved in the water-dominated case to produce the same amount of energy as produced in the steam-dominated case. Because of this, steam-dominated operations at the Geysers in California can be used to evaluate the hydrothermal situation. Several years of well-kept data involving up to 14 electrical generating plants varying in size from 11 to 106 MW are available for analysis.^{2,3} The treatment below reflects information about spills that is required to be reported to California authorities. These spills do not include operational spills that were contained entirely within a plant facility/yard or well-pad.

The dominant types of spills at a hydrothermal development will be brine water related (see Table I). Sometimes this water will be extremely hot ($> 166^{\circ}\text{C}$), as when it escapes from production pipelines, but more often it will be just warm or hot ($< 100^{\circ}\text{C}$), as when the heat has been extracted and it is in the cooling tower or reinjection stage of the overall process. These spills can be as large as 250,000 gals (multiply by 3.785 to convert to liters). Small, infrequent spill types (iron sludge, sodium hydroxide, etc.) are related to the hydrogen sulfide abatement system and are listed under the "Others" category in Table I.

TABLE I
COMPOSITION OF REPORTABLE SPILLS^{a,b}

<u>Spill Liquid</u>	<u>Events</u>	<u>% of Events</u>	<u>% of Total Volume</u>	<u>Maximum Size (gals)</u>
Very Hot Brine	3	5.3	13.2	132 000
Brine Condensate	46	80.7	86.6	250 000
Others	8	14.0	0.2	1 000

^a Data from CRWQCB and covers data from the Geysers, CA from 1974 through 1980.

^b This data represents occurrences for a steam-dominated thermal reservoir.

The dreaded blowout of a well can occur. This has indeed occurred at the Geysers. No information is available in the California Regional Water Quality Control Board (CRWQCB) records about spills in the past few years on which to estimate the extent of any effects from it. The blowout well had not been capped in mid-1980 when we saw it and had obviously and negatively affected an area several hundred feet in diameter. The steam-domination of the thermal reservoir has probably kept the total amount of effluent down. A similar occurrence in a water-dominated system should affect a larger area and thus be more serious.

A new source of "spillage" appears to be developing as berms are being constructed around the electrical plants to prevent spills of the types mentioned above. These new releases have been dubbed "orange rains" at the CRWQCB since the drainage near several plants sometimes appears as an orange liquid after rain storms. The sizes of these rain-associated effluents have not been estimated. "Orange rains" are speculated to be related to the most frequent spill type, i.e., the spent or cooling tower brine, but the relationship has not been established.

The major single cause of spills at a geothermal operation will probably be broken or corroded pipes (see Table II); the next major causes are auto-control/equipment failures and improper operating procedures. If the four "plugged-pipe" events listed in the "Others" category are included under equipment failures, these three causes account for 89% of all the spills tabulated in Table I.

TABLE II
CAUSES OF REPORTABLE SPILLS^a

Cause	Occurrences	
	Number	Percent
Broken or corroded pipes	17	30
Auto-control or equipment problems	15	26
Improper operating procedures	15	26
Others	10	18

^a See footnotes in Table I.

The frequency distribution of spills at the electrical generating plants at the Geysers is somewhat Gaussian (see Fig. 1). Thus two-thirds of the lifetime of a plant contains years that are spillfree or contains only one spill. Similarly, however, two or more spills are likely to occur yearly during one-third of the lifetime. Generally, 0.687 spills can be expected yearly just for an electrical generating plant. Less data are available to evaluate the frequency distribution associated with the steam production phase. Dividing the total number of spills by the number of plant-years associated with the spills gives a frequency of 0.17 spills for steam generation per plant per year. The frequency during 1979 and 1980 was 0.32 spills/plant-year. The overall total, long-term frequency for an electrical generating plant including steam generation is thus 0.86 spills per year.

The size distribution of spills at the Geysers appears to be multimodal (see Fig. 2). During the period 1974-1977, the distribution was bimodal with no small spills recorded. The two peaks occurred around 10,000 gals and 100,000 gals. More recently, 1978-1980, these peaks have shifted to lower values, 4,000 gals and 40,000 gals. In addition, many small spills have been recorded. As berms are built, new

Fig. 1. Frequency distribution of spills at the Geysers plants.

data often reflect only the amount that escapes the plant yard and thus spills automatically become smaller. In any event, the decrease in spill sizes reflects a conscientious effort by the industry to prevent these spills from reaching the environments.

