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Biomedical and Environmental Research Program
of the
LASL Health Division
January through December 1973

Compiled by

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Contributions from the staff are indicated by group.

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This report presents the status of the Biomedical and Environmental Research Program at LASL. The four most recent reports in this series, unclassified, are:

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LA-4923-PR

LA-3848-MS

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ABSTRACT

This report summarizes research and development activities of the Los Alamos Scientific Laboratory's Biomedical and Environmental Research Program for calendar year 1973. We have included information on organization of the participating groups within the Health Division, research interests of the staff, and supporting facilities available at the Los Alamos Scientific Laboratory.

In February 1973, the Biomedical Research Group was disbanded and reorganized into four new groups. Previous annual reports of this series related to the now defunct Biomedical Research Group are LA-4923-PR (1971) and LA-5227-PR (1972). This report is the first to reflect the overall activities of all the research and development conducted by all six groups within the Health Division that are supported by the Division of Biomedical and Environmental Research of the U. S. Atomic Energy Commission.

Although the technical portion of this report is based upon major areas of research conducted by each group, we feel the total effort reflects the multidisciplinary approach to problem solving which still represents a very basic ingredient of our research philosophy.

The format is akin to that of Science with the goal of transmitting a maximum of information in a concise manner with a minimum of technical detail. Work which has been published or submitted for publication has not been duplicated in this report. A list of publications for 1973, which follows as an appendix, allows the reader to consult the published literature for additional specific technical detail.

TEMPORARY APPOINTMENTS

ASSOCIATED WESTERN UNIVERSITIES, INC.

The Los Alamos Scientific Laboratory, as a participant in the Associated Western Universities, Inc., serves the intended purpose of the U. S. Atomic Energy Commission of providing support for education and training of advanced students and faculty participation appointees. The function of AWU is to promote and coordinate orientation and training on projects of a regional nature that require inter-university cooperation. AWU is particularly concerned with organization and management of programs and facilities that cannot be undertaken with equal effectiveness by a single institution. One such area of interest is the promotion of orientation and training in recent and specific trends in the biological sciences.

It is felt that increasing opportunities for collaboration would serve the best interests of the universities, LASL, and the biological sciences. University personnel benefit by increased opportunities for training and teaching, by access to unique or specialized LASL facilities, and by use of Los Alamos as a focus, or catalyst, for multi-institutional cooperative efforts. Los Alamos biologists benefit from greater contact with university faculties and graduate students. All biologists participating in this program benefit from mutual collaborative efforts, cross-fertilization of ideas, and by sharing specialized expertise, unique capabilities, and costly equipment.

A truly functional relationship between LASL and other AWU members is of great potential value to research, continued professional development, and graduate training. Following is a list of participants in the AWU-LASL biomedical research program for 1973.

AWU Summer Student Trainee

D. A. Freeman (Group H-10)
University of Houston
Houston, Texas

AWU Faculty Participant

H. B. Knowles, Ph.D. (Group H-10)
Washington State University
Pullman, Washington

AWU Graduate Students

P. Lowry (Group H-5)
University of Arkansas
Little Rock, Arkansas
E. L. McGuire (Group H-5)
University of Arkansas
Little Rock, Arkansas
A. W. Harmon (Group H-11)
Oklahoma State University
Stillwater, Oklahoma

POSTDOCTORAL RESEARCH PROGRAM

A limited number of research assignments are available for young individuals with a Ph.D. degree at the Laboratory. These LASL fellowships are awarded on a competitive basis for one year with the possibility for renewal for a second year. The following is a list of postdoctoral appointees who participated in the LASL biomedical research program during part or all of 1973, with their previous academic affiliations and major research interests.

Group H-9

G. F. Strniste, Ph.D., Pennsylvania State University (1971)
Identification of molecular effects of various ionizing radiations upon carefully characterized model systems

Group H-10

H. A. Crissman, Ph.D., Pennsylvania State University (1971)
Application of cytology and histochemistry techniques to high-speed, flow-system cell analysis instrumentation
J. M. Crowell, Ph.D., The Johns Hopkins University (1973)
Automated cytology: Computer-controlled cell sorting

UNDERGRADUATE CO-OP STUDENTS

Several persons participated in the Organic and Biochemical Synthesis Group's (H-11) activities during 1973 as part of the Laboratory's Undergraduate Co-Operative Program. The participants are hired as pairs and spend six-month periods between the participating university and LASL. Attempts are made to continue periodic employment for successful candidates from the beginning of the sophomore year

through graduation. The current participants, E. G. Adame, M. Gonzales, D. W. Montoya, and M. A. Nevarez, are matriculated at Highlands University at Las Vegas, New Mexico, and function as chemistry technicians. Mr. Gonzales and Mr. Montoya were at the LASL during the March to September quarter and are now at Highlands University. Mr. Nevarez and Mr. Adame are at LASL for the September 1973 to March 1974 quarter.

LOS ALAMOS SCIENTIFIC LABORATORY

A rather spectacular aerial photograph shows the bridge over Los Alamos canyon which links the major Laboratory sites (right of bridge) and facilities with the Health Research Laboratory-Los Alamos Medical Center complex (1) and town site (left of bridge). To the east (top) of the bridge are located the airstrip on the left and the Clinton P. Anderson Meson Physics Facility (LAMPF) on the right (2) of the prominent Los Alamos canyon. The Occupational Health Laboratory (3) which houses Groups H-5 and H-8 of the LASL Health Division is the first facility on the right side of Pajarito

Road which connects the main Laboratory technical area complex with the White Rock residential area (top, right). The large-animal radiation exposure facility which houses several ^{60}Co sources and the large-animal holding facility are in the last site complex (4) shown on the left side of the road to White Rock. Within the area shown are reactors, accelerators, sophisticated computing facilities, machine shops, and all the related equipment and technology associated with a large, well-established laboratory concerned with research in the physical sciences.

HEALTH RESEARCH LABORATORY

Two aerial views of the Health Research Laboratory, which houses the Mammalian Radiobiology Group, Cellular and Molecular Radiobiology Group, Biophysics and Instrumentation Group, and Organic and Biosynthesis Group of the Health Division, are shown above. The left photograph shows the close proximity of the Health Research Laboratory to the bridge which spans Los Alamos canyon. The main multistoried building was constructed in 1952, and the single-level annex used to house experimental animals was added in 1960. Immediately above the Health Research Laboratory is shown the 84-bed Los

Alamos Medical Center, administered by the Lutheran Hospital and Home Society of Fargo, North Dakota, which serves Los Alamos and surrounding communities. The close physical relation between the Los Alamos Medical Center and the Health Research Laboratory fosters the exchange of ideas and interests among respective staff. Shown in the right photograph is the Health Research Laboratory and the Los Alamos Medical Center complex and its spatial relationship to the main centralized Laboratory complex across Los Alamos canyon.

ANIMAL HOLDING FACILITY

The new animal holding and isolation facility at TA-51 was put into use in March 1974. The facility contains five spacious animal rooms and a laboratory/treatment room. The animal rooms are designed to handle a variety of species from large

farm animals to small rodents. The remote location of the building makes it an ideal quarantine area for incoming animals and an isolation facility for animals belonging to off-site investigators.

OCCUPATIONAL HEALTH LABORATORY

The Occupational Health Laboratory of the LASL's Health Division, shown in the above photograph, contains approximately 26 000 gross square feet of space on the ground floor plus some usable

basement space. This facility is currently shared by the Industrial Hygiene Group (H-5) and the Environmental Studies Group (H-8).

CLINTON P. ANDERSON MESON PHYSICS FACILITY (LAMPF)

Upon initial full operation, a high-current (1-milliampere) beam of protons will be accelerated through a linear structure located in a tunnel below the long building. The beam will enter the building complex at the far end of the structure with an

energy of 800 million electron volts. Mesons and other secondary particles will be produced in larger quantities than ever before and will make possible a new approach to nuclear, radiochemical, defense, and biomedical research.

EXPERIMENTAL AREA AT CLINTON F. ANDERSON MESON PHYSICS FACILITY (LAMPF)

Plan of the experimental area at the Clinton P. Anderson Meson Physics Facility (LAMPF) which is operated by the Medium Energy Physics (MP) Division of the Los Alamos Scientific Laboratory. The structures are or will be located at the far end (right) of the nominally 2800-foot-long accelerator structure. The high-intensity, 800-million electron volt proton beam enters the area from the left, is guided by a system of vacuum plumbing and magnets, and can be distributed among several targets and beam stops. Pi and mu mesons originating from

targets in beam area A will be formed into secondary beams and used in experiments. A proton spectrometer, neutron beams, isotope production facility, biomedical research facility, and classified research area (planned) will be used by the scientific community in a diversified program of basic research at this unique national facility of the AEC. During 1973, work continued on the construction of the biomedical facility where preclinical radiobiological and dosimetric studies with negative pions will be conducted.

LABORATORY RESOURCES

Although the Los Alamos Scientific Laboratory had its beginning as a weapons laboratory during the second World War, about half of the Laboratory's effort currently is directed toward basic research in the physical and biological sciences. The Laboratory has some unusual research tools available to all Laboratory personnel, graduate students, and visiting scientists.

LIBRARIES

LASL maintains a system of libraries to meet specialized needs of the various research groups. The main library, with its branches, constitutes the largest technical library between the Mississippi and Pacific coast. The Medical Branch Library of the Health Research Laboratory has complete holdings of about 750 current periodicals in biochemistry and biophysics, radiation biology, cell biology, medicine, etc., including clinical medicine, through the Library's affiliation with the Los Alamos Medical Center serving the community.

STABLE ISOTOPE PROGRAM

LASL has unexcelled facilities for the production of large quantities of highly enriched stable isotopes: nitrogen-15, oxygen-17, and carbon-13. It is anticipated that sulfur-33 production will begin in the near future. Since these are all magnetically active nuclei, they can be detected by magnetic resonance techniques as well as by conventional mass spectrometry. A program of biochemical studies utilizing these isotopes and organic and biological syntheses, in conjunction with sophisticated nuclear magnetic resonance spectrometers and other advanced techniques of physical biochemistry, is just beginning to gain momentum.

RESEARCH REACTOR

The 8-megawatt Omega West Reactor is located in Los Alamos canyon conveniently near to the Health Research Laboratory. It is often used by Health Division investigators in various research programs.

ACCELERATORS

Several electrostatic accelerators, including a 16-MeV tandem Van de Graaff, an 8-MeV vertical Van de Graaff, a 25-MeV cyclotron, and an 800-KeV Cockcroft-Walton for producing 2.5- and 14-MeV

neutrons, are available for radiation experiments. Available particles include electrons, protons, deuterons, neutrons, and alphas. The Van de Graaff accelerators can be used to produce large quantities of high-energy neutrons.

CLINTON P. ANDERSON MESON PHYSICS FACILITY (LAMPF)

A unique accelerator capable of producing an 800-MeV proton beam of unusually high intensity (1 mA average current) which, in turn, will be used to produce negative pions is now under construction. A biomedical research building is under construction in the target area for investigating theoretically promising medical applications of these particles for cancer therapy. Preclinical radiobiological studies using negative pions are in the planning stage, and some comparative studies and work on exposure systems are in progress.

INSTRUMENT DEVELOPMENT

LASL maintains a large and unusually competent electronics group for adaptation and creation of instrumentation needed for today's highly specialized research. The main machine shop, foundry, glass fabrication unit, and branch shops constitute one of the nation's unique experimental facilities. A branch shop with full-time machinists is available within the Health Research Laboratory.

COMPUTER FACILITIES

One of the largest computer installations in the world, including three CDC 6600's, two CDC 7600's, and several smaller machines, is available to all Laboratory personnel as are programming and other support services. A special computer, MANIAC II, also is available. In addition, five smaller computers are in use in the Health Research Laboratory. Three of them (a PDP 11/20, PDP 8/F, and PDP 8/I) are dedicated as multichannel analyzers and data processors for flow microfluorometric (FMF) and gamma-ray spectrometry. An 8/E is dedicated to chromosome karyotyping and other digitizing problems, and a PDP 11/40 is used for data processing and analysis. The Health Research Laboratory also has a remote terminal communicating directly with one of the CDC 6600's at the Central Computer Facility to handle jobs which are too large for the local machines.

THE BIOMEDICAL AND ENVIRONMENTAL RESEARCH PROGRAM AT LOS ALAMOS

PAST HISTORY AND ACHIEVEMENTS

A significant historical event which altered the course of mankind occurred on February 23, 1941, when Dr. G. T. Seaborg and colleagues discovered the element plutonium in room 301 of Gilman Hall at Berkeley. The isotope of mass-238 and not the more familiar mass-239 was first discovered at that time; the plutonium isotope of mass-239 was not isolated until the spring of 1941, and element 94 remained unnamed until March of the following year. On March 28, 1941, 0.5 μ g of plutonium-239 was fissioned by thermal neutrons, and the enormous effort to produce plutonium-239 in quantity for military purposes was begun. The potential toxicity of plutonium was recognized soon after its discovery and availability in quantities for biomedical research.

It is worth recalling that only extremely small quantities of this precious material were available for experimentation during the early 1940's. As an example, the following memorandum written to the Director of the Health Division of the Metallurgical Laboratory is indicative of the early concern about the potential toxicity of plutonium. The memorandum states, in part: "It has occurred to me that the physiological hazards of working with plutonium and its compounds may be very great. Due to its alpha radiation and long life it may be that the permanent location in the body of even very small amounts, say 1 milligram or less, may be very harmful. The ingestion of such extraordinarily small amounts as some few tens of micrograms might be unpleasant, if it locates itself in a permanent position. In the handling of the relatively large amounts soon to begin here and at site Y (Los Alamos), there are many conceivable methods by which amounts of this order might be taken in unless the greatest care is exercised. In addition to helping set up measures in handling so as to prevent the occurrence of such accidents, I would like to suggest that a program to trace the course of plutonium in the body be

initiated as soon as possible. In my opinion, such a program should have the very highest priority." The writer of this memorandum was Dr. Glenn T. Seaborg, and the date was January 1944.

The biomedical research program at Los Alamos has evolved from its conception in 1943 as a small Health Group established to protect the health of the workers, to develop safe working procedures, and to establish tolerance levels for exposure to radioactivity, plutonium, and other radionuclides. In 1944, once significant amounts of plutonium began to accumulate at Los Alamos, the Laboratory Director, Dr. J. Robert Oppenheimer, at the request of the Health Group, authorized the temporary establishment of a group of four people to initiate a research program designed to develop tests for setting exposure limits for plutonium. Several months later, this small group was absorbed by the Health Group as a Biochemistry Section, and the Laboratory's biomedical research program was born. In 1945, the Section moved into a small building of its own, and its members established the urine assay procedure for diagnosing exposure of Laboratory personnel to plutonium. Experiments were conducted which led to the first successful labeling of a biologically important compound (nicotinic acid) with reactor-produced carbon-14. The first measurement of carbon-14 by scintillation counting procedures was accomplished here and formed the basis for the present generation of commercially available liquid scintillation counting systems.

By 1948, the Health Group was a Division in the Laboratory, and the Biochemistry Section became Group H-4, the Biomedical Research Group. In October 1952, the group moved from temporary wooden structures (Fig. 1) into its present building and by the end of that decade had established itself in both national and international circles as an authority on the effects of radiation from nuclear weapons, worldwide fallout, and the physiology and toxicology of tritium and plutonium.

Fig. 1. Makeshift building used for biomedical research activities during the war years (photographed about 1950 showing three additions to the original structure).

The Biomedical Research Group pioneered in and became a recognized authority on liquid scintillation counting, synthesis of isotopically labeled organic compounds, use of radioactive tracers in biology and medicine, and whole-body counting techniques and applications to biomedical research. By utilizing the development of large-volume liquid scintillation detectors, the group contributed significantly to the field of anthropometry through its capability to measure total-body potassium by quantitating the natural level of potassium-40 within the human body. By exploiting the whole-body counting systems designed for both research animals and man and making use of the Laboratory's significant computer capabilities, the Biomedical Research Group contributed significantly to the field of radiation protection by conducting studies on the uptake, distribution, and excretion of radioisotopes by animals and man. The interest in metabolic kinetics was also applied to the emerging field of nuclear medicine during the late 1950's.

Shortly after the discovery in 1955 of the presence of cesium-137 in man from worldwide nuclear fallout, measurements were begun on a controlled population of subjects residing in the Los Alamos area. These studies have continued to the present time and represent perhaps the most meaningful documentation of the temporal changes in man of a radioactive material released to the environment.

Beginning in the early 1960's, more emphasis was placed on the fundamental research aspects of the biomedical research program. Although investigations continued in the Mammalian Metabolism and Mammalian Radiobiology Sections related to the response of higher organisms to ionizing radiations and radioactive materials, a major emphasis was directed toward research in the fields of molecular and cellular biology.

The late 1960's also marked the start of a research program related to the question of the probable biological effects resulting from non-uniform dose distribution of alpha-emitting particulates in the lung. Interestingly, this very difficult problem has received considerable attention but little resolution since the mid-1940's. This particular problem is now one of the highest priority because of the vast potential applications of the element plutonium as regards breeder reactors, space nuclear power systems (radioisotopic thermoelectric generators), medical applications such as the heart pacer and artificial heart, as well as production, transportation, and deployment of this material for national defense. It is interesting in a sense that some Health Division personnel are now actively working on the problem which relates to plutonium efforts conducted some 20 years previously at LASL.

The 1970's have witnessed the emergence of an interest in the stable isotope program designed to exploit the use of stable elements in the general field of biomedical research. In 1971, an Isotope Applications Section was formed within the Biomedical Research Group to concentrate on the biomedical aspects of the ICONS (Isotopes of Carbon, Oxygen, Nitrogen, and Sulfur) program which involves several groups within the Los Alamos Scientific Laboratory. More recently the vast potential application of these naturally occurring non-radioactive isotopes to such diverse areas as environmental pollution and agronomy has created much enthusiasm at the LASL.

Our molecular radiobiologists are engaged in a variety of organic synthesis procedures to assemble polynucleotides having known base sequences. Certain enzymes that catalyze polynucleotide synthesis are not only being isolated and purified but are also being studied as biofunctional proteins

participating in information transfer reactions. The structure, function, and metabolism of both acidic and basic nuclear proteins, believed to be involved in readout of genetic specifications and thus differentiation, are being investigated.

Biologists and biochemists in the cellular radiobiology research area are investigating the temporal sequence of a variety of cellular processes in relation to specific phases of the cell life cycle using synchronized cultures of mammalian cells. Methods have been developed in this Laboratory for producing relatively large quantities of cultured mammalian cells synchronized with respect to period in the life cycle. Using these cultures, mechanisms controlling synthetic processes, energy metabolism, recovery from ionizing radiation, cell-surface phenomena, and mathematical methods of cell growth are being investigated. Several staff members associated with molecular radiobiology research are using synchronized cultures to examine the synthesis, turnover, and structural alterations of nuclear and cytoplasmic basic proteins. In addition to studies on animal cells, investigations are in progress on survival of microorganisms exposed to ultraviolet and ionizing radiations and the biochemistry of bacterial genetic transformation.

Activities in the biophysics and instrumentation area are mainly concerned with development and improvement of instrumentation for cell biology research. In collaboration with the cellular radiobiologists, electronic instruments have been developed for high-speed electronic cell counting and cell sizing. A high-speed sorter has been invented which can physically separate living cells in suspension according to cell volume. A fluorescent cell spectrometer has been developed which utilizes a laser to provide the high intensity and collimation of the light of excitation required to determine rapidly and quantitatively the DNA content of individual cells of a cell population. Both cell sensing and sorting efforts are currently being extended to other optical and multiparameter methods. The biophysicists also provide general electronics, mechanical engineering, radiological physics, and computer science support for the group.

On 1 July 1972, the Pion Radiobiology Group (H-9) was disbanded and incorporated into the Biomedical Research Group (H-4) to consolidate the

research effort on applications of negative pions to radiation therapy and pretherapy radiobiological research. This action added four people to H-4.

In September 1972, an Agricultural Biosciences Group (H-6) was formed within the Health Division, resulting in the loss of one person from the Biomedical Research Group.

On 1 February 1973, the Biomedical Research Group was disbanded after being in existence for about 26 years. Personnel from the defunct organization were reorganized into four new groups: Mammalian Radiobiology (H-4); Cellular and Molecular Radiobiology (H-9); Biophysics and Instrumentation (H-10); and Organic and Biosynthesis (H-11). These groups plus the Industrial Hygiene Group (H-5) and the Environmental Studies Group (H-8) represent the organizations within the LASL Health Division which contribute to the AEC's Division of Biomedical and Environmental Research Program.

CURRENT EFFORTS

The biomedical and environmental research program at the LASL currently includes research efforts in all major funding areas within the U. S. Atomic Energy Commission's Division of Biomedical and Environmental Research (DBER) RX program: Health Studies, Environmental Studies, Biological Studies, Physical and Analytical Studies, and Heart Devices. This program currently involves six groups [Mammalian Radiobiology (H-4); Industrial Hygiene (H-5); Environmental Studies (H-8); Cellular and Molecular Radiobiology (H-9); Biophysics and Instrumentation (H-10); and Organic and Biosynthesis (H-11)] within the Health Division and groups within the Chemistry-Nuclear Chemistry (CNC) Division, the Chemistry-Materials Science (CMB) Division, and the Medium Energy Physics (MP) Division.

In the Health Studies category are included three high priority studies related to plutonium and the transuranium elements and the development of radiation protection guides. These programs have developed in response to the needs of the AEC and other agencies as regards information on the fate and potential biological effects of plutonium and other transuranium elements which man will encounter as a by-product of the ever-expanding plutonium economy. The project on high specific-activity plutonium dioxide particles in the lung is

part of an integrated AEC program involving biological studies at several other laboratories and at the LASL. The other programs are related to the development of metabolic information for use in radiation protection guides and to the establishment of environmental levels of various radioactive and nonradioactive metals in human tissues. Another program dealing with short-lived isotopes is centered in CNC Division.

The project in the Environmental Studies funding area is titled "Ecological Investigation of Radioactive Materials in Waste Discharge Areas at Los Alamos." Essentially all this DBER-supported work and other programs (funded by AEC divisions other than DBER) related to the waste management of plutonium are conducted in areas other than the Health Research Laboratory. Of special interest in this program is the use of the honeybee as an indicator species for estimating contamination from radioactive materials. We are developing these program areas in response to environmental considerations at LASL and in response to developing efforts related to energy production.

A major research effort under the Biological Studies funding category is related to the effects of external radiation on living organisms. Our major interest is in the area of comparative biological effects of whole-body radiation dose protraction on various mammalian species. We are most interested currently in dogs and subhuman primates (monkeys). These projects have developed in response to the current high priority assigned by the U. S. Atomic Energy Commission's Division of Biomedical and Environmental Research to the general area of the effects of external radiation delivered at low dose rates to man and other animals. This project consists of continuing efforts designed to obtain data required to better understand the effects of ionizing radiation and to provide a rational basis for extrapolation of dose-rate dependence of whole- and partial-body radiation effects on man in the low and very low dose-rate range. This project is programmatically oriented toward assisting in protection and management of personnel and realistically predicting the short- and long-term effects of exposure to radiation in those practical situations or missions in which radiation exposure may not be restricted to

maximum permissible exposure levels (e.g., troop deployment in the event of nuclear war, radiation accidents, manned space operations, and civil defense applications).

Also under the Biological Studies funding category are the LASL studies in molecular and cellular radiobiology. The main research efforts in this program area center on three areas: regulatory and control mechanisms, cellular interactions, and properties of genetic materials. Test systems range systematically from models constructed at the molecular level with as close as possible control of structure and conditions to corresponding organized systems of increasing complexity represented by living cells in culture. This program includes projects on the synthesis and radiosensitivity of model DNA, radiation and genetic information transfer, response of histone metabolism to x irradiation, biological effects of radiation on cells, and chromosome structure and function. These projects are all directly or indirectly related to understanding the effects of irradiation and other environmental stressing agents on biological cells or cellular moieties such as chromosomes. A parallel effort in this funding category is related to electronic cell sensing and sorting of biological cells. This project reflects the interdisciplinary approach to solving biomedical problems at LASL as it involves the application of laser systems and other exotic biophysical instrumentation to the solution of both basic and mission-oriented problems.

Still another research area under the Biological Studies category is the biological and agricultural applications of stable isotopes. The application of stable isotopes (e.g., carbon, oxygen, nitrogen, and sulfur) to biological, medical, and environmental research is particularly exciting, and the potential gain to the AEC and other agencies from the exploitation of these non-radioactive isotopes is particularly large. The development of a national center for stable isotopes at LASL would allow the optimum utilization of these nonradioactive isotopes in many areas such as clinical medicine, biochemistry, biology, agriculture, and environmental pollution. Such a center might provide the transfer of this developing technology to the general scientific community in

perhaps a decade instead of a generation.

Under the Physical and Analytical Studies funding category are included projects in the area of radiological and chemical physics, health protection, and instrumentation technology. Included are studies on the biological applications of negative pions to biological studies and eventually human clinical investigations, applied aerosol studies that are related to AEC health, safety, and air-cleaning requirements, and the development and calibration of radiation detectors for measuring plutonium and other transuranium elements in man. Some of this research is conducted by the Medium Energy Physics (MP) Division.

Research and development related to the Heart Device Studies are conducted in another division (CMB) and have only recently been funded via the AEC's Division of Biomedical and Environmental Research. These efforts will not be included this year in this report.

FUTURE CONSIDERATIONS

About three decades after the discovery of plutonium, the President of the United States proclaimed the Liquid Metal Fast Breeder Reactor (LMFBR) as a national goal. More recent concern about meeting the nation's energy needs (Project Independence) and increased importance of the light water and breeder reactors for producing electrical power, coupled with our nation's concerns about the

effects of pollutants on man and the quality of his environment, make broad based programs such as ours both timely and relevant.

Basically, our Biomedical and Environmental Research and Development Program is directed to man and society for, in the final analysis, the betterment of man and society is one of the main reasons we conduct research. Our philosophy is to combine basic and mission-oriented efforts whenever possible as we believe that they are both necessary and compatible entities that should not be isolated, segregated, or assigned a relative scientific pecking order. Both are necessary as they satisfy different interests and demands of the investigator and those who in the end "pay the way" -- that is, society. As scientists, we have an obligation to meet societal needs in the Biomedical and Environmental Research area both for the short term (current generations) and for the longer term (future generations). It is only by prudently exploiting our scientific resources, both basic and mission-oriented, that we can efficiently attack the many problems facing the nation in the area of producing energy for society with the minimum attendant biological cost to man and his environment. We look forward to participating actively with the AEC and other agencies in this immensely important and interesting scientific and technological challenge.

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INTRODUCTION

The activities of the Mammalian Radiobiology Group (H-4) consist primarily of programmatic research with major emphasis on effects of radiation from both external and internal sources on mammals and appropriate extrapolations to man. Investigations on the effects of radiation from external sources are concerned largely with the injury and repair of radiation damage to bone marrow produced by sublethal and lethal radiation exposure. Current emphasis is on low dose rate irradiation. Rodents, dogs, and monkeys are used in studies that are oriented programmatically toward civil defense, manned space flight, military, and emergency radiation exposure situations. Research activities on the effects of internally deposited radioactive materials include interspecies correlations (rodents, dogs, monkeys, and extrapolations to man) of uptake, deposition, and excretion of radioisotopes. Much emphasis currently is on the response of various body tissues (especially the lung) to high specific-activity particulate transuranium radio-nuclides (such as plutonium-238 and plutonium-239 oxide). One additional programmatic objective is

to establish radiation protection guides for internally deposited radioisotopes in man. These activities are programmatic to the control of worker exposure to radioactive materials. Other research efforts are related to animal radiobiology studies of negative pions for preclinical therapy work to be conducted at the biomedical addition at LAMPF.

EFFECTS OF INTERNAL RADIATION ON LIVING ORGANISMS
(HOT PARTICLE PROGRAM)

Introduction

An objective of this research is to establish quantitative relations between soft tissue damage and the distribution function which describes the fraction of the cell population which is exposed to a given local radiation dose. To date, attention has been focused on the lung. Because of a primary interest in low-level exposures to a small number of insoluble particles, we have chosen a basic exposure method using static particles and, in the first exposures, the particles were injected at low concentrations so that they would be separated by distances that are large as compared with the alpha range. Under these conditions,

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microdosimetric calculations are straightforward even when the complications of lung structure are included. Considerable inefficiency in the production of biological damage was anticipated because of overkill in the intense radiation fields close to the particles. This was taken into consideration in selection of the plutonium levels, and a 1000-fold range of dose was covered in the original exposures in an attempt to bracket the region of interest in spite of possible errors in the predictions. Continued failure to produce significant lung damage, including malignancies, has led us to make additional exposures under conditions which produce a somewhat more limited dose distribution. A current objective is to reproduce the results of Little *et al.*,¹ who obtained high tumor incidence with short induction times in hamsters exposed to insufflated solutions of polonium-210, and to use this as a point of departure in the study of the inefficiency of localized point sources of plutonium in producing lung cancer.

Exposure of Animals

(E. C. Anderson, G. A. Drake, L. M. Holland, J. E. London, J. D. Perrings, and J. S. Wilson)

Plutonium. To review briefly, the original experiments begun in the spring of 1971² consisted of eight groups each of 60 hamsters with 2000 microspheres of plutonium-loaded ZrO_2 ceramic per animal. Specific activities ranged from 0.07-59 pCi/sphere, giving total lung burdens of 0.14-119 nCi. About 1% of the lung was irradiated, and the median dose rate to those cells within alpha range of a sphere was estimated to be 20-1800 rad/day (for methods of dosimetry calculation, see below). During the latter half of 1971, an additional 418 animals were injected with larger numbers of spheres: 6000-20 000 per animal. Thus, the fraction of lung irradiated increased 3.5-8.2% with median dose rates from 6-1800 rad/day. During 1972, the exposures were extended to still larger numbers of spheres: 6000-1 000 000 per animal, the latter irradiating over 98% of the lung. Lower specific activity spheres were used, and median dose rates ranged from 6-25 rad/day. During calendar year 1973, another 740 animals have been injected, bringing the total number to 1864. Sphere numbers ranged from 50 000-900 000, and the range of sphere specific activity

Fig. 1. Distribution of exposure groups with respect to number of spheres per animal (ordinate) and specific activity of spheres (abscissa). The lines are loci of constant lung burden and are labeled with nCi of plutonium per animal. Symbols indicate year of injection: (●) 1971; (■) 1972; and (▲) 1973.

was extended downward to 0.015 pCi/sphere. Lung burdens were 0.86-117 nCi, and median dose rates were 1.3-320 rad/day.

The distribution of all exposure conditions is summarized in Fig. 1 in which the ordinate is the number of spheres per animal (scale on left) or fraction of lung irradiated (scale on right), and the abscissa is sphere specific activity. The diagonal lines are loci of constant plutonium dose and are labeled with the lung burden in nCi. The special interest in burdens between 10 and 100 nCi is occasioned by the reports of Little *et al.*,¹ that a high tumor incidence develops rapidly in hamsters exposed to these levels of soluble polonium.