The experiences found above have been combined to form a prediction of the "reportable" spills that are likely for the 30-yr operating period of a hydrothermal-energy, electrical-plant complex. The predictions are based on the spill-size distributions for plants 53 MW in size and for all plants regardless of size. Assuming 0.687 spills per year at the electrical plant and 0.17 spills per year for the supply-plus-reinjection components, the total number of spills at a hydrothermal complex over a 30-yr period will be 26. A summary of the distribution of these spills according to size is given in Table III. We believe that the 53-MW data are much less influenced by the recent berm emplacements and thus reflects a truer distribution of actual spill sizes

than do the "all-plant" data. The "all-plant" data, however, probably better reflect the behavior of a conscientious, precautionary, and experienced industry. We predict that 9 to 14 large (> 10,000 gals) spills of predominately spent, but still hot, brine will occur during the lifetime of each electrical plant utilizing hydrothermal energy. The effectiveness of berms or other control measures will determine how much of this material will actually affect the environment.

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TIME-SIZE DISTRIBUTION OF ALL GEYSERS SPILLS

Fig. 2. Size distribution of spills at the Geysers.

Table III

A PREDICTION OF REPORTABLE SPILLS FOR A 30-YEAR PLANT OPERATION^a

Type	Mean Size (gal)	53-MW Plant Experiences		All-Size Plant Experience	
		Spills/yr	Spills/30 yrs	Spills/yr	Spills/30 yrs
Very Small	200	0.141	4	0.313	9
Small	3 200	0.253	8	0.253	8
Large	32 000	0.312	9	0.229	7
Very Large	160 000	0.151	5	0.062	2
Total		0.857	26	0.857	26

^a Forecast based on Geysers data for PG&E and Union spills

Activities of the Los Alamos National Environmental Research Park, FY 81

[K. V. Bostick]

The Los Alamos National Environmental Research Park (LA/NERP) was established as a field laboratory for ecological research, to study the environmental impacts of energy development, and as a source of public information on environmental issues. The emphasis of research on the park is to develop criteria that facilitate energy development in ways that are least harmful to the environment. The requirements in the Charter for the Life Sciences at Los Alamos National Laboratory also guide activities of the LA/NERP.

The LA/NERP encompasses approximately 111² km of DOE land at Los Alamos. The steep elevation gradient (1500 m in 25 km) and canyon/mesa terrain give the Research Park a wide spectrum of southwestern habitat types in a compact area. Another unique feature of the LA/NERP is that some areas within the park have been protected from activities such as agriculture, lumbering, or mining for nearly 40 years.

Studies on the LA/NERP are conducted by laboratory staff, and by graduate and undergraduate students from regional universities. Some of the work is conducted in cooperation with federal and state agencies, such as the National Park Service and the New Mexico Department of Game and Fish. Research projects sponsored by this program are selected by the LA/NERP Advisory Committee. Members of the committee include representatives from Life Sciences, Health, Earth and Space Sciences, and Engineering Divisions.

Much of the work sponsored by the LA/NERP are related to the 1977 La Mesa fire that burned 60 km² of Department of Energy, National Park Service, and United States Forest Service land. Reports on these and other LA/NERP sponsored studies follow this section.

In June 1981, Richard Bowker worked at Los Alamos under a guest agreement with LA/NERP to study thermoregulation in lizards in the park. His immediate objective was to combine the results ob-

tained on LA/NERP with previous data obtained throughout the United States and Africa in order to group lizards according to their ability to thermoregulate and to relate thermoregulatory behavior to ecological niche. Ultimately, he will extend this research to obtain energy balances on lizards and to investigate metabolic rates, again with the objective of relating this information to foraging strategy and ecological niche.

Mr. Bowker's research was funded in 1981 by an NSF grant. The work will continue in FY-82 under LA/NERP funding. During 1982 he hopes to gather data on more species of lizards and to begin preliminary investigations into the use of radioisotopes in determination of metabolic rates.

The report "Status of the Flora of the Los Alamos National Environmental Park" received the 1981 Technical Communication Award for excellence in topical reports. This award was given by the East Tennessee Chapter of the Society for Technical Communication.

Biotelemetry Studies on Elk--Updates

[G. C. White and D. K. Thiel]

The movements of Rocky Mountain elk (*Cervus elaphus nelsoni*) in the eastern Jemez Mountains of north-central New Mexico have been studied from 1978 to 1981. Seventy elk have been trapped, marked, and released; sixty of these animals were radio collared. The results of the study through 1980 are published in "Biotelemetry Studies on Elk" (Los Alamos National Laboratory report LA-8529-NERP, 1981). A movie based on the study, "Elk Biotelemetry at the Los Alamos Environmental Research Park" (Y-355), was also produced.