Promethium-147. To obtain exposures to a lower LET radiation delivering a more nearly uniform dose to the entire lung and with minimal dose to other

TABLE I
PROMETHIUM-147 EXPOSURES

Specific Activity (nCi/sphere)	Number of Spheres	Lung Burden (μCi)	Dose Range 1-3 Quartile (rad/day)	Number of Animals
0.07	6000	0.42	0.3-1.4	62
	50 000	3.50	6-14	61
	300 000	21.00	82-123	52
0.45	10 000	4.50	2.7-164	65
	50 000	22.00	41-82	62

organs, a series of animals were injected this spring (1973) with microspheres loaded with promethium-147 (half-life 2.6 y, maximum beta energy 225 KeV). The exposures are summarized in Table I. The dose range (column 4) was calculated by a Monte Carlo method, assuming random sphere distribution in a uniform lung (see below).

Polonium-210. We are currently exposing hamsters by intratracheal insufflation with solutions of polonium-210 in saline by the method of Little *et al.*¹ Weekly doses of 75 nCi per animal will be given. Because of concern over excretion of the activity, a pilot experiment with 20 animals is being performed inside a glove box to determine the magnitude of the contamination. It is hoped that this can be controlled well enough to permit a larger experiment to be performed outside the glove boxes (as for the insoluble microsphere experiments).

Retention Measurements

(E. C. Anderson, P. N. Dean, G. A. Drake, L. M. Holland, J. E. London, and J. S. Wilson)

Previously reported retention measurements have been continued and extended. A summary is given in Fig. 2 (all curves are based on *in vivo* measurement of the 120-KeV gamma ray from a cobalt-57 tag of the microspheres). The upper curve is for the previously reported group of animals (initially eight) which received intravenous injection of 2000 microspheres of Level 6 specific activity (60 pCi plutonium-238 per sphere). Data now extend to 500 days, at which time only three animals survive. The

Fig. 2. The retention of microspheres as a function of time: (—■—) intravenous, 2000 microspheres; (---▲---) intravenous, 300 000 microspheres; and (---●---) intratracheal, 500 000 microspheres.

results give a biological half-time of the order of 7000 days, corresponding to an excretion rate of 0.01% per day after the initial drop of 5% (which may be due to leaching of cobalt-57 from the surface of the spheres, since direct measurement of plutonium in the excreta did not indicate any large initial loss).

The middle curve is a similar experiment in which six animals were injected with 300 000 spheres of Level 2A (0.4 pCi plutonium-239 per sphere). The slope corresponds to about 0.025% per day or a half-time of some 2800 days. The initial drop is not well documented, and there is a possibility of a calibration shift during the first month. It is also possible that, due to the much larger number of spheres injected, some 20% may really have escaped the lung.

The bottom curve is for intratracheal insufflation of 500 000 spheres tagged with cobalt-57 but without any plutonium (the first 100 days of this curve were reported previously). Twenty-four animals were initially used; six remain (some were sacrificed). The halftime of the curve is 244 days, with no evidence of change with time.

The constant excretion rates observed in all three experiments indicate that the lungs are sustaining no damage which would cause increased loss of microspheres, nor are the spheres being encapsulated in any manner which would decrease their elimination rate.

Survival Curves

(E. C. Anderson, P. N. Dean, G. A. Drake, L. M. Holland, J. E. London, and J. S. Wilson)

The fraction of animals surviving as a function of time is useful as an indicator of the health of the population and as a nonspecific signal of possible radiation damage. The hamsters used in our experiments generally show a mean survival time (MST) of about 700 days, indicating an unusual longevity. Typical histograms for cumulative deaths out to 1000 days are given in Fig. 3 for the original experimental groups receiving 2000 spheres with lung burdens of 0.1-120 nCi. The numbers at the beginning of each curve identify the plutonium level in the spheres. The line labeled "C" is for a control group receiving no spheres, and the line

Fig. 3. Survival curves for hamsters receiving 2000 microspheres intravenously. The number of each curve identifies specific activity level; (O) nonradioactive spheres and (C) control group, no spheres.

labeled "0" is for a group receiving nonradioactive spheres. Only the four extremes of the exposed groups are drawn for comparison. There seems to be no significant correlation of MST nor of pattern of deaths with the specificity of the particles. The shortest survival time is shown by Level 5, while the highest dose level (curve 6) is indistinguishable from the controls. The longest survivals are shown by Levels 2 and 3 (similar reports of life lengthening are not uncommon in connection with low levels of radiation exposure; the present result is not regarded as statistically significant). The remaining dose levels fall within the range of those plotted. The data suggest that MST values vary by some ± 100 days under the conditions

Fig. 4. Examples of bimodal survival curves.

Fig. 5. Mean survival time as a function of sphere number. Arrows labeled "C" denote control groups receiving no spheres. The number to the left of each point identifies specific activity level, and the numbers to the right give month/year of injection. Broken vertical lines connect the double survival times for bimodal distributions.

of our experiments; this large span is due, in part, to the small group sizes (60 animals). In many cases, MST curves are obtained which appear to be bimodal with a high death rate at around 200 days which later declines. Additional examples of such curves are shown in Fig. 4 for one control group (with no spheres) and two exposed groups (Level 2, with 6000 spheres, and Level 4, with 2000 spheres). The slopes of the initial portions of the curves would extrapolate to MST values of 370, 430, and 520 days. The slopes diminish after 20-30% deaths, and 50% survival is actually reached at 620-690 days.

A summary of current data on MST values as a function of sphere number and specific activity is given in Fig. 5 (time is again age of the animal; spheres were administered at age 100 days). The low MST values shown for the 1973 groups are based on extrapolations from survivals which still exceed 70%; therefore, it is possible that bimodal curves may develop with much longer MST values. Life shortening by the large numbers of spheres or by changes in environmental conditions or animal susceptibility seems unlikely. We are attempting to identify the cause of the early deaths but so far without success.

Dosimetry

(E. C. Anderson and P. N. Dean)

To provide a basis for understanding the approximate distribution of lung cells with respect to radiation dose, Monte Carlo calculations have been performed for random distributions of spheres with both alpha and beta radioactivity. The sphere concentrations covered the range of interest from isolated individual radiation fields with no overlap up to the highest levels which seem biologically reasonable. The results can be logically separated into two parts: the fraction of lung cells which receive a finite dose and the dose distribution for this irradiated fraction.

Fraction of lung irradiated. An important criterion in determining the type and extent of damage to the lung by nonuniform radiation fields is the fraction of cells included in the radiation fields. With radiation of sharply defined range such as alpha particles, it is easy to calculate the lung fraction which is beyond the range limit for randomly distributed point sources in a uniform medium. The effects of lung structure (on which we have previously reported)³ will, of course, introduce complications which are not included in the present approximation.

For microspheres distributed at random, the probability $p(n)$ of finding n spheres in a given sample volume is given by the Poisson distribution

$$p(n) = \frac{N^n e^{-N}}{n!}, \quad (1)$$

where N is the average number of spheres in the given volume. The probability of finding no spheres ($n = 0$) within alpha range R_0 of a given point is the same as the probability of finding no spheres in a volume $V_0 = 4/3 \pi R_0^3$ and is, therefore,

$$p(0) = e^{-cV_0}, \quad (2)$$

where c is the average concentration of spheres in the medium. This probability is, of course, equal to the fraction of the lung receiving zero dose.

If the density of the lung is taken as 0.25, the critical volume V_0 is 0.0171 mm^3 for an alpha range of 0.004 mg/cm^2 . In the groups so far exposed, the zero dose fraction ranges from 1.4 to

99.2% of the lung volume. In a structured lung, this fraction may be less because the airways permit some of the alphas to reach greater distances from the microspheres.

Equation 2 also served as one test criterion for the Monte Carlo calculations of dose distribution.

Dose distribution in uniform medium. A computer code MULDOS has been written to calculate the dose distribution due to random spacing of radioactive spheres in a uniform medium with special reference to overlapping radiation fields. Two versions of the code are in use: one for beta and one for alpha activity. A Monte Carlo method of calculation is used; for each iteration, spheres were distributed at random locations around the test point, and the total dose rate at the point was calculated on the basis of inverse square distance attenuation and dE/dx . For the beta version, dE/dx was calculated by a truncated Loevinger formula.^{4*} For the alpha version, dE/dx was assumed to be constant. The current versions of the code do not include the effects of lung structure. These will be added from the results of our previous digital analysis lung images when such refinement is justified by the associated biological results.

The results of MULDOS calculations for alpha particles of range $160 \mu\text{m}$ in a uniform medium of density 0.25 are shown in Fig. 6, which gives a general picture of dose rate as a function of sphere number at three quartiles of the dose distribution in comparison with the rate calculated for uniform irradiation. At low concentrations, the dose distribution is independent of concentration because the radiation fields of the spheres do not overlap. At high concentrations, the dose distribution narrows as shown by the converging quartiles. According to this simplified model, the third quartile dose is very close to the uniform dose for all sphere concentrations above 100 mm^{-3} . Over the concentration range covered here, the dose rate to the half of the population between the first and third

* Compared with the theoretical calculations of Cross⁵ and Berger,⁶ the Loevinger formula gives a dose rate which is about 10% high at $0.5 R_{\text{max}}$. The error rises to a factor of 3.0 at $0.7 R_{\text{max}}$. Thus, the MULDOS beta distributions are somewhat too narrow and should extend to lower doses.

Fig. 6. Dose rate vs sphere concentration at three quartiles of the dose distribution curve compared with rate for uniform activity distribution.

quartiles (at any given concentration) varies by factors of 2-3.

Some actual dose distribution curves, smoothed from histograms calculated with this model, are given in Fig. 7. All the curves are truncated at unit dose, which is the minimum possible dose (i.e., that delivered by a single sphere from a distance

Fig. 7. Logarithmic differential dose rate distribution curves for four sphere concentrations (mm^{-3}) indicated by the number on the curve. Fraction is calculated for those cells which are within alpha range of a sphere, not for entire lung. Unit dose rate is minimum possible rate from one sphere at maximum alpha range.

equal to the alpha range). For very low concentrations (not shown), the curve is monotonically decreasing with increasing dose. At a concentration of 32 mm^{-3} , a bump due to irradiation by two spheres begins to appear. As the concentration rises, a peak develops which moves to higher dose rates as more and more spheres are found in the critical volume. Statistical variability decreases and the peak slowly narrows, but the distribution is still quite wide at 750 mm^{-3} . At this concentration, a hamster with a lung volume of 4000 mm^3 would have 3×10^6 spheres. If the concentration of capillary segments per g of lung is similar in the hamster to that estimated by Weibel⁷ for the human (namely, 3×10^{11} per kg), then a 1-g hamster lung would have roughly 3×10^8 segments, and this sphere number would plug something like 1% of them. Therefore, one might expect that sphere doses much larger than this would produce significant infarction.

These dose distributions are not approximated by any simple mathematical function. The integral distribution somewhat resembles a truncated log-normal function as shown in Fig. 8. The number on each curve identifies the corresponding sphere concentration. The curve labeled "limit" is the analytic solution for sphere concentrations approaching zero; in which case, there is no overlap, and the fraction $F(D)$ of the irradiated cells receiving a dose less than D is determined simply by the volumes within concentric spheres around the source:

Fig. 8. Integral distribution of dose rates (log scale) for several sphere concentrations. "Limit" refers to the limiting curve as sphere concentration approaches zero.

$$F(D) = 1 - (D/D_0)^{-3/2},$$

where D_0 is the minimum possible dose from a single sphere. No Bragg peak is included in this calculation, nor is the effect of lung structure on broadening the range distribution.

Computer Support

(E. C. Anderson and P. N. Dean)

A considerable effort is being devoted to the development of computer support systems based on the PDP 11/40 which was recently acquired for use by the several groups working in the Health Research Laboratory. Data processing codes are being transferred from the large machines at the Central Computer Facility with a significant saving in personnel time and gasoline consumption. Turn-around time is less, and job submittal and retrieval are more convenient. In addition, we are developing a data base to receive information generated in the Hot Particle Project and to provide for rapid recall, selection, tabulation, and analysis. Portions of the data currently are transcribed to punched paper tape and processed by subroutines in order to obtain operating experience with some aspects of the proposed system (survival data reported herein were treated in this way). Full implementation will require magnetic tape and disc hardware which are being purchased this fiscal year. In addition to the time savings resulting from elimination of much manual bookkeeping, the benefits of the system are expected to include closer control over the experiments because of rapid return of current status analyses and increased efficiency in preparation of reports.

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Biological Results

(S. G. Carpenter, G. A. Drake, L. M. Holland, J. E. London, J. R. Prine, J. S. Wilson, and R. H. Wood)

Most of the animals placed on study early in the program have reached the end of their normal life span without developing significant pulmonary lesions. During the past few months, we have observed some histological changes in the lungs of very long-term animals (15-20 months). In these animals, an extension of bronchiolar epithelium into the alveolar ducts and alveoli has occurred. In some cases, the alveoli are lined with cuboidal or columnar epithelial cells (Fig. 1). This lesion has been observed almost entirely in the higher activity levels (levels 4-6) and in animals given relatively small numbers of spheres (2000-6000). An interesting recent observation has been the identification of a similar lesion in animals from a lower activity group (level 3) which had been given larger numbers of spheres of approximately 60 000 (Fig. 2). This group of animals has been exposed only about 6 months. A consistent observation of this lesion after drastically different induction times could lead to speculation that the amount of tissue irradiated is an important element in timing of the tumorigenic response. There has been no increase in frank tumors observed within the past year; however, the epithelial changes described above could be considered as precursors of peripheral adenomas.

The most commonly observed neoplasm during the past year has been an adrenal adenoma. The incidence of this lesion has increased markedly in animals nearing the end of their life span and is regarded as a nonexperimental spontaneous change.

Fig. 1. Epithelialization of alveoli in the neighborhood of a microsphere 15 months post-injection. The sphere is from the highest activity level (59.4 pCi/sphere).

Fig. 2. Alveolar epithelialization near a microsphere from activity level 3 (0.91 pCi/sphere) 7 months post-injection.

Last year¹ we reported on a group of rats which had been injected with about 6000 microspheres of activity at level 4 (4.3 pCi/sphere). These animals are approaching 2 yr post-injection, and several animals have died from causes unrelated to the presence of microspheres. While most of the losses have been from phenomena often observed in aged rats, only one case of chronic respiratory disease (CRD) has been observed in over 2 yr.

During 1973 we committed a small group of animals to a study in which the lung had been damaged by infarction with tetrachloroethane. The microspheres containing the PuO_2 were injected by our normal procedures and, after a 2-week period, the lungs were infarcted by intravenous injection of the tetrachloroethane. This method creates multiple ischemic areas in the lung which subsequently undergo necrosis and healing with a resultant scar. The amount of lung affected is dependent upon the dose of tetrachloroethane. It has been shown histologically that, in areas bordering the infarcts, some neo-epithelialization occurs. Tissue damage of this sort, coupled with new epithelial growth, may increase the incidence of neoplastic change.

Another study has been initiated in which the lung is infarcted with sym-difluorotetrachloroethane which seems to enhance the epithelial proliferation. It has been reported by Stanton and Stouffer² that the damage created by this method increases the effect of many chemical carcinogens.

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Microradiography

[S. G. Carpenter, L. M. Holland, J. R. Prine, and R. H. Wood, with L. E. Bryant and J. R. London (M-1)]

Our efforts at determining sphere distribution within the lung have been reported previously. Last year¹ we reported use of ultrasoft x-radiography

Fig. 3. The lung parenchyma from a hamster injected intravenously with ~ 2000 microspheres.