As of October 1981, twenty-four collared animals were being monitored. The others have been hunter-killed or poached, radio failures, or unknown disappearances. There were two significant occurrences during 1981. Due to a timber sale on the eastern slopes of Cerro Grande and Pajarito Mountain, and a minor outbreak of the disease *Elaeophora schneideri*, there was a change in migration routes (see Fig. 1).

Fig. 1. Change in animal migration routes.

Nutritional Ecology of Elk at the LA/NERP

[D. A. Kovacic]

The Los Alamos National Environmental Research Park (LA/NERP) covers 111 km², one-third of which contains ponderosa pine. In 1977, the La Mesa fire destroyed 6 km² of ponderosa pine including 1.2 km² on LA/NERP land. The La Mesa burn and other burns in the Los Alamos vicinity provide a unique opportunity to investigate succession and nutrient dynamics following fire in the ponderosa pine ecosystem.

Thirty-eight million acres of ponderosa exist in the west, 12 million of which are in New Mexico, Colorado, and Arizona. The ponderosa pine forest is an important resource, not only for timber but also because it provides forage for cattle and wildlife, wildlife winter range, and watershed.

Little is known about the effects of fire on vegetation and soil nutrient dynamics in New Mexico. Studies show that fire exerts divergent effects on the ecosystem nutrient status. Ecosystem dynamics following fire are not clearly understood due to the lack of knowledge concerning nutrient transport and transformation processes. Basic knowledge of nutrient dynamics and the use of fire may help to reduce the need for energy-intensive fertilizer use.

Our present study is a continuation of work begun by Gary White (Los Alamos National Laboratory) to study the elk population dynamics on LA/NERP lands following the La Mesa fire. In this phase of the ongoing project, we are studying post-fire successional and nutrient dynamics. We will determine how these dynamics relate to elk population carrying capacity and animal condition.

In March 1981, preliminary understory biomass data was gathered to study the trends in understory succession by comparing three successively older burns. Preliminary forage digestibility was also determined for grasses and forbs. This data has not been analyzed fully and is not yet ready for publication. From June through August of 1981, a total of 40 sites at burns of various ages and intensities were located. On each of these sites, fifty 1- x 2-ft quadrates were read in order to determine species composition and percent biomass cover at each site. We also completed an estimate of tree size and density for each site. This data will be used to compare and classify various sites.

Presently we are clipping vegetation on the 40 sites. The information we gather will be used to estimate winter standing biomass and elk forage. Digestibility samples are also being collected, as well as soil samples which will be analyzed for total nitrogen, nitrate, and ammonium. This quantitative effort will provide us with information that will help us to understand the vegetative dynamics of the ponderosa pine ecosystem and, in turn, to understand elk population dynamics in New Mexico.

Investigation of Radionuclide Concentrations in Tissues of Elk Utilizing Los Alamos National Laboratory Lands

[S. Meadows and J. Salazar]

Five Rocky Mountain elk (*Cervus elaphus nelsoni*) were collected on Los Alamos National Laboratory land and various tissues were sampled and analyzed for concentrations of ¹³⁷Cs, ⁹⁰Sr, ²³⁹Pu, and U. Samples of elk tissue were also collected from hunter kills in Cuba, New Mexico and similarly analyzed to provide background data for comparison with the Laboratory samples. Expected doses (mrem) to a human consumer from elk meat were then calculated using the reported concentrations (Tables I and II).

Plutonium concentrations were so low that differences between Laboratory and Cuba samples were negligible and uranium concentrations fell below the detection limit of the counting apparatus. Significant differences ($p < 0.05$) in nuclide concentrations between the two sets of samples were found with respect to ¹³⁷Cs and ⁹⁰Sr. Lung tissue

of Cuba samples contained significantly more ^{137}Cs than lung tissue of Lab samples. This difference is presumed to result from differences in fallout patterns in the two areas. Bone of Lab elk contained significantly more ^{90}Sr than bone of Cuba elk. However, since only small amounts of ^{90}Sr are released by the Laboratory in an area which receives little or no use by elk this difference was presumed to be a result of differences in fallout patterns or mineral content of the respective environment.