Fig. 4. The lung parenchyma from a hamster injected intravenously with ~ 200 000 microspheres.

of helium-dried lung tissue to visualize the microspheres *in situ*. This technique allows the microspheres to be seen in relationship to air passages

from large bronchi down to the small bronchioles. With this method, the effect of dose size (number of spheres) can be shown. In the animals injected intravenously with about 2000 microspheres, the distribution is fairly uniform throughout the lung, with a small nearest-neighbor effect. As the number of spheres injected is raised, the distribution becomes less uniform, and clumping and entraining become more pronounced (Figs. 3 and 4).

This radiography technique also allows demonstration of the distribution of microspheres around the termination of airways when the particles are administered intratracheally. The diameter of the spheres (~10 μ m) used in this study undoubtedly precludes deposition of many in the very deep lung but, as illustrated in Fig. 5, does allow the microspheres to reach the terminal bronchioles.

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Fig. 5. The lung parenchyma from a hamster which received microspheres by intratracheal instillation. Note that the microspheres are grouped around the termination of an airway.

EFFECTS OF EXTERNAL RADIATION ON LIVING ORGANISMS

(J. F. Spalding, L. M. Holland, P. M. LaBauve, J. E. London, J. R. Prine, and O. S. Johnson)

Introduction

Persistent biological effects of relatively large doses of ionizing radiation, delivered acutely or chronically to man, are not well known. Reasonable estimates of the magnitude of the persistent radiation injury component, referred to as the "irreparable" or long-term residual lesion, are difficult to make because there are few experimental data on large animals and reports based on rodents are frequently conflicting. Thus, guidelines and recommendations for human exposure to ionizing radiation have a tenuous basis.

Research projects in progress and reported here are programmatically designed to meet the objectives of the whole-body exposure (from external sources) program. These objectives are to obtain and react to problems of the Division of Biomedical and Environmental Research and, thus, to obtain knowledge about the nature and magnitude of the persistent lesion induced by exposure to radiation under different total-dose and dose-rate conditions.

It is well known that dose rate influences the nature of the immediate and persistent biological response to radiation injury. However, the comparative biological effects of dose protraction by continuous exposure at different dose rates and by the acute fractionation method in a given mammalian species and among different species are not well known. Earlier investigations have clearly demonstrated that the method used to protract a given dose of radiation may significantly influence the nature and magnitude of the biological effect.^{1,2}

Investigations at this Laboratory are programmatic in nature and are designed to obtain needed acute and low dose-rate data in species with different radiation response characteristics. Because the primate (monkey) is a relatively long-lived animal that has not been used widely in low dose-rate investigations, it is of primary interest as a research species in our long-range program.

The dog and monkey differ widely in their response to single high dose-rate exposure and to low dose-rate continuous exposure; therefore, comparative dose-rate studies of the two species under the

same exposure conditions should yield data that would be helpful in better understanding the kinetics of radiation injury and recovery. Radiation effects data from these two species should also help to make extrapolations of the prompt and long-term effects from animals to man more meaningful.

Projects being investigated include (1) large-animal studies using dogs and monkeys to compare response to gamma-ray exposure at different dose rates and residual injury that may reflect the species long-term effects and (2) small-animal studies in which mice are used to observe the extent and nature of the long-term lesion from radiation in partial- and whole-body gamma-ray exposed animals.

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Recovery Rate and Persistent Injury in Monkeys Given Large Doses of Gamma Rays by Fractionation

Two basic theories about the nature of the long-term radiation lesion (LTRL) persist in the confusion of conflicting reports. The LTRL is expressed as (a) dose-related irreparable injury that does not affect the recovery or repair mechanism or rate¹⁻⁵ or as (b) a dose-related injury to the repair mechanism only.^{6,7} This investigation is being done to obtain dose-rate effects data on the nature and magnitude of the LTRL in monkeys and to evaluate the two theories mentioned above in practical terms.

Sixteen monkeys (*Macaca mulatta*), 6 males and 10 females, approximately 6-8 yr old were used. Ten of them (3 males and 7 females) were given 24 separate 100-rad doses of gamma rays at approximately 35 rad/h, administered 56 days apart. Eighty-four days after the 24th exposure, we irradiated these monkeys, together with 6 nonirradiated controls (3 males and 3 females), to death during continuous gamma-ray exposures at 24 rad/23.5-h day.

To test the hypothesis that the LTRL may decrease the recovery rate and be related to total

exposure, we determined hematopoietic response to the 24 successive acute 100-rad gamma-ray doses at 3, 6, 13, 27, and 51 days after each exposure. We tested the two-component concept of radiation injury as a reduction in mean survival time and hematopoietic response [packed cell volume (PCV) and white blood cell count (WBC)] during continuous gamma-ray exposure at 24 rad/23.5-h day, starting 84 days (three suggested repair halftimes for man⁸) after the 24th 100-rad exposure. The PCV remained within 80% of the preexposure level throughout the fractionated radiation regime. After the first five exposures, lymphocytes and neutrophils dropped to approximately 52 and 30%, respectively, of pre-exposure levels.

We investigated the extent and nature of the hematopoietic LTRL using PCV and WBC analyses 6 and 51 days after each exposure. The mean PCV plots are shown in Fig. 1, and similar WBC plots are presented in Fig. 2. A straight-line fit through the 6th day PCV values revealed a slope expressed by the function $Y = 45.644 - 0.085x$. The t-test ($t = 1.86$) showed the slope to be significant at the 10% but not at the 5% level. The slope for the 51st day PCV values ($Y = 47.25 - 0.141x$) was significant ($t = 2.69$) at the 5% level. Thus, the PCV did appear to be decreasing significantly with each successive exposure or with increasing dose (Fig. 1).

To determine the relationship between decreasing PCV and successive 100-rad exposures at 56-day intervals, we determined the magnitude of recovery from the 6th to the 51st day after each exposure and fitted it to a straight line. The regression function representing these data was $Y = 1.036 - 0.0012x$, and the slope was not significantly different from zero ($t = -1.15$). The two observations stated above, that (1) a significant residual injury was shown by a progressive decrease in PCV values on the 51st day after each of 24 successive 100-rad exposures and (2) no significant difference in percentage of recovery occurs between the 6th and 51st days after each of the exposures, suggest that some sort of dose-related residual lesion was provoked in the monkeys by injury from ionizing radiation but that it is not expressed as a decreased recovery rate. Numbers for WBC plotted on the 6th and 51st days after each 100-rad exposure are shown in Fig. 2. There was an obvious drop after the first four

Fig. 1. Fitted line plots through PCV values of 10 monkeys at 6 and 51 days after each of 24 gamma-ray doses of 100 rad.

Fig. 2. Fitted line plots through WBC counts of 10 monkeys at 6 and 51 days after each of 24 gamma-ray doses of 100 rad.

exposures (4-24). The equation for 6th day values was $Y = 3572.24 + 26.19x$. Neither the 6th nor 51st day slope differed significantly from zero. Thus, after the striking effect following the first four separate 100-rad exposures, the leukocytic series showed no further residual injury with increasing dose.

The fractionation part of this study showed hematopoietic injury and recovery characteristics more consistent with Blair's hypothesis (namely, a radiation-induced irreparable injury component with a constant repair rate) than with the thesis that an irreparable lesion adversely affects the repair mechanism, thus changing the recovery rate.

According to the effective residual dose (ERD) concept of radiation injury kinetics recommended for man,⁸ monkeys accumulating 2400 rad of gamma rays

from 24 100-rad exposures at 56-day intervals would have the equivalent of 360 rad of injury immediately after the 24th exposure and approximately 255 rad 84 days later. This 255-rad theoretical ERD would be a significant part (39%) of the acute LD_{50}^{30} of the monkey and should be recognized as causing a reduced mean survival time (MST) in a radiation field intense enough to stress continuously the hematopoietic system.

We subjected the gamma-ray conditioned and nonconditioned monkeys to continuous 24 rad/23.5-h day gamma-ray exposure until they died. Survival time distributions for both groups are plotted in Fig. 3. Mean survival times for conditioned (2438 ± 267 h) and nonconditioned (2261 ± 260 h) monkeys did not differ significantly, and curve fits of the survival time data showed essentially the same distribution for both groups (Fig. 3). These data failed to reveal the "irreparable" or long-term residual lesion predicted by the theoretical ERD.⁸

Necropsies were performed on the monkeys as they succumbed to the lethal effects of continuous gamma-ray exposure. Females apparently had normal menstrual cycles during conditioning and challenge exposures. At death, the ovaries of all females appeared normal and showed all stages of follicular and oocyte development. Without exception, the male gonads were devoid of germinal activity. Seminiferous tubules contained only Sertoli cells. We found no areas of epithelial atrophy in the gastrointestinal tract, and the crypts of Lieberkuhn

contained cells with mitotic figures of a normal frequency and pattern for animals of this age. Occasional petechial hemorrhages were found at necropsy; however, we found no frank hemorrhagic suffusions. The death of all monkeys, both conditioned and nonconditioned, was attributed to hematopoietic hypoplasia.

This investigation suggests that, under these radiation conditions, the recovery rate from radiation injury is independent of total accumulated gamma-ray doses and that the LTRL from gamma-ray conditioning doses totaling 2400 rad is not measurable as a reduction in MST in a radiation field of 24 rad/23.5-h day.

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Comparative Hematopoietic Effects of Dose Protraction by Fractionation in Beagles and *Macaca mulatta* Monkeys

Beagles and monkeys (*Macaca mulatta*) were exposed simultaneously to 13 acutely delivered 100-rad gamma-ray doses at 28-day intervals (dose rate approximately 16 rad/h). Blood samples were obtained from all animals at 7-day intervals during the time course of the investigation (approximately 1 yr). Hematopoietic response of the two species to this radiation regime was compared.

White blood cell (WBC) and packed cell volume (PCV) were plotted as percent of preexposure values

Fig. 3. Survival time distributions of conditioned and nonconditioned monkeys continuously exposed to gamma rays at 24 rad/23.5-h day.

just prior to each exposure. These plots are shown in Fig. 1. Bone marrow response to the first eight acutely delivered 100-rad exposures was quite similar in both species. PCV values remained within clinically normal levels, and WBC values dropped progressively with each exposure, with dogs being somewhat more seriously affected than monkeys.

Dogs were unable to cope with radiation injury beyond the 8th exposure, as evidenced by a progressive decline in PCV and WBC counts to critical levels (Fig. 1). Six of the 12 dogs exposed died as a result of radiation injury, while only one of the 12 monkeys succumbed during the radiation regime.

In the past, dose protraction by fractionation has been assumed to be equivalent (in terms of biological response) to dose protraction by continuous low dose-rate exposure, with time and total dose in each case being equal. Earlier investigations with mice¹ have shown that these assumptions are not warranted in all cases. The dose rate in this fractionation radiation regime is equivalent to a dose rate of 3.57 rad/day of continuous exposure.

Norris and Poole² have reported that beagles given a dose of 5 rad/day can survive at least twice as long as the dogs in this investigation, thus confirming the results of the mouse study that dose protraction by fractionation cannot be assumed to have the same biological effects as equivalent doses given during a continuous exposure.¹

Fig. 1. Packed cell volumes (PCV) and white blood cell counts (WBC) in dogs and monkeys prior to each of 13 acute 100-rad gamma-ray exposures (expressed as percent of preexposure values).

Animals which survived this fractionated exposure regime will be allowed 84 days for recovery, at which time residual injury will be measured as a reduction in mean survival time in a continuous gamma-ray field with a dose rate of 37 rad/23-h day.

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Recovery and Residual Injury Observations [Equivalent Residual Dose (ERD) Assumptions]

The National Committee on Radiation Protection and Measurements¹ has suggested the equivalent residual dose (ERD) concept of radiation injury and recovery to regulate daily exposure to radiation in an emergency. This concept assumes that approximately 10% of any given dose of radiation absorbed by man will be retained as an irreparable lesion, with the remaining 90% being eliminated by repair with a recovery halftime (RT_{50}) of 28 days. Observations on one or more of these ERD assumptions have been reported earlier.²⁻⁴ In this observation a plot of the theoretical build-up of the ERD was made for the 13 acutely delivered 100-rad gamma-ray exposures. From this plot the accumulated dose and ERD were determined at the time of death of those animals dying during the radiation regime. Gamma-ray exposures and ERD assumptions are presented in Table I. A plot of the residual dose during the 13 exposures is shown in Fig. 1. The time of death of the monkeys and dogs is indicated on the plot (Fig. 1). Only one (8%) monkey died from radiation injury, this death occurring at an ERD of approximately 260 rad. Six (50%) dogs died at ERD levels ranging from 270-310 rad.

The acute LD_{50} of the monkey is approximately 600 rad, while that of the dog is about 250-300 rad. With the exposure conditions used, the ERD assumptions would predict the death of approximately 50% of the dogs at some point after the 8th exposure (accumulated dose ranging between 800 and 1200 rad). Few, if any, monkeys would be expected to die during the 13-exposure series because the ERD does not approach the LD_{50} of that species.

TABLE I
SURVIVAL OF ADULT BEAGLE DOGS AND MONKEYS (*Macaca mulatta*) DURING A FRACTIONATION RADIATION REGIME
(INCLUDING 13 100-RAD GAMMA-RAY
EXPOSURES AT 28-DAY INTERVALS)

Exposure No.	Accumulated Dose (rad)	ERD (rad)	Number of Survivors Dogs Monkeys
1	100	100	12 12
2	200	155	12 12
3	300	188	12 12
4	400	209	12 12
5	500	224	12 12
6	600	237	12 12
7	700	249	12 12
8	800	259	12 12
9	900	270	12 11
10	1000	280	12 11
11	1100	290	11 11
12	1200	300	9 11
13	1300	310	8 11
(28) ^a	1300	220	6 11
(56)	1300	175	6 11
(84)	1300	152	6 11

^aDays after the 13th exposure.

Fig. 1. Theoretical equivalent residual dose (ERD) in dogs and monkeys during and following 13 acute 100-rad gamma-ray exposures.

This investigation is still in progress. However, with the radiation exposure factors used, the injury and recovery assumptions of the ERD concept are consistent with experimental observations.

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MAMMALIAN METABOLISM

Introduction

Research activities on interspecies correlations in uptake, deposition, and excretion of radioisotopes and response of the various tissues of the body to such internally deposited radioactive materials are continuing and should provide metabolic data useful in establishing maximum permissible levels for extrapolation to man. Calculation of cesium-137 levels in a normal New Mexico population is of interest as a radioactive fallout nuclide and also as a guide in the study of pollutants of the atmosphere.

Disposition of Cesium-137 during Potassium Depletion in Rats

(J. E. Furchner and G. A. Drake)

The maintenance of low levels of fallout cesium-137 in a human population has caused renewed speculation over a possible long-lived compartment in the bone,^{1,2} as well as some long-lived external source of chronic contamination. If the bone is indeed a small compartment ($5\%^{1}$ to $0.005\%^{2}$) with a long half-time, it should be possible to demonstrate bone burdens during and after chronic feeding. Because a low potassium diet increases cesium retention,³ such a regimen may also augment the deposition of cesium in bone.

After a preliminary three-week period of adjustment to powdered food, 24 rats were given a synthetic low potassium diet, and 12 rats were maintained on a normal diet of commercial laboratory animal food. The drinking water of both groups of animals was contaminated with cesium-137 (0.8 nCi/ml) for about 40 days. Thereafter the concentration has been maintained at 0.5 nCi/ml. At 39 days, four of the experimental animals and two controls were sacrificed. Tissues were assayed for cesium-137 activity. Whole-body activity and body weight

were determined periodically. The results to date are presented in Table I and in Figs. 1 and 2.

The synthetic diet appeared to be less palatable to the rats, who consumed (and spilled) about 20 g/day per rat while each control rat used about 30 g/day. It may be that the differences in weight shown in Fig. 1 may be due, in part, to the differences in food consumed.