The highest expected dose to a human consumer from elk meat was 0.37 mrem (from ^{137}Cs in Cuba elk muscle). This constitutes only 0.07 percent of the NCRP dose limit (500 mrem/year) for a member of the public. Generally, concentrations of radionuclides were not significantly different in the tissues of elk utilizing Los Alamos National Laboratory land and elk that did not. In all cases, the expected dose to a human consumer from elk meat, liver, or heart was negligible.

TABLE I

A COMPARISON OF RADIONUCLIDE CONCENTRATIONS (pCi/g ASH) IN ELK TISSUES^a

	^{137}Cs ^c		^{238}Pu ^c		^{239}Pu ^c		^{90}Sr ^c	
	C	L	C	L	C	L	C	L
Heart	2.4 ⁽⁴⁾ ^b ± 2.1	1.4 ± 0.89						
Lungs	3.1 ± 2.1	-1.1 ± 2.9	0.00048 ± 0.0016	0.011 ± 0.027	0.00094 ± 0.0042	-0.0016 ± 0.0053	0.37 ± 0.58	0.16 ± 1.08
Liver			0.00042 ± 0.0021	-0.00092 ± 0.0020	0.0012 ± 0.0005	0.00044 ± 0.0015		
Kidneys			0.00080 ± 0.0024	-0.0013 ± 0.0012	0.0006 ± 0.003	0.0010 ± 0.0005		
Rumen Content	3.8 ± 4.7	1.9 ± 1.1	-0.0005 ± 0.0020	0.00083 ⁽³⁾ ± 0.00076	0.017 ± 0.08	0.015 ⁽³⁾ ± 0.0078		5.86 ⁽³⁾ ± 1.06
Leg Bone			-0.00024 ± 0.0010	-0.022 ± 0.044	0.00018 ± 0.0012	-0.008 ± 0.020	0.76 ± 0.21	1.40 ± 0.19
Jaw Bone			0.00010 ⁽³⁾ ± 0.00044	0.00014 ± 0.00068	0.00037 ⁽³⁾ ± 0.000058	-0.00074 ± 0.002	0.84 ⁽³⁾ ± 0.26	1.0 ± 0.19
Muscle	3.5 ⁽³⁾ ± 3.5	1.3 ± 1.2	0.00037 ⁽³⁾ ± 0.0011	-0.00036 ± 0.00072	0.0051 ⁽³⁾ ± 0.0044	0.00092 ± 0.00090		
Tendon			-0.0023 ± 0.0032	0.00032 ± 0.0027	0.0034 ± 0.0037	0.011 ± 0.005	0.022 ⁽⁴⁾ ± 0.016	0.0086 ± 0.0034

^a Elk tissues collected near Cuba, NM (C) and elk tissues collected on Los Alamos National Laboratory land (L) establish little difference. Mean concentrations ± one standard deviation.

^b () sample size other than five.

^c Samples were also analyzed for uranium but all results were below the detection limit.

TABLE II
FIFTY-YEAR DOSE COMMITMENT TO MAXIMUM ADULT FROM EATING ELK^a

Radioactive Isotope	Critical Organ	Dose (mrem) from Eating Heart of 1 Elk in 1 yr		Dose (mrem) from Eating Muscle of 1 Elk in 1 yr ^b		Percent of RPS ^b
		Cuba	Lab	Cuba	Lab	
¹³⁷ Cs	Total Body	2.5 x 10 ⁻⁶	1.5 x 10 ⁻⁶	3.7 x 10 ⁻⁴	1.4 x 10 ⁻⁴	0.07
		Dose (mrem) from eating liver of 1 elk in 1 yr				
²³⁸ Pu	Bone	1.8 x 10 ⁻⁸	0.0	8.4 x 10 ⁻⁷	0.0	< 0.001
²³⁹ Pu	Bone	6.5 x 10 ⁻⁸	2.4 x 10 ⁻⁸	1.2 x 10 ⁻⁵	2.1 x 10 ⁻⁶	0.002
⁹⁰ Co	Bone	1.8 x 10 ⁻⁷	3.4 x 10 ⁻⁷	9.8 x 10 ⁻⁶	1.8 x 10 ⁻⁵	0.004

^a From eating muscle, liver, and heart from 1 elk in 1 yr based on concentrations of certain radioactive elements in 5 elk collected near Cuba, NM and 5 elk collected on Los Alamos National Laboratory land near Los Alamos, NM.

^b Highest dose calculated as a percentage of the Radiation Protection Standard of 0.5 rems/person/yr.

^c Based on concentration in elk bone; the concentration in elk muscle and liver were calculated using a transfer ratio of bone to muscle and liver.

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

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

A study of winter diet quality and nutritional status of elk (*Cervus elaphus nelsoni*) was conducted in the Jemez Mountains, New Mexico during January - April 1980. A recently burned montane area, including Bandelier National Monument and Los Alamos National Laboratory lands, and an unburned upper montane area in the Valles Caldera were selected for winter range comparisons. Botanical composition of elk diets, determined from fecal analysis, was markedly different between the two areas. Grasses dominated diets in the burned area ($\approx 90\%$), but were consumed in nearly equal proportions with browse in the unburned area ($\bar{X} = 52\%$, grasses; $\bar{X} = 41\%$, browse). Forbs were a uniformly minor dietary component. Grasses were more digestible than browse, but protein was similar between forage classes. Comparisons of forage protein and *in vitro* digestible organic matter (IVDOM) revealed no differences between locations. Dietary proteins ranged from 3.5 - 7.2% in burned and unburned areas, respectively, and was consistently higher in the unburned area. Diet IVDOM increased from 36 to 48%

in the burn and from 33 to 39% in the unburned area from January to April. Compared with the unburned area, diet IVDOM of elk on the burn increased more rapidly and was superior in 5 of 6 periods. Observed differences between areas in diet quality were caused primarily by differences in diet composition, rather than in forage quality. Increases in diet quality during the study were highly correlated with increases in quality of grasses consumed by elk. A simulation model of ruminant energy and nitrogen balance was used to predict effects of diet quality on elk nutritional status. Greater energy deficits were predicted for elk in the unburned area, effecting greater losses of fat and lean body in this area. Superior dietary protein was insufficient to compensate for these losses.

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

Physiologic Response of Elk to Variations in Winter Range Quality

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

Thirty-six elk were live-trapped during February to April 1980, approximately 2.5 years after the La Mesa fire. Twenty elk were captured on Burnt and Escobas Mesas at Bandelier National Monument (BURN). The remaining elk were caught on the Valle San Antonio (BACA), an unburned winter range. Comparisons of body conditions between elk wintering on the BURN and those wintering on the BACA were made using a 3-way analysis of variance (location, age, sex) model that incorporated the covariates of day and time of capture.

All elk captured were radio-collared and tracked. The locations obtained substantiate the use of discrete winter ranges and use of a common summer range on the Valle Grande and surrounding areas. Comparisons of winter weights from the two areas yielded substantially higher values for elk from the BURN. Average weight for elk calves, adult females, and yearling males exceeded the average weight of BACA elk by 17.7, 36.0, and 25.1 kg, respectively ($P \geq 0.01$). The thickness of back fat was highly variable between all elk, but was detected on only one BACA elk, while 14 of the 18 elk sampled on the BURN had discernible fat levels. Blood metabolites were measured and produced highly significant differences ($P \geq 0.01$) between locations for alkaline phosphatase, triglycerides, anion gap, total bilirubin, and mean corpuscular volume (MCV). Significant differences ($P \geq 0.05$) were found for serum CO_2 and albumin levels and for total monocyte counts. These metabolic differences suggest that the diet of elk wintering on the BURN was substantially higher in available protein and energy.

All adult female elk captured were diagnosed as pregnant by rectal palpation and serum progesterone levels. No yearling females were captured on the BACA so interpretations on the effect of winter range condition on this younger age class are limited. However, differences in elk body condition from adult female elk sampled on the BURN and BACA may elicit changes in subsequent calf production and survival between the two groups.

Habitat Types in the Pinon-Juniper Woodland of Northern New Mexico

[F. J. Barnes and G. L. Cunningham]

Pinon-juniper woodland is a major vegetation type in the United States, covering over 75,000 square miles of the southwest. The distribution of the trees species comprising pinon-juniper woodland has been documented, but the adaptive physiological mechanism that might explain such distributions has not been studied. The work that will be completed in this study will lay the groundwork for such an ecophysiological investigation into the distribution of *Pinus edulis* and *Juniperus monosperma*, the two dominant tree species in the northern New Mexico pinon-juniper woodland.