The differences in retention (Fig. 2) appear to be more dramatic and are clearly due to potassium deficiency, since the water consumption per rat was the same for the two groups. The rats on the normal diet appear to have been near equilibrium when the contamination level was decreased.

Although there were large differences in the sizes of the body burdens, there were only small differences in the distribution of the body burdens among the various tissues at 39 days of chronic exposure (Table I). Only muscle, bone, and kidney appear to have higher concentrations in the potassium-deficient animals than in the normal animals. There appear to be little or no differences among the other tissues of the two groups. Animals will be sacrificed at later times.

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TABLE I
AVERAGE TISSUE DISTRIBUTION OF CESIUM-137 IN NORMAL AND POTASSIUM-DEPLETED RATS (AT 40 DAYS)

Tissue	Body Burden (%)		Body Burden (%/g)	
	Normal	Potassium Deficient	Normal	Potassium Deficient
Muscle	72.8	76.8	0.259	0.304
Bone	4.4	5.4	0.231	0.326
Pelt	8.3	7.3	0.089	0.084
Gut	4.1	2.6	0.124	0.151
Liver	4.4	3.9	0.249	0.223
Lung	0.4	0.3	0.127	0.150
Heart	0.3	0.3	0.290	0.277
Kidney	0.8	1.6	0.267	0.419
Spleen	0.1	0.1	0.110	--
Testis	0.7	0.7	0.230	0.217
Brain	0.2	0.2	0.147	0.116
Blood	0.5	0.6	0.035	0.033

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Fig. 1. Average whole-body weight changes for rats on normal and potassium-deficient diets. The deficient diet was adequate for growth but at a less than normal rate.

Fig. 2. Average whole-body cesium-137 activity changes for rats on normal and potassium-deficient diets. There was a four-fold increase in retention caused by the deficient diet.

Retention of Arsenic-74 after Oral, Intraperitoneal, and Intravenous Injection in Rats

(J. E. Furchner and G. A. Drake)

There is an extensive literature dealing with toxic and therapeutic effects of arsenic and arsenic compounds. Retention and tissue distribution have been determined for various species, but few investigators have reported interspecific comparisons. Moreover, retention of the trivalent state appears to differ from retention in the pentavalent state.¹ The following represents the beginning of an attempt to determine the relevance of these factors to radiation protection guides.

Arsenic-74 decays by electron or positron emission with associated gamma rays of about 8.0 fJ energy (0.5 MeV). The 18-day half-life is adequate for relatively short-term studies. One-year-old male Sprague-Dawley rats weighing 490-540 g were injected with an activity of about 1.85×10^4 dis/sec (0.5 μ -Ci) of arsenic-74 in the form of sodium arsenate (pentavalent). Six animals were given intraperitoneal injections without anesthesia, and of 12 animals anesthetized with ketamine hydrochloride six were given intravascular injections by tail vein and six were dosed by intragastric intubation. Within 30 min of injection, whole-body gamma activity of the animals was measured in a liquid scintillation

detector, HUMCO III,² and then at convenient intervals during the following five weeks. The data collected up to this time were analyzed and fit to the sum of two exponentials. The best fit to all the data for each group is given in Table I, as well as the best fit to the data for each animal in the oral group.

Only small differences in effective retention were found among the three groups. The intravenous and oral groups could be fit by one retention function, but the intraperitoneal group showed small differences. It remains to be determined if the use of an anesthesia influenced the amount lost with a halftime less than one day -- the greatest difference found.

The halftimes given are effective halftimes and include the physical decay of arsenic-74. The time required to reach one-half the initial activity is ~15 days. The biological halftime may be calculated from $T_b = \frac{T T_p}{T_p - T}$, where T is the effective halftime (15 days), T_p is the physical halftime (18 days), and T_b is the biological halftime. The result of 90 days is only about one-third of that given for the whole body by the International Commission on Radiation Protection.³ This value is derived largely from Hamilton's work using rats and dogs.^{4,5}

TABLE I
EFFECTIVE WHOLE-BODY RETENTION PARAMETERS: ARSENIC-74 IN RATS

	a_1^a	k_1^a	$T_{1/2}$ (days)	a_2	k_2	$T_{1/2}$ (days)	EF^b
Intraperitoneal	16.15	2.762	0.3	83.85	0.0464	14.9	18.1
Intravenous	27.00	3.049	0.2	73.00	0.0461	15.0	15.9
Intragastric	28.69	2.288	0.3	71.31	0.0451	15.4	15.9
<u>Individual Rats (Intragastric)</u>							
1	26.46	2.150	0.3	73.56	0.0459	15.1	16.2
2	30.65	2.141	0.3	69.35	0.0457	15.2	15.3
3	25.65	1.843	0.4	74.35	0.0451	15.4	16.6
4	29.00	2.705	0.3	71.00	0.0436	15.9	16.4
5	29.99	2.084	0.3	70.01	0.0493	14.1	14.4
6	28.29	2.722	0.3	71.71	0.0448	15.5	16.1
Mean	28.34	2.274	0.3	71.66	0.0457	15.2	15.8

^a a is the intercept constant (in percent of injected dose), and k is the rate constant (in reciprocal days) in the retention function $R_t = \sum_{i=1}^n a_i (\exp k_i t)$, where t is in days.

^bEF is the time integral of the retention function.

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Cesium-137 Activity in a Normal New Mexico Population

(J. S. Wilson and J. E. Furchner)

A number of normal laboratory employees have been used as a representative New Mexico population for the purpose of detecting low-level, whole-body, gamma-ray radioactivity. About 10 individuals have been counted on a weekly basis and about 30 on a monthly basis. Results for earlier periods have been reported.¹⁻⁴ Values for all subjects have fallen from a maximum of about 4.4×10^{-1} ds⁻¹/g potassium in 1964 to a value of about 7.4×10^{-1} ds⁻¹/g potassium. This latter value has persisted with minor fluctuations for about five years. These levels and similar low levels in food⁵ suggest a source of radiocesium with a slow turnover rate, perhaps with a similarly slowly changing small pool in man. Documentation of the activity levels of cesium-137 as a function of time furnishes a useful basis for assessment of radiation exposures following new pollutions of the biosphere. Whole-body burdens of fallout cesium-137 and natural potassium levels in a normal New Mexico population are reported herein for the period November 1972-October 1973.

The collection and analytical procedures have been reported.⁶ The average monthly values are given in Table I. A biannual seasonal peak-valley relationship seen in previous years was less evident during the past year. Similar low levels, persisting for a number of years, also have been noted in other laboratories.^{7,8}

Average values for the whole-body burden cesium-137 activity in 1971, 1972, and 1973 were 96.2, 94.0, and 85.8 ds⁻¹, respectively. These levels produce⁹ annual doses of 4.3, 4.2, and 3.9 μ J kg⁻¹, respectively, in a nominal 70-kg man, or less than 1/30th of the dose delivered by 120 g of potassium in a 70-kg man.

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TABLE I

MONTHLY AVERAGE VALUES FOR BODY WEIGHTS, POTASSIUM AND CESIUM-137 IN A SAMPLE NEW MEXICO POPULATION

	November	December	January	February	March	April	May	June	July	August	September	October
Body Weight (kg)	67.2	69.5	68.8	68.1	68.8	68.1	68.6	67.3	66.7	67.5	67.7	67.0
Potassium (g)	125.8	128.0	125.2	116.9	123.5	121.9	122.2	110.9	119.1	123.1	125.1	120.1
Potassium (g/kg)	1.86	1.84	1.79	1.76	1.75	1.79	1.77	1.66	1.78	1.82	1.83	1.77
Cesium-137 (ds ⁻¹)	96.9	98.1	101.0	96.2	89.9	92.1	85.8	87.0	57.0	70.3	71.0	83.6
(nCi)	(2.62)	(2.65)	(2.73)	(2.60)	(2.43)	(2.49)	(2.32)	(2.35)	(1.54)	(1.90)	(1.92)	(2.26)
Cesium-137 (ds ⁻¹ /g K)	0.78	0.78	0.74	0.84	0.77	0.78	0.72	0.85	0.48	0.58	0.58	0.74
(pCi/g K)	(21.0)	(21.1)	(20.1)	(22.8)	(20.7)	(21.1)	(19.4)	(22.9)	(13.1)	(15.8)	(15.7)	(19.9)
Cesium-137 (ds ⁻¹ /kg)	1.44	1.42	1.46	1.57	1.33	1.36	1.25	1.28	0.88	1.05	1.06	1.26
(pCi/kg)	(39.0)	(38.5)	(39.4)	(39.6)	(36.0)	(36.8)	(33.9)	(34.7)	(23.7)	(28.3)	(28.6)	(34.1)

ANIMAL COLONY ACTIVITIES

Introduction

The major function of the LASL animal colony has been the support of any research involving live animals. While daily animal inventories have shown some increases in the past year, the main change has been the addition of breeding groups of C3H/HeJ and DBA/2J mice for use in transplantable tumor experiments.

The new animal facility reported last year is nearing completion at TA-S1 and will provide flexible space to house several species ranging in size from small rodents to swine. The building contains five multiple-use animal rooms and a laboratory-equipped room which can be used by visiting investigators utilizing the biomedical beam at the LAMPF.

Disease Surveillance

(P. M. LaBauve, L. M. Holland, and M. R. Brooks)

Surveillance for disease problems within the animal colony is a continuing effort. A constant monitoring program for the detection of *Pseudomonas* "P.", coupled with treatment of drinking water, has been highly successful in the mouse colony.

All incoming rodent species are subjected to bacteriological testing during quarantine in an effort to minimize the possibility of disease introduction. Because of the increased demand for primates, the acquisition and quarantine of monkeys

have become more of a problem. All primates are subjected to a minimum of 90 days quarantine, during which extensive testing for tuberculosis, observation for Herpes lesions, and bacteriological examination for gut pathogens are carried out.

A program of surveillance for various viral agents, especially in the rodent populations, has been instituted this year. Serum samples are taken at random throughout the colony and sent to a consulting laboratory where they are tested for relevant virus antibodies according to species.

Pathology and Support Activities

(S. C. Carpenter, L. M. Holland, P. M. LaBauve, J. R. Prine, R. H. Wood, J. E. London, and J. S. Wilson)

The pathology effort has increased each year in support of the various research programs utilizing animals. Complete pathology work-ups were performed on over 1000 animals in the first 11 months of 1973. The increased number of animals used in the external radiation studies and the high volume of animals used in the hot particle study are the major sources of pathological material.

Two dogs from the LASL breeding colony have died of spontaneous neoplastic disease over the past year. One animal died as a result of an aortic body tumor (heart base tumor) and the other dog as a result of a generalized malignant lymphoma. Neither animal had a significant radiation history.

One of the more important support activities has been the performance of all hematology and blood chemistry in conjunction with the external radiation programs. Results of this effort are detailed elsewhere in this report.

Compliance with Federal Law

The LASL animal colony remains in compliance with all federal laws governing the use of animals in research. Our accreditation has been renewed by the American Association for the Accreditation of Laboratory Animal Care after a site visit in late 1972.

Research
INDUSTRIAL HYGIENE GROUP* (H-5)

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* Personnel assigned part or full time to AEC/DBER-funded programs.

** Currently on a two-year AEC governmental assignment to the Radiological Assessment Branch, Directorate of Licensing, U. S. Atomic Energy Commission, Washington, D. C.

INTRODUCTION

In addition to purely biomedical problems, the Los Alamos Scientific Laboratory is engaged in the investigation of a number of problems related to the environment and particularly the working environment. Several of these funded in Group H-5 by the Division of Biomedical and Environmental Research include studies on sampling, characterization and control of aerosols, and analyses of tissues from autopsies of persons in the general public and those exposed to materials associated with AEC operations. In the aerosol field, a study is being made of the collection efficiency of filters operated under intermittent cyclic flow simulating conditions occurring when a filter is used in a respirator. Some preliminary results indicate differences between cyclic flow efficiencies and steady flow efficiencies for certain particle sizes, but these require more detailed study. Another project in this field is a study of agglomeration of particle dispersions used for in-place filter testing. Coagulation rate constants have been measured on dioctyl phthalate aerosols. Fresh and agglomerated

aerosols were used in testing both new and damaged filters, and agglomeration did not result in significant changes in the filter evaluations.

A computer model has been developed for assessing the effects of various parameters on air cleaner performance especially in terms of factors of biological and physical significance such as visibility degradation and inhalation risk. Another study which is just beginning involves resuspension of aerosols from various surfaces. These should relate to both theoretical and practical aspects of the redistribution of radioactive and nonradioactive particles. An investigation is being made of the theory underlying design of centrifugal aerosol spectrometers. The resulting equations are being solved by computer to indicate the optimum design. Because the escape of radioactive aerosols from controlled operations does not produce a homogeneous dispersion, it is important to locate air samplers correctly to yield meaningful data from the standpoint of hazard evaluation or control. Studies are being made of the distribution of particulates under actual working conditions. Many aerosol investigations involve

particle size analysis of fine particles requiring electron microscopy, and it is necessary to transfer collected material from a filter to an electron microscope grid. A new technique has been developed which avoids the losses encountered in earlier methods.

In the tissue analysis program, analyses for plutonium content have been made on tissues from 60 occupationally exposed persons and several hundred having no occupational exposure to plutonium. The highest concentrations found were in the lymph nodes with liver, lung, kidney, and bone following in order to decreasing concentration. Reanalysis of gonadal tissue obtained in a limited number of cases revealed concentrations lower than reported previously. A statistical study of lung and liver tissue analyses for plutonium in the general population shows an apparent correlation of liver concentration with age at death. The lung data do not show such correlation. An investigation has been initiated on the use of neutron irradiation of bone sections mounted on thin plastic film to determine the distribution of minute amounts of plutonium in bone and marrow.

Some tissue samples are analyzed for uranium, although the method of analysis is not sensitive enough to detect quantitatively the amounts in samples from persons having no known occupational exposure. Only eight sets of tissues were available from persons having any occupational exposure. Portions of the ashed remains of most of the samples collected under the tissue program are available for analyses of other metals and environmental pollutants. All tissues obtained for analysis are screened for gamma and x-ray activity, and measurable amounts of several isotopes, in addition to naturally occurring potassium-40 and nuclear fallout cesium-137, have been found.

AEROSOL CONTROLS AND HEALTH PROTECTION

(H. J. Ettinger, M. I. Tillery, C. I. Fairchild, and P. Lowry)

Filtration Efficiency of Respirator Filter Media with Cyclic Flow

Minimum acceptable performance of filters used in respirators to protect against inhalation of radioactive and toxic particles is defined in terms of steady flow operations.¹ In actual use, flow

through the filter medium is cyclic with the frequency and tidal volume dependent on the work rate of the individual. To determine the relationship between filter performance under steady and cyclic flows for various work rates and to define better the mechanisms of filter collection and retention, an experimental program has been established. Collection efficiencies of filtration media and filter cartridges used on respirators are measured for a variety of particle sizes using a cyclic flow pump to simulate different human breathing patterns. During cyclic flow, collection efficiency varies as a function of instantaneous velocity; therefore, there are several possible definitions of efficiency. In the initial experimental program, the instantaneous efficiency was measured a minimum of 10 times during the inhalation portion of the breathing cycle, and the average of these efficiencies was used to represent filter performance during the complete cycle.² Because maximum flow rate occurs only once during the inhalation cycle, this method biases the results toward those existing during low flow rates. In the current test program, efficiency is measured in terms of the integrated total mass penetrating the filter, as compared with the mass concentration upstream of the filter during the complete cycle.

The challenge aerosol used consists of monodisperse polystyrene latex particles generated from an aqueous suspension with a compressed air nebulizer. The solution concentration is adjusted to provide a large fraction of singlets as confirmed by electron microscopy. Upstream and downstream concentrations are measured continuously with forward light-scattering aerosol photometers, with automatic valving designed to provide flow through the filter only during the inhalation portion of the cycle.