During 1980-81, we have completed the field work for a vegetational analysis of the pinon-juniper woodland from its upper limit, at about 2250 m where it intergrades with ponderosa pine forest, down to its lower ecotone with sagebrush-grassland at about 1650 m elevation. The study area was primarily in the Los Alamos National Environmental Research Park (LA/NERP), with additional study plots located on adjacent United States Forest Service and United States National Park Service lands. The LA/NERP was an ideal place to locate our study for the following reasons: (1) the entire range of pinon-juniper woodland seems to be expressed in the LA/NERP, a geographically continuous area whose underlying geology has been well studied; (2) the LA/NERP lands have been closed to disruptive management and public access for over 30 yrs, and consequently the understory of the woodland is in relatively pristine condition; (3) the area is deeply dissected by steep canyons and it was possible to find stands of woodland with a diversity of aspects and environmental conditions.

Sampling was aimed at obtaining unbiased data from stands representing the full range of environmental conditions in the study area. Our choice of stands was restricted only by the preconditions that they show minimal impact (with respect to evidence of logging, grazing, and recreational use) and be located reasonably close to roads. Our major emphasis was recognized homogeneous stands representative of all aspects of frequency and

distribution of pinon and juniper, and to select stands without a preconceived bias about their position in a classification array. Plots (25- x 15-m) were located to represent the stands in terms of undergrowth and tree composition, density of species, and topography. A total of 40 plots have been established and studied. Density and cover of pinon and juniper were recorded by maturity classes. Canopy cover for each understory species was determined using a grid of 50 microquadrants (50 cm x 25 cm) on each plot.

The field data is being analysed using synthesis tables, ordinations, and cluster analysis. We anticipate that the data analysis will reveal indicator species in the understory stratum that will differentiate between the various habitats of the woodland. The series of stands that will result from the analysis will be the basis for our subsequent ecophysiological study.

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

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

Problems of ponderosa pine regeneration have been of concern in the Southwest for years. When sources of seeds are removed either by fire or logging, problems of regeneration become paramount. In 1977, a major wildfire (La Mesa fire) burned over land under control of the National Park Service, United States Forest Service, and Department of Energy. Each of these agencies, by virtue of their mandates, have a different concept as how to maintain the burned land. A study was designed to look at natural regeneration of pine after fire as related to the various types of land management and restoration processes.

The study had various aspects. (1) Using aerial photo coverage, the total area burned by the La Mesa fire was calculated and the amount of area devoid of seed trees was determined. The total acreage burned was 5.74 km² with a total of 2.08 km² (36.3%) devoid of seed trees. (2) Leaf water potentials (LWP) of three grass species, two seeded (Agropyron trachycaulum and Festuca ovina) and one naturally occurring (Muhlenbergia montana), were measured periodically. Soil samples at two sites were taken from 10-, 20-, 30-, 40-cm depths whenever LWP was measured. The results indicate that

ponderosa pine seedling would experience severe competition for water from F. ovina early in the growing season, and continued competition with A. trachycaulum through the summer. M. montana seems to have a water usage pattern and phenology that coordinates well with known requirements for ponderosa pine seedling survival. The results indicate that artificial or natural regeneration of pine seedlings can be facilitated by seeding undercover species that will stabilize soils yet have niche requirements that are complimentary to those of regenerating tree seedlings. (3) A population of 1431 seedlings, with heights of 1- to 150-cm, were examined for microsite characteristics such as soil texture litter depth, litter composition, living ground cover, phylogeny of ground cover, growth form of ground cover, vertical canopy, potential tree shade, seedling proximity, need for protective objects, relation to trees, distance to trees, slope and slope direction.

The best ponderosa pine seedling survival was in fine soils with conifer litter less than 1 cm, sparse ground cover and some shade, or ground cover of bearberry or mountain muhly and no shade. Overhead canopy or proximity to protective objectives is not required. There was a high association of the common native grass mountain muhly (Muhlenbergia montana) and bearberry (Arctostaphylos urvaursi). The relationship by which bearberry acts as a nurse plant is deserving of further investigation.

Nitrogen-Nutritional Strategies of Plants, in Natural Ecosystems and in Agriculture

[V. P. Gutschick]

The quartz-microbalance detector for ambient parts-per-billion levels of ammonia in air has been tested. The principles of microbalance operation and electronic stability have been verified. The high responsiveness of the NH₃-selective coating (L-glutamic acid hydrochloride) claimed in the scientific literature by G. G. Guilbault¹ has been found to be deficient. Alternative coatings are to be sought in conjunction with researchers in solid-state chemistry; specifications of thermodynamic properties and of crystallography are being developed.

The experiments to discover and quantify the evolved strategies of selective root growth for

mineral nutrition are well advanced. The preliminary experiment was successful; rapid, physiologically normal growth was achieved in hydroponic culture without problems of disease, algal contamination (which can add uncontrolled amounts of fixed nitrogen), trace-element stress, or root-temperature control. The preliminary growth patterns of roots and of whole plants according to joint variations of nitrogen and phosphorus supplies are well-differentiated. Chemical and physical methods of analyzing nutrient usage, tissue nutrient content, and tissue growth rates have been tested and verified. For the full scale experiment to be performed next growing season, I found that high-capacity pumping of nutrient solutions with controlled delivery rate and concentrate mixing is required. The pumps and electronic controls are nearly completed; auxiliary instrumentation to record ambient temperatures and incident intensity of photosynthetically active radiation has been designed and is under construction.

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Surficial Contamination of Vegetation by Soil [M. Dreicer]

Numerous studies have cited surficial contamination of vegetative surfaces as a primary source of toxic and radiotoxic contaminants measured in plants. This study was conducted to assess the importance of resuspension of soil onto plant surfaces by raindrop splash as a pathway for contaminants to enter the food chain. The experiments have been described in past annual reports. Data from this study provides evidence that an equilibrium quantity of soil is retained by plant foliage when it is splashed up by rainfall.

This past year we completed the data analysis of two sections of this study: resuspension and retention of soil onto tomato plant surfaces. It was found that no soil particles $> 105 \mu\text{m}$ in diameter were detected on plants after exposure to four natural rainstorms. This could have been due to the lack of raindrop splashup of the soil or to the rapid loss of larger soil particles that occurs

Very little soil was detected on plant foliage higher than 40 cm above the ground surface. The

retention section of this experiment showed that soil on the upper and lower strata of the plants were retained in the same manner. Therefore the lack of soil on the upper strata of the plant was due to no soil being splashed higher than 40 cm.

Relationships were found between different storm characteristics and the quantity of soil resuspended and retained on the plants. However, further study is needed to properly define the relationship.

The soil loss seen through time on the retention plot was lower for the control plants. This shows that rainfall caused greater soil loss than weathering due only to gravity and experimental procedure.

The results of the plants sampled after chronic exposure for both the accumulation and retention plot is shown on Fig. 1. After exposure to two storms the quantity of soil detected on plants in both plots reaches a common plateau at approximately 2 mg soil per grown plant (wet). This plateau can be interpreted as the equilibrium level where the quantity of soil lost equals the quantity resuspended on the foliage.

Given the concentration of ^{239}Pu in a soil size fraction and the equilibrium quantity of soil found on the plants, we can calculate the concentration ratios due to the splashup mechanism. In comparing these values with past studies, in some cases, almost all the ^{239}Pu found in plant samples is due to this process. Therefore, we can conclude that

Fig. 1 Results of plants sampled after chronic exposure for both the accumulation and retention plot.

under certain conditions, this pathway can be very important in food chain analysis.

This mechanism would be understood better if further studies varying rainfall (simulator), soil composition, and type of plants were conducted.

Checklist of Vascular Plants of the Pajarito Plateau and Adjacent Jemez Mountains

[T. S. Foxx and G. D. Tierney]

During the past three years, several studies have been carried out to determine the presence of rare, endangered, or threatened plant species within the confines of the Los Alamos National Environment Research Park. The first portion of the study was published in LA-8050-NERP Volume 1, "Status of the Flora of the Los Alamos National Environmental Research Park" (1980). The second volume of the study is in press. The third and final volume of this study was the compilation of a checklist of vascular plants of the Pajarito Plateau and adjacent Mountains. Using information gathered by Foxx and Tierney and other information sources, a checklist of 1000 species for the described area has been compiled. Presently, the information is being typeset and checked for accuracy. It is hoped that both publications will be available in FY 92.

Piceance Basin Mule Deer Study

[R. A. Garrott and G. C. White]

This report summarized the results of the first year field work on the Piceance Basin mule deer study. The primary objectives of the study, which is funded by the United States Department of Energy, are to determine migration pathways, seasonal movements, and mortality of mule deer inhabiting areas being developed as Federal Prototype Oil Shale leasing sites in the Piceance Basin of northwest Colorado. There is a clear need for the information that will be forthcoming from this study because the potential impact of full-field development of oil shale resources on Piceance deer is a sensitive political public concern. The Piceance Basin is the location of the largest migratory population of mule

deer in the world. Information on deer migration patterns and movements are needed to determine critical relationships to habitat that may be impacted by oil shale development and to provide a rationale for mitigating impacts, should they occur.