Experimental data have been collected on Whatman 41 filtration media which are often used for sampling but not used in respirators: American Optical R-30 filtration medium, a resin impregnated medium used for dusts, smokes, and mists; and American Optical R-1040, a medium used for disposable respirators for dusts and mists.

To compare the two methods for calculating efficiency, one filter medium (Whatman 41) has been tested both ways. Table I shows these results for four particle sizes. The bias toward the low flow

TABLE I

CYCLIC FLOW EFFICIENCIES FOR WHATMAN 41 FILTRATION MEDIUM

Particle Diameter (μm)	% Penetration (Total Mass) 415 kg-m/min Work Rate	% Penetration (Average Value) 415 kg-m/min Work Rate	% Penetration with Steady Flow	
			$\sim 0.00037 \text{ m}^3/\text{sec}$ ($\sim 22 \text{ l./min}$)	$\sim 0.00027 \text{ m}^3/\text{sec}$ ($\sim 16 \text{ l./min}$)
0.126	10.75	5.00	18.00	---
0.312	14.39	9.10	14.70	35.00
0.790	3.02	22.50	4.80	8.00
1.011	3.68	17.50	2.80	3.00

rates with the averaging method overrates performance of the filter for small particles and under-rates performance against large particles, as the smaller particles are more efficiently collected by diffusion at these lower velocities. At higher instantaneous velocities, where inertial collection predominates, these small particles are not removed as efficiently from the air stream.

The Whatman 41 data shown in Table I also indicate that steady flow data taken at a flow rate equal to the timed average flow rate during the inhalation portion of the breathing cycle (above $0.00037 \text{ m}^3/\text{sec}$ or $\sim 22 \text{ l./min}$) approximate the actual (total mass) cyclic flow efficiency. Similar results were obtained with the American Optical R-30 medium. However, one of the primary collection mechanisms for this medium is electrostatic so that the importance of flow velocity on the diffusion and inertial collection mechanism is reduced. The American Optical R-1040 medium has been tested at two work rates for five particle sizes (Table II). The only filter holder available made it necessary

to carry out these tests on somewhat less than half of the filtration area; therefore, the efficiencies obtained may be somewhat higher than achieved under actual use conditions. These results indicate that efficiencies are higher at higher work rates for small particles. For larger particles the trend is not clear, but there seems to be little difference in collection efficiency with work rate. All these data are preliminary and will be expanded in the near future.

Additional experimental data will help define those parameters that primarily affect cyclic flow filtration efficiency and will permit development of reliable tests to predict performance of filters under these operating conditions. Experimental data will be obtained for different breathing rates and tidal volumes so that a model can be developed adapting Davies' filtration equations³ to cyclic flow.

REFERENCES

1. Federal Register 37, 59, Part 11.140-7 and Part 11.140-11 (1972).
2. R. G. Stafford, H. J. Ettinger, and T. J. Rowland, "Respirator Cartridge Filter Efficiency under Cyclic and Steady-Flow Conditions," Amer. Ind. Hyg. Assoc. J. 34, 182 (1973).
3. C. N. Davies, "The Separation of Airborne Dusts and Particles," Inst. Mech. Eng. Proc. B, 185 (1952).

Aerosol Agglomeration and Filter Testing

In-place testing of high efficiency particulate (HEPA) filters is normally carried out with air jet-generated dioctyl phthalate (DOP) aerosols to determine the integrity of gasket seals and to provide an index of expected filtration efficiency.¹ In most facilities, the aerosol generators are located outside the filter plenum and the undiluted

TABLE II

CYCLIC FLOW EFFICIENCIES FOR AMERICAN
OPTICAL R-1040 FILTRATION MEDIUM

Particle Diameter (μm)	% Penetration (Total Mass) 415 kg-m/min Work Rate	% Penetration (Total Mass) 622 kg-m/min Work Rate
0.126	33.00	24.00
0.312	19.00	18.00
0.500	6.30	4.70
0.790	4.10	4.10
1.011	1.40	4.40

aerosol is piped to the filter bank to be tested. As the aerosol passes through the lines, there is an increase in median size and a decrease in number concentration due to aerosol agglomeration. These changes depend on the original particle size, concentration, and transit time. To determine the magnitude of this change and the effect it has on in-place testing, coagulation rate constants have been determined by measuring the change in particle size distribution with respect to time using a Royco particle counter and an Andersen cascade impactor. For contact times of up to 538 sec, coagulation rate constants of 0.15×10^{-9} to 0.66×10^{-9} cm^3/sec have been measured. Theoretical rate constants for these aerosols would be approximately 0.4×10^{-9} to 0.9×10^{-9} cm^3/sec . Count median diameter and volume median diameter increased approximately 40% under these test conditions.

The effect of this change on in-place testing was then determined by measuring filter penetration with the original aerosol and with the agglomerated aerosol. No significant differences were noted when

testing new, perfectly sealed filters. To determine the effect of these changes in aerosol size in evaluating damaged filters or leaky gaskets, a filter was damaged with 0.115-cm-diameter pinholes and tested with both aerosols. Table I, summarizing these results, shows that the change in test aerosol size due to agglomeration does not result in any significant change in in-place test filter performance and that transport time from the generator to the test plenum is not critical.

In an attempt to improve the sensitivity and accuracy of in-place HEPA filter testing, a new DOP air jet generator was designed and built to increase the DOP output. Increased output resulted in a larger particle size distribution with the actual aerosol number concentration slightly decreased. As the light-scattering devices used to detect the aerosols in in-place testing are more closely related to number concentration, this generator offered no advantages, and further development was dropped.

TABLE I
PENETRATION OF HEPA FILTER WITH PINHOLES BY AEROSOLS WITH DIFFERENT SIZE DISTRIBUTIONS^a

Generator Pressure = 6894 pascals (10 psi)		Generator Pressure = 13,788 pascals (20 psi)		Generator Pressure = 20,682 pascals (30 psi)	
Initial Aerosol	Agglomerated Aerosol	Initial Aerosol	Agglomerated Aerosol	Initial Aerosol	Agglomerated Aerosol
CMD ^b = 0.57 μm σ_g = 1.58	CMD = 0.78 μm σ_g = 1.78	CMD = 0.50 μm σ_g = 1.68	CMD = 0.64 μm σ_g = 1.69	CMD = 0.50 μm σ_g = 1.69	CMD = 0.64 μm σ_g = 1.61
Penetration (%)	Penetration (%)	Penetration (%)	Penetration (%)	Penetration (%)	Penetration (%)
0.16	0.18	0.14	0.15	0.16	0.14
0.17	0.13	0.17	0.14	0.18	0.17
0.14	0.15	0.16	0.11	0.17	0.17
0.16	0.16	0.17	0.14	0.14	0.14
0.16	0.13	0.14	0.16	0.17	0.17
0.14	0.18	0.14	0.16	0.16	0.18
<u>0.15</u>	<u>0.14</u>	<u>0.16</u>	<u>0.16</u>	<u>0.15</u>	<u>0.17</u>
Average 0.155	0.153	0.154	0.148	0.164	0.162

^aSize distributions measured with a Royco Model 220 aerosol particle monitor; flow rate of $0.00708 \text{ m}^3/\text{sec}$ (15 cfm); pressure drop across filter of 112 pascals (0.45 in. of water).

^bCMD = aerosol count median diameter and σ_g = aerosol geometric standard deviation.

REFERENCE

1. C. A. Burchstedt and A. B. Fuller, "Design, Construction, and Testing of High-Efficiency Air Filtration Systems for Nuclear Application," Oak Ridge National Laboratory report ORNL-NSIC-65 (January 1970).

Computer Assessment of Air Cleaner Performance in Terms of Mass, Number Concentration, Haze Formation, and Inhalation Hazard

The performance of commercial air cleaners is usually defined in terms of mass collection which is consistent with current emission standards expressed in units of mass per unit time or mass per unit volume of air. The relation between mass collection efficiency and other parameters defining physical characteristics of the effluent have been determined for idealized electrostatic precipitators, fibrous filters, and mechanical cleaners (cyclones and scrubbers) using a computer model. The effluent parameters investigated are number concentration, haze formation, and inhalation hazard.

In the model, challenge aerosols having different log-normal size distributions are passed through each air cleaner to determine its efficiency as defined by the various parameters of interest. The challenge aerosols can be varied over a wide

range of median diameters and geometric standard deviations. Reference performance of the air cleaner is determined by assigning the mass collection efficiency for the challenge aerosol. Characteristic efficiency curves for each type air cleaner are used to determine the fraction of each particle size that penetrates the cleaner. The particle size distribution of the effluent aerosol is then compared to the challenge aerosol to determine the performance of the cleaner with respect to the other efficiency parameters of interest. The relation between mass collection efficiency and these parameters is strongly dependent on the particle size of the challenge aerosol. The results for one efficiency curve for a mechanical cleaner (cyclone) are shown in Table I. The tabulated median volume diameters are for different challenge aerosols, and the geometric standard deviation for all these is 2.25. Table II shows the results for challenge aerosols having a median volume diameter of 10 μm and varying geometric standard deviations. In both tabulations, the respirable volume is based on the American Conference of Governmental Industrial Hygienists¹ definition of particulates which penetrate to the nonciliated portions of the lung and provides a more realistic estimate of the

TABLE I

REDUCTION OF CHALLENGE AEROSOL PARAMETERS BY A MECHANICAL AIR CLEANER WITH RESPECT TO PARTICLE SIZE

Median Volume Diameter (μm)	Geometric Standard Deviation (σ_g)	Total Particulate Volume	% Reduction of Particle Number	% Reduction of Respirable Volume	Total Projected Surface Area
20	2.25	82	32	43	67
15	2.25	77	25	40	61
10	2.25	68	16	36	50
5	2.25	50	5	27	30

TABLE II

REDUCTION OF CHALLENGE AEROSOL PARAMETERS BY A MECHANICAL AIR CLEANER WITH RESPECT TO PARTICLE SIZE

Median Volume Diameter (μm)	Geometric Standard Deviation (σ_g)	Total Particulate Volume	% Reduction of Particle Number	% Reduction of Respirable Volume	Total Projected Surface Area
10	3.50	65	0.4	25	29
10	2.25	68	16	36	50
10	1.50	70	57	54	66

inhalation hazard to the individual than provided by measurement of total volume (or mass). Similarly, measurement of total projected surface area better relates to visibility degradation, while particle number concentration and its association with nucleation potential relate to some weather effects. These limited results indicate that, for many aerosols and cleaners (especially where small particles are present), mass collection efficiency is a poor index of pollutant impact on environmental conditions and health concerns. Extensive comparative calculations are planned, and these should indicate appropriate criteria to define better the performance of these air cleaners.

REFERENCE

1. American Conference of Governmental Industrial Hygienists, Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Environment, Cincinnati, Ohio (1973).

Aerosol Resuspension from Surfaces

To predict the probable spread or migration of surface contaminants, it is necessary to know what parameters affect aerosol resuspension. A program currently is being established to determine the primary parameters of interest. Major efforts during 1973 were directed to the design of the test system based on theoretical consideration of the principal physical and chemical parameters of concern, a literature survey of previous resuspension studies, and discussions with persons currently involved in related programs. Current plans include the use of an existing wind tunnel that will provide air velocities up to 25 m/sec. Particle size distribution and concentration of the resuspended aerosol will be monitored with a Royco particle size analyzer. The system initially will be set up and calibrated using simple surfaces such as glass or metal with relatively large particles (10 to 20 μm) of glass or metal oxide. Subsequent tests will be conducted with more realistic surfaces (soil, grass, and other vegetation) and particles (such as PbO_2 and fly ash). The effect of mechanical agitation such as saltation and scraping will also be investigated. The major parameters of concern will include aerosol size distribution with special attention directed to the importance of variations in aerosol standard deviation, surface velocity, relative depth of the fluid

boundary layer and particle diameter, holding surface roughness, and contact time. Data obtained will help define the resuspension mechanism as a secondary source of pollutants. Potential applications would include input data to accident analysis for postulated reactor fires and SNAP reentries, evaluation and control of strip mining contaminants, and assessment of secondary pollutant sources from radioactive waste disposal, fossil fuel power plants, and industrial operations.

AEROSOL SIZE CHARACTERIZATION AND SAMPLING

(H. J. Ettinger, M. I. Tillery, L. McGuire, and L. Ortiz)

Aerosol Size Spectrometer Performance

Aerosol size spectrometers are valuable tools for many studies of small airborne particulates. Although there are several types of these instruments, the spinning spiral channel first developed by Stober¹ is the most useful and operates over the widest range of particle sizes. There are two general types of these instruments, one having a channel of constant height and the other a channel that expands in the direction of deposition along the length of the channel. Because of the shape of the channel, it has been impossible to determine theoretically the deposition points for specific particle sizes in these instruments, but this has not been a problem as the instruments provide excellent resolution and are easily calibrated by empirical methods. The design of new or special purpose instruments must be based on modifications of existing designs and involves a significant amount of extrapolation based on previous instrument designs. As the expanding channel is more expensive to fabricate, it is necessary to determine what advantages this design offers over the nonexpanding channel.

Because of the complicated shape of the spiral channels, a simpler model assuming a duct of rectangular cross section with the deposition surface located along the wall of a right circular cone was assumed to represent the spiral channel. The axis of rotation is taken as the axis of the cone. For a channel of constant height, the top of the channel is parallel to the deposition surface. For the expanding channel, the angle between the axis of

rotation and the top of the channel is smaller than the angle between the axis of rotation and the deposition surface. The trajectory of particles entering at the top of the channel near the axis of rotation can be determined by equating the centrifugal force driving the particles toward the deposition surface with the Stokes drag that the air exerts on the particles. By combining several channels having different angles with the axis of rotation, it is possible to create a model representative of aerosol spectrometers currently in use. The analytic expressions developed to describe the trajectory of particles in these models are:

For an expanding channel

$$dR = \left[\sin\theta + \frac{\rho C d^2 \omega^2 R b}{18 \eta Q} (h_0 + l \sin\phi - l \cos\phi \tan\theta) \right] dl$$

For the nonexpanding spiral

$$dR = \left[\sin\theta + \frac{\rho C d^2 \omega^2 R b}{18 \eta Q} h_0 \right] dl$$

where R = radial distance

l = distance along the deposition surface

h_0 = initial height of the channel

b = width of channel

η = coefficient of viscosity for air

Q = volumetric flow rate

ρ = density of particle

C = slip factor for particle

d = diameter of particle

ω = rotational velocity

θ = angle between collection surface and axis of rotation

ϕ = angle between top of channel and axis of rotation

As there are several simplifying assumptions made to arrive at these equations, the results will not be exact. These equations will be solved by computer and then compared with empirically derived calibration curves for the spectrometers to determine if the model is sufficiently representative. These equations do indicate that the expanding channel will require a greater length for deposition as the top of the nonexpanding channel drives the particles into a region of high centrifugal force. This suggests that the simpler (nonexpanding) design is preferable from both a cost and usefulness point of view.

REFERENCE

1. W. Stober and H. Flachsbarth, Environ. Sci. Technol. 3(12), 1280 (1969).

Air Sampling for Hazard Assessment

Air sampling in the vicinity of glove boxes should provide an accurate assessment of the potential hazard to workers in these areas in case of a containment system leak, as well as provide a continuing indication of proper operation of the glove boxes and associated control equipment. Optimum sampler location to provide these data must take into account air flow patterns in the room containing the glove boxes, probable location of workers in the work area, location of the most probable contaminant release points, and nature of the leaks (i.e., steady or pulsating, and particle size). To determine optimum sampling locations and to permit proper sampling data interpretation, the effect of room ventilation and worker location on sampling results is being measured in a specially constructed room that permits control of the ventilation rate and its orientation relative to the leak source. A dioctyl phthalate (DOP) tracer aerosol is used in conjunction with a forward light-scatter photometer to monitor relative contaminant aerosol concentration as a function of distance from the leak source, ventilation conditions, and time. These are then related to aerosol concentrations at the breathing zone of the worker and in those locations where static or personal samplers may be located.

Initial progress on this program included calibration of the ventilation flow equipment and characterization of the size distribution of the DOP tracer aerosol with respect to time and location within the room. These tests indicate that the tracer aerosol size distribution is constant as a function of time and sampler location, confirming that light-scatter photometer response is directly related to aerosol concentration. Preliminary data indicate variations of approximately one order of magnitude between aerosol concentrations in the breathing zone of the worker compared to those existing in typical static sampler locations. These data also suggest that personal samplers may overestimate the aerosol concentration breathed.

Electron Microscopy of Filter Samples

Membrane filters provide a simple method for efficiently sampling relatively large volumes of air for submicron particulates. Although analysis of these samples is generally directed toward measuring total activity or mass concentration, some particle size information can be obtained using conventional oil immersion optical microscopy. However, in many cases, the particles of primary interest are sub-micron particles which require use of an electron microscope. Techniques that have been used to examine these small particles include collection by electrostatic or thermal precipitators, replication of the surface of the filter, and transfer of the material to an electron microscope grid. Precipitators used to deposit particles directly on electron microscope grids usually sample at low flow rates (up to $17 \times 10^{-6} \text{ m}^3/\text{sec}$). Replication of the surface is a fairly lengthy process involving several opportunities to lose small particles, and the very small particles that penetrate into the filter matrix will not be resolved. Early techniques used for transferring the particles to electron microscope grids led to the loss of many small particles.

A new technique has been developed for transferring the sample to electron microscope grids. This technique is faster than previous methods and minimizes the loss of small particles. Using this

Fig. 1. Transfer of chrysotile asbestos from a Millipore-MF filter to an electron microscope grid.

Fig. 2. Transfer of fly ash from a Millipore-MF filter to an electron microscope grid.

technique, the membrane filter is fused with acetone vapor, shadowed with a heavy metal (chromium and carbon), and then dissolved with acetone while supported as a carbon-coated electron microscope grid. Particle losses are less than 10% and are not size-dependent. Figures 1 and 2 show typical electron micrographs from transfers of chrysotile asbestos and fly ash. This technique provides a means for obtaining particle size information from membrane filter samples for all sizes of particles.

This work is funded jointly by the AEC's Division of Biomedical and Environmental Research and the National Institute for Occupational Safety and Health.

PLUTONIUM AND ENVIRONMENTAL METALS IN MAN

(E. E. Campbell, J. F. McInroy, W. D. Moss, B. C. Eutsler, and H. F. Schulte)

Since 1957, selected tissues from deceased humans have been analyzed for plutonium by the Industrial Hygiene Group (H-5). These include persons whose potential exposures resulted from their occupations and a much larger group from the general public having no occupational exposures. Until 1970, almost all samples were from the Los Alamos area, but recently samples have also been obtained from several other areas. Some analyses have also been made for other metals, including uranium.

Plutonium in Occupationally Exposed Workers

Autopsy tissues from 60 former Laboratory employees have been analyzed for plutonium. Concentrations in the lung, pulmonary lymph nodes, liver, kidney, and bone were measured routinely. The cases were divided into a potential high exposure group including those employees who had the potential for direct exposure to plutonium in the working environment and a potential low exposure group of those who did not work directly with plutonium but had potential exposures through handling materials or equipment which may have been in a plutonium processing area. The data obtained were used to estimate both the whole-body distribution and total-body concentrations of plutonium and to assess the reliability of the plutonium body burden estimates based on urinary excretion data. The counting and analytical procedures have been reported elsewhere.¹

Work history records for each employee were reviewed to obtain such health physics information as plutonium contamination incidents, nose swipe activity measurements, working environment air monitoring results, and plutonium urine assay results. Table I lists the comparison of whole-body content estimated by tissue analysis and by urinalysis (PUQFUA)² for those employees having measurable body concentrations of plutonium. The extrapolations, based on the tissue plutonium content, used to estimate whole-body content were made using the measured concentration of plutonium in the organs analyzed and correcting for the aliquots of tissue solution used in the analyses. No assumptions have been made as to the concentration of plutonium in other tissues such as skeletal muscle, heart, and adipose tissue, which should increase the total estimated whole-body content of plutonium. In

TABLE I
COMPARISON OF THE ESTIMATES OF BODY PLUTONIUM CONTENT DERIVED FROM TISSUE AND URINE ANALYSES

Case	Date of Death	Time from First Exposure to Death (yr)	Date of Last Urine Sample	Number Urine Samples Analyzed	PUQFUA Body Burden (nCi)	Body Burden (Tissue Data) Whole-Body (nCi)	Systemic-Body (nCi)	Lung and Lymph Node (nCi)
1-039	1959	13	1958	71	24.6	21.0	18.3	2.60
2-030	1962	15	1961	58	10.0	8.8	4.2	4.50
2-064	1962	NA ^a	1958	1	0.0	0.05	0.02	0.02
2-068	1962	14	1954	1	0.0	0.01	0.005	0.01
2-100	1962	12	1961	13	2.8	0.28	0.27	0.01
2-126	1962	8	1962	23	2.4	0.46	0.0	0.45
2-130	1962	7	1962	18	3.2	1.20	0.95	0.25
3-014	1965	22	1955	1	0.0	5.15	5.15	0.0
3-016	1965	19	1965	4	3.4	0.87	0.68	0.19
3-028	1966	8	1966	7	0.0	0.0	0.0	0.0
3-088	1968	4	1964	1	0.0	0.0	0.0	0.0
5-064	1970	10	1969	8	0.23	0.01	0.0	0.01
5-076	1970	17	1964	16	0.22	0.05	0.04	0.01
5-080	1970	15	1957	4	0.16	0.02	0.01	0.01
5-150	1971	22	1970	14	2.7	0.01	0.0	0.01
7-006	1971	14	1970	12	0.11	0.01	0.01	0.0

^aNA = data not available.

the case of the skeleton, the extrapolation was more difficult as the distribution of plutonium in bone was known to be nonuniform and since only a small fraction of the system was available for analysis. The skeletal burdens were estimated using the ponderal method, as discussed by Durbin,³ which attempts to account for the variable skeletal mass and nonuniform plutonium distribution in the skeletal system. The equation used in this determination was:

$$Pu_{sk} = \frac{bw \times fsk \times fb_i \times Pu_i}{fr_i}$$

where Pu_{sk} = total skeleton plutonium

bw = body weight (kg)

fsk = fraction of total body weight contributed by the skeleton

fb_i = fraction of skeletal weight contributed by the individual bone

Pu_i = measured plutonium concentration in bone i

fr_i = fraction of the skeletal radionuclide contributed by bone i

The body weight and Pu_i are measured quantities. Data on fsk and fb_i are available from various studies and a limited number of dissections of the human body. Unfortunately, no information is available at this time on specific plutonium values for fr_i , the fraction of total skeletal plutonium concentration contributed by a given type of bone. For the purpose of the present calculations, values recommended by Durbin were used for skeletal distributions of radium-226 and strontium-90 in man. It was assumed that plutonium would follow the distribution of these bone-seeking radioisotopes. However, differences in distribution are known to exist for these isotopes, and we anticipate that these estimates can be improved as information on plutonium distribution in skeletal tissue becomes available.

The whole-body contents shown in Table 1 and estimated from tissue analysis data were divided into lung and lymph node concentrations and a "systemic" body concentration. This was necessary to distinguish between the internal distribution of plutonium and that part that was in the lung air spaces and, therefore, essentially external to the body (as regards urinary loss from the body but not from the standpoint of radiation dose). In theory,

the estimates of plutonium body burdens based on the urine data should be indicative of the former or systemic concentrations. However, this does not seem to hold true. Estimates based on urine data generally tend to overestimate the body burden calculated from tissue concentrations, and from the radiation protection point of view the urine estimates would result in conservative protective measures being initiated.

This program is undoubtedly the best source available to obtain data on the distribution patterns of plutonium in the human body from inhalation of airborne contamination. Intensive efforts currently are being made in this direction using autopsy tissue obtained from a long-time employee who was estimated to have a body burden of approximately 60 nCi of plutonium at time of death. Continued efforts will be made to obtain data and additional tissue specimens from this population to gain additional insight on the estimation of body burden from bioassay procedures.

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Plutonium in Tissues of the General Population

Scientific, industrial, and public interest in environmental concentrations of radioactive and non-radioactive pollutants has increased in the past few years. Knowledge of plutonium metabolism, distribution, and existing baseline concentrations in the general population is essential for many reasons. The proposed speed-up in design and installation of nuclear-fueled power plants of conventional and breeder types requires that the potential risk and effects of radioactive contamination of the general population be evaluated. As a starting point, baseline tissue concentrations of ubiquitous plutonium resulting from worldwide fallout following the

atmospheric testing of nuclear weapons must be determined.

Autopsy tissues from nonoccupationally exposed persons have been collected from areas in New Mexico, Colorado, Georgia, South Carolina, and New York. Analytical procedures for the analysis of plutonium in these tissues have been described previously, and the results have been documented.¹ Median concentrations of plutonium in tissues collected and analyzed prior to 1972 are shown in Table I. The

highest concentration of plutonium in order of decreasing levels was observed in pulmonary lymph nodes, liver, lung, kidney, and bone. The largest amount of plutonium in the body was deposited in the skeletal system, which is not surprising as it is known to be a bone-seeking radionuclide. The mode of intake for this population was primarily via inhalation of insoluble oxide forms of plutonium. Residence times of plutonium oxide in lung and the associated lymphatic tissue are very long, thus

TABLE I

50TH PERCENTILE DISTRIBUTION OF PLUTONIUM IN HUMAN TISSUE FOR THE NONOCCUPATIONALLY EXPOSED (1959-1971)

<u>Location</u>	Disintegrations per Second per Kilogram					
	<u>Lung</u>	<u>Liver</u>	<u>Lymph Nodes</u>	<u>Kidney</u>	<u>Vertebrae</u>	
Los Alamos	0.022 (57) ^a	0.018 (58)	0.083 (52)	0.002 (54)	0.007 (35)	b
New Mexico and United States	0.017 (76)	0.015 (73)	0.067 (66)	0.003 (66)	0.008 (41)	b
Colorado	0.008 (66)	0.028 (60)	0.033 (46)	0.023 (45)	0.015 (65)	0.020 (23)
New York	0.007 (26)	0.028 (26)	b	b	0.033 (25)	0.017 (26)
Savannah River	b	b	b	b	b	b
All populations	0.013 (217)	0.023 (217)	0.050 (164)	0.010 (163)	0.010 (156)	b

^aNumber of samples received.

^bSamples not requested.

TABLE II

50TH PERCENTILE DISTRIBUTION OF PLUTONIUM IN HUMAN TISSUE FOR THE NONOCCUPATIONALLY EXPOSED (1972-1973)

<u>Location</u>	Disintegrations per Second per Kilogram					
	<u>Lung</u>	<u>Liver</u>	<u>Lymph Nodes</u>	<u>Kidney</u>	<u>Vertebrae</u>	<u>Gonad</u>
Los Alamos	0.013 (8) ^a	0.027 (5)	0.583 (4)	0.003 (5)	0.027 (5)	b
New Mexico and United States	0.007 (17)	0.012 (10)	0.333 (15)	0.020 (10)	0.007 (16)	0.025 (9)
Colorado	0.012 (29)	0.030 (25)	0.250 (22)	0.050 (25)	0.018 (25)	0.023 (14)
New York ^c	0.005 (34)	0.023 (31)	b	b	0.012 (32)	0.015 (34)
Savannah River	0.007 (20)	0.020 (14)	0.667 (6)	0.036 (11)	0.012 (12)	b
All populations ^d	0.010 (74)	0.025 (54)	0.417 (47)	0.025 (51)	0.012 (58)	0.007 (30)

^aNumber of samples received.

^bSamples not requested.

^cNew York data for 1972 include all data analyzed.

^dAll population data for 1972 do not include the New York data analyzed during 1972.

resulting in the high concentrations reported in these tissues. The reticuloendothelial system of the liver undoubtedly filters colloidal or plutonium aggregates from the blood and lymphatic systems. Statisticians from LASL have estimated that 100 autopsy cases are required for a given variable such as geographic area, age, and sex to establish statistically the baseline concentrations. Analyses of an additional 15 to 25 autopsy cases/yr for the same variable would be sufficient to observe trends in changes of tissue concentrations.

Table II shows the concentrations of plutonium in tissues analyzed since publication of "Plutonium in Autopsy Tissue."¹ No statistical differences in lung, liver, vertebrae, and gonad concentrations were observed within each tissue type. Differences were observed within the lymph node and kidney concentrations. When compared with pre-1971 data (Table I), the lymph node concentrations increased from 5 to 7 times for the various geographic groups represented. Much of this can be attributed to improved techniques in excising the nodes from surrounding adnexal tissue. The average weight of all lymph node specimens¹ was approximately 20 g. The average weight of lymph nodes analyzed since that time was approximately 4 to 5 g. The presence of any adnexal tissue included in the analysis has resulted in reduction of the apparent plutonium concentration reported for that tissue. Therefore, the increased concentrations observed in 1972-1973 undoubtedly are more representative of the true concentration present in pulmonary lymph nodes of the general population. The kidney concentrations observed in the 1972-1973 data are higher in most cases by a factor of 2. No explanation for this difference is readily available at the present time.

Gonad concentrations from the Colorado specimens reported previously¹ have been revised. Replicate analyses of these tissues using larger aliquots taken from solutions saved following the first analysis indicate that gonadal concentrations are generally below our minimum detectable limits. The first results reported for these tissues used an internal spike of plutonium-242 as a tracer to determine the efficiency of plutonium recovery. All previous analyses had used plutonium-236 as the internal tracer. Plutonium-242 was judged superior

to the plutonium-236 tracer, since the lower energy portion of the plutonium-236 spectrum complicated evaluation of the lower energy isotopes of plutonium. Plutonium-242, having an alpha energy lower than either plutonium-239 or plutonium-238, did not interfere with the spectrometric analysis of these isotopes. However, the first samples of plutonium-242 obtained were contaminated with small amounts of plutonium-239 and plutonium-238. Approximately 1.85% and 2.0% of the total plutonium-242 alpha activity showed up in the plutonium-238 and the plutonium-239 channels, respectively. No correction was made at that time to subtract out this background contamination. Because the samples were spiked with a plutonium-242 activity of approximately 33.3×10^{-3} dis/s and counted for 60×10^3 sec, the contributions of plutonium-238 and plutonium-239 from the tracer would be approximately 0.67×10^{-3} sec⁻¹ for each isotope, assuming 100% recovery. The average weight of the gonads received was roughly 50 g, and a 10% aliquot of this was analyzed. This meant that, if only contaminated plutonium-239 were present, the 0.67×10^{-3} sec⁻¹ per aliquot observed when extrapolated to activity per sec/kg of tissue gave 0.133 sec⁻¹/kg of gonad as the minimum amount detected. This resulted in the 0.167 sec⁻¹/kg median value reported. Recalculation of these data, subtracting out the contamination and the analysis of a 50% aliquot of the solution remaining from each gonad sample, indicates a median concentration of ~ 0.02 sec⁻¹/kg for gonadal tissues from Colorado.

Current activities in this program have included a statistical evaluation of analytical data using the propagation of errors method, taking into account variations in chemical recovery, counting, backgrounds, and detector efficiencies. As suspected, chemical recovery is the greatest contributor of error in our present analytical system, and efforts currently are being directed toward reducing the variance from this function.

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Concentration of Plutonium Found in Human Lung and Liver Tissue Collated from the General Population

Data from the analysis of human lung and liver samples, expressed as activity/kg wet weight, were studied to determine correlation as a function of (1) year of death; (2) residence at time of death; (3) sex; and (4) age at death. Only data from cases received during the period 1969-1973 were used in this study. Those cases which were born after 1945 were excluded. The frequency distribution (Table 1) for each tissue type (130 lung and 113 liver samples) showed that the results were skewed to the right and were assumed to follow a log-normal distribution. The median concentration of plutonium per sec/kg of wet lung and liver tissue was found to be 0.015 and 0.026, respectively. The standard statistical t-test for differences in the means was calculated from the logarithms of the data to compare sets of results for the variables listed. No significant differences were found at the 1% confidence level for year of death, residence at time of death, or sex.

The lung and liver data sets were grouped according to age by pooling each 10-yr age span. When the median concentration for each age group was plotted vs the concentration of plutonium in

TABLE 1
MEDIAN CONCENTRATION OF PLUTONIUM IN HUMAN
LUNG AND LIVER TISSUE AS A FUNCTION OF AGE AT DEATH^a

Age	Number of Cases	Lung	Number of Cases	Liver
26-35	7	0.013	8	0.015
36-45	13	0.009	10	0.013
46-55	20	0.011	16	0.022
56-65	29	0.013	24	0.026
66-75	33	0.012	32	0.034
76-85	21	0.014	18	0.030
86-95	7	0.013	5	0.043
Significant slope hypothesis probability =		85%		99%
Calculated t-test value =		1.58		6.73

^aDis/g wet tissue.

Fig. 1. Median plutonium concentration per g wet lung and liver tissue vs age at death.

the lung and liver, the slope of the regression line for the lung was significant at the 0.15 level but not significant at the 0.1 level. The significance of the slope for the liver data is clearly seen from Fig. 1.

Plutonium Deposition in Human Bone: Fission Track Analysis

A preliminary investigation has been initiated to determine the possibility of using thermal neutron irradiation of bone sections mounted on polycarbonate plastic film to calculate the percentage of distribution of plutonium within human bone and bone marrow. This procedure, developed by Jee *et al.*,¹ has been used successfully for relatively high concentrations of plutonium in beagles and rats. Detailed neutron-induced autoradiographs produce both fission fragment tracks and a corresponding bone image, allowing one to identify the plutonium deposited on the bone surface or buried within the bone and that found in the bone marrow cells. Bone sections prepared from tissue taken at autopsy from a former LASL employee having an occupational exposure to plutonium have been irradiated at the Ames Laboratory Research Reactor (ALRR) and at the Massachusetts Institute of Technology Research Reactor (MITRR). The total neutron fluence on the first slides to be examined was approximately 2×10^{17} neutrons. At this exposure, a few fission tracks

were evident; however, it is estimated presently that, for the levels of plutonium observed in human bone, the neutron fluence will have to be on the order of 10^{20} neutrons to give images with sufficient tracks to allow identification of deposition sites.

Dog bone tissues have been irradiated at the LASL Omega West Reactor (OWR). Excellent results were obtained after a 30-h exposure in a thermal neutron field of $\sim 6 \times 10^{10}$ neutrons/cm²·s. Human bone slides have been prepared and will be irradiated for a longer period of time to obtain the higher fluence required. Attempts will be made to adapt the present equipment to enable these tissues to be placed closer to the reactor core in a higher thermal neutron flux to reduce the time required to obtain the exposure needed. The major problem in this modification of the procedure is maintenance of a temperature ($< 333^\circ\text{K}$) that will not damage the resulting track and bone images by annealing the polycarbonate film on which the tissue sections are mounted. Spark counting techniques² will be investigated as a means of determining the total plutonium present on each slide.

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Uranium in the Tissues of Occupationally Exposed Workers

The purpose of the uranium analysis program is to determine whether methods for evaluation and control of uranium exposures are achieving their objective. Tissues from several hundred persons have been analyzed for uranium, but only eight of these were from persons having an occupational exposure. The fluorometric method used in these analyses is not sufficiently sensitive to detect uranium at the concentration level prevailing in the general population. Small amounts of uranium were found in a few isolated tissues, but no patterns were detected

TABLE I
URANIUM CONCENTRATIONS IN THE TISSUES
OF OCCUPATIONALLY EXPOSED WORKERS

Case Number	Occupation	Uranium in Tissue (μg/kg)			Years of Exposure
		Lung	Kidney	PLN ^a	
1-54	Machinist-Foreman	0	-	240	6
1-58	Machinist	833 ^b	113	545 ^c	8
1-128	Technician	21	26	-	4
1-150	Technician	38	-	17	8
2-4	HP-Monitor	10	3	-	11
2-30	HP-Monitor	9	8	5	13
2-98	Physicist	18	-	25	16
7-16	Machinist	88	-	54	26

^aPLN = pulmonary lymph nodes.

^bIncludes pulmonary lymph nodes.

^cHilar lymph nodes.

that could link these results to a source of exposure.

Tissue analyses from the eight occupationally exposed workers are shown in Table I. The exposures in cases 1-54, 2-4, 2-30, and 2-98 were only incidental and should have been very light. In case 1-54, the presence of uranium in the lymph nodes rather than in the lungs may indicate a very early exposure before he became a foreman. Routine urinalyses for uranium had been made on only three cases. Too few data are available to draw any quantitative conclusions about the relationship between urine assay and tissue deposition at this time.

Other Isotope Screening (Diagnostic)

Each tissue is screened for gamma and x-ray activity. In addition to cesium-137 and potassium-40, a number of other isotopes have been identified including indium-113m, cobalt-60, iodine-131, and cesium-134. The tissue is screened for gamma activity upon receipt, while still frozen, and again after ashing in the prescribed manner. A standard sodium iodide detector and a phoswich (thin cesium iodide/sodium iodide) detector are used. Cesium-137 is normally near the detectable limit and varies significantly from case to case, as well as from organ to organ for a given case. Values as high as 600 pCi ¹³⁷Cs/g K have been observed.

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INTRODUCTION

The Los Alamos Scientific Laboratory's radioecology program was initiated in 1972 to supply information on the environmental impact of the Laboratory's radioactive liquid waste disposal practices. The scope and objectives of this program, including the design of specific research projects, appear in our first annual report to the AEC's Division of Biomedical and Environmental Research.¹ The purpose of this report is to summarize briefly 1973 progress on two research activities (namely, the environmental resources inventory and the radioecological studies). A detailed account of our research efforts will appear in the near future.²

ENVIRONMENTAL RESOURCES INVENTORY

(T. E. Hakonson and W. C. Hanson)

A preliminary description of the Laboratory environment, including a listing of some of the associated plants and animals, was presented elsewhere.¹ Current environmental resource data include a map (1:24 000 scale) of the vegetation types based on the dominant overstory species for a 310-km²

portion of Los Alamos County, including the 120 km² occupied by the LASL. Color infrared aerial photographs supplemented with field observations were used in identifying at least seven major vegetation types in the county. These types, from east to west, include juniper (Upper Sonoran life zone); pinon-juniper, ponderosa pine/pinon-juniper, ponderosa pine-fir (Transition life zone); and limber pine-fir, fir-aspen (Canadian life zone). In addition, an open shrub-grass type of variable species composition occurs mostly within the Transition zone.

Many of the woody and herbaceous species associated with the seven vegetation types have been identified; however, our herbarium contains nearly 350 individual plant species, attesting to the diversity of vegetation in the area and indicating the need for further work.

National Environmental Research Park Proposal

(W. C. Hanson)

A proposal to designate LASL properties as a National Environmental Research Park was drafted and presented to the LASL Director in November 1973.

The proposal would provide for a comprehensive policy of land use, coordinate LASL engineering and research projects, and foster complementary environmental investigations within LASL boundaries by other universities or agencies.

The designation of National Environmental Research Parks is a major new thrust of the U. S. Atomic Energy Commission. The principal objective of the Parks is to preserve representative parcels of land at each of the major AEC contractor installations within the United States for long-term studies of various ecosystems. Such Parks have been proposed previously at Savannah River, South Carolina, and at Oak Ridge, Tennessee.

The outstanding attributes of LASL that recommend its preservation and study as a National Environmental Research Park are (1) the impressive diversity of ecosystems contained within its Upper Sonoran, Transition, and Canadian life zones; (2) the three major canyons (Acid-Pueblo, DP-Los Alamos, and Mortandad) that have received liquid radioactive wastes, especially cesium-137 and plutonium, during three different periods of time over the past 30 yr; and (3) the recent designation of the Ecology Section within the Environmental Studies Group of the Health Division in July 1973. Radiation ecology studies at the LASL began in July 1972, some 20 yr later than those at several other USAEC installations, and have a challenging future.

A unique feature of the Park would be preservation for study of ecosystems that contain plutonium of various "environmental ages" in which studies of changing mobility of the element may be sustained. The principal reservoir of plutonium is within alluvial soils in the canyon ecosystems; therefore, characterization of soils and radionuclide distribution within the various soil fractions is an important consideration in their description.

Characterization of Soils

(J. W. Nyhan and F. R. Miera)

Many of the alluvial soils in the waste discharge areas exist in deep canyons which have formed in the Bandelier tuff since Pleistocene times. The original ash-flow tuff deposits, consisting of rhyolite ash and pumice with small amounts of quartz and sanidine crystals, have weathered to form the alluvial soils in the study areas.

Ten core samples were taken of the alluvium at each of 11 stations in Mortandad, DP-Los Alamos, and Acid-Pueblo Canyons during the period May-July 1973. These core samples were frozen, cut into four segments (0-2.5, 2.5-7.5, 7.5-12.5, and 12.5-30 cm), oven dried, and characterized as to their particle distribution, cation exchange capacity, and soil pH. More than 500 of these soil samples were mechanically separated into six size fractions: silt and clay (< 53 μ m diameter); very fine sand (53-105 μ m diameter); medium and fine sand (105-500 μ m diameter); coarse sand (500-1000 μ m diameter); very coarse sand (1000-2000 μ m diameter); and coarse fragments (2-23 mm diameter). Severely eroded and moderately eroded soil profiles in all canyons contained less than 1-2% silt and clay in the top 2.5 cm of soil and less than 3-4% silt and clay in the remainder of the profile. Severely eroded soils contained up to 70% coarse fragments, whereas the dominant size fraction (up to 46% by weight) in most moderately eroded soils was very coarse sand. Only four of the 33 stations contained soils with minimal water erosion; these soil profiles contained up to 54% silt and clay, with increasingly smaller amounts of larger sized fractions. They usually contained less than 10% coarse fragments (2-23 mm diameter).

The importance of adsorption and exchange of radioactive cations by the soil colloidal complex is well recognized; therefore, the cation exchange capacity of about 150 alluvial soil samples was determined. In general, increases in the cation exchange capacities of soil samples were correlated with increases in the smaller sized fractions, especially soil particles smaller than 53 μ m diameter. Thus, severely eroded, moderately eroded, and minimally eroded soil profiles usually demonstrated cation exchange capacities of 2-4, 4-10, and 11-21 milli-equivalents/100 g soil, respectively. Cation exchange capacity generally increased with soil depth, reflecting increased percentages of silt and clay beneath the 0-2.5 cm level in the profile.

Soil pH fluctuated as a function of distance above and below the waste outfall areas, partially due to the dumping of alkaline waste solutions. For example, values in Mortandad Canyon increased from about 5.7 at the pre-outfall station to a maximum value of 9.2 320 m below the outfall, and then decreased to near the pre-outfall values at stations

farther downstream from the outfall. Very little change in soil pH was found with depth in any soil profile.

Arrangements have been made for a formal soil survey of the LASL environs to be completed by U. S. Department of Agriculture Soil Conservation Service scientists during early 1974. This will provide a detailed data base for future radiation ecology studies and for engineering and waste management operations.

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RADIOECOLOGICAL STUDIES

Honeybees as Biological Indicators of Radionuclide Contamination

(T. E. Hakonson and K. V. Bostick)

Experimental results obtained during 1973 demonstrated that caged honeybees do not concentrate tritium (as H_2O) above the levels measured in a supplied food (i.e., $\frac{pCi\ ^3H/cm^3\ \text{bee moisture}}{pCi\ ^3H/cm^3\ \text{food moisture}} \leq 1$). Therefore, the bees which exhibited unexpectedly high tritium concentrations in body moisture during 1972¹ probably acquired the radionuclide from a source which was at least equally contaminated with tritium. The effluent liquid waste water in Acid-Pueblo and Mortandad Canyons did not contain sufficient tritium to account for the levels of the nuclide observed in bees from the respective canyon environs.¹ Conversely, tritium concentrations in DP Canyon water consistently exceeded the levels measured in a nearby bee colony. The fact that the bees were not equilibrating with the tritium in effluent water from any of the canyons indicated that another source of the radionuclide existed in the environment.

In addition to bees and effluent water, vegetation samples were collected routinely since May

1973, both in the canyons and on the adjacent mesas where bees actively gathered nectar and pollen, to determine the potential of flowering vegetation as a source of tritium to bees. Significant quantities of tritium are released in gaseous effluents from the Laboratory,² and it was anticipated that the vegetation might reflect these releases. In most cases, vegetation moisture samples contained tritium concentrations which were at least equivalent to the levels measured in bee samples collected at the same time and in the same general location.

An increase from about 35-3500 pCi $^3H/cm^3$ in Mortandad Canyon bees during October 1973 could not be attributed to a corresponding increase in tritium content of vegetation moisture in the general area around the canyon or to the liquid effluent entering the canyon. However, a large number of bees were observed in a stand of flowering white clover (*Melilotus albus*) in a fenced solid-waste burial ground about 600 m from the Mortandad bee colony. Clover samples from the burial ground were measured to contain as much as 10^6 pCi $^3H/cm^3$ water.

The feasibility of using honeybee colonies as bio-indicators of tritium in the environment will depend on several factors. First, the nature of the tritium release will determine the potential pathway(s) of the material to the insects. At Los Alamos, where tritium-contaminated liquid and gaseous effluents are both released to the environment, a gaseous effluent-vegetation-bee pathway is indicated by the data. Other pathways are possible (e.g., surface water-bee) and, under a given set of environmental conditions, may become primal.

Experiments with caged bees at Los Alamos demonstrated that the insects rapidly reflect changes in the tritium content of a food source in response to a measured biological half-time of the radionuclide in bees of about one day. Consequently, sampling intervals need to be relatively short in order to detect rapid fluctuations in tritium content of any source.

The local climate in the geographical area will determine the length of time that the bees will actively gather food materials. In Los Alamos, where we have a cool mountain climate, honeybees remain active for a 6-7 month period, depending upon weather conditions. The presence of flowering vegetation and natural or supplied surface water are

requisites for establishing a honeybee colony. The foraging activities of a bee colony may cover as much as 190 km², depending on the availability of food items.³ At Los Alamos, foraging bees have been observed in abundance nearly 2 km from the nearest hive.

Radiation Ecology Studies of Liquid Waste Disposal Areas

(T. E. Hakonson, W. C. Hanson, J. W. Nyhan, K. V. Bostick, and F. R. Miera)

The chronic input of low-level radioactive liquid waste into Mortandad Canyon over the last 10 yr has resulted in plutonium-238 and plutonium-239 concentrations which are 2-3 orders of magnitude higher than corresponding pre-outfall samples. Maximum concentrations in most samples occur within 160 m post-outfall and steadily decline with distance downstream. Concentrations approaching pre-outfall levels were measured at distances greater than 5120 m below the waste discharge point.

Highest concentrations