1. Trapping. During the autumn of 1980, a total of 256 mule deer captures were made within the 5000-acre C-b tract oil shale lease located between Stuart Gulch and Willow Creek drainages (Table I). Forty-eight modified Clover traps baited with alfalfa were used for a total trapping effort of 1716 trap nights. Deer were physically restrained, sexed, and aged (adult or fawn), and 53 animals were equipped with radio collars operating on the 191-Mhz frequency.

2. Winter Movements. Locations of radio-collared deer were obtained once each week throughout the winter period (December through April) for a total of approximately 600 fixes. All winter tracking was conducted on foot using hand-held directional antennae. Triangulation using two or more bearings taken at reference points throughout the winter study area provided an estimate of an animal's location. Movements of individual deer were generally restricted to a small area near the trapping location (Fig. 1). Deviation from this general winter movement pattern was observed for nine deer (4 adults and 5 fawns) which traveled from the C-b tract area to the head of the Stuart Gulch drainage where they remained for varying lengths of time before returning to the tract. Deer were distributed throughout C-b tract, moving readily across roads and well pads, and occupying areas within 100 m of the main mine shafts.

3. Spring Migration. Movements of 29 collared deer surviving the winter were monitored weekly through aerial tracking during the migration from winter to summer ranges. The majority of the deer left winter range areas during the last week in April and the first week of May. Duration of the migration for individual deer ranged from one to three weeks with the first deer arriving on summer range the last week of April and the last deer arriving the first week of June (Fig. 2). Twenty-five of the 29 deer migrated south through the Stuart drainages to the Roan Plateau (Fig. 3).

TABLE I

MULE DEER TRAPPED, RADIO COLLARED, AND RELEASED ON C-b
OIL SHALE TRACT DURING THE WINTER OF 1980-1981

Age-Sex Class	Number Trapped	Number Collared and Released	Number Collared Animals Recaptured
Fawn-Female	83	28	48
Fawn-Male	87	0	0
Adult-Female	82	25	18
Adult-Male	4	0	0
Totals	256	53	66

Fig. 1. Movements of two adult female deer illustrating the migration routes and the relatively small summer (lower) and winter (upper) ranges utilized by each.

Fig. 3. Migration routes of deer radio collared on Tract C-b. The widths of the arrows approximately represent the numbers of deer utilizing each route.

Fig. 2. Chronology of deer migration during the spring of 1981.

Two deer migrated to the southeastern edge of the Piceance Basin and the remaining two deer moved eastward, crossing Highway 13 and ending their migration along the periphery of the White River National Forest. Straight line distance traveled by collared deer during the migration ranged from 20 to 60 kilometers (12 to 35 miles).

4. Summer Movements. Summer movements of radio-collared deer were monitored weekly using a fixed-wing aircraft. Twenty-three of the 29 deer summered in the upper Parachute Creek drainage from Garden Gulch on the west, across the Parachute-Piceance

Divide to Anvil Points on the east. Two deer summered in the Clear Creek area and two in the Cow and McCarthy drainages of Piceance Divide to Anvil Points on the east. One of the two deer which migrated across Highway 13 summered 3 miles east of Rio Blanco and the second deer summered on the south fork of the White River near Bailey Lake. Approximately 400 fixes from May through July indicate that the deer remain relatively sedentary with only occasional movements of over 1.5 miles observed (Fig. 1). Seventeen of the deer on summer range were consistently located in close proximity to at least one other collared deer indicating that deer may maintain social aggregations during the summer. Four groups of 2 and three groups of 3 were identified with all 7 surviving deer collared as fawns during the winter associated with one of these groups.

5. Mortalities. Radio-collared deer mortalities were detected with special motion sensing devices inside the transmitter package. Mortalities were investigated immediately upon detection in an effort to determine cause of death before predators and scavengers destroyed the carcass. All mortalities occurred during the winter, predominantly from

mid-January to mid-April (Fig. 4). Twenty-one of the 28 collared fawns died during this period for a 78% mortality rate. Two of 25 adults died for an 8% mortality rate. All deaths were from natural causes with the exception of one fawn which was apparently poached. Predation by coyotes accounted for 13 of the 23 natural mortalities. The remaining ten carcasses were fed on heavily by coyotes but we could not determine whether the deer were killed by these predators.

Fig. 4. Mortality of radio-collared deer during the 1980-81 winter. Twenty-eight fawns and 25 adults were radiocollared.

APPENDIX
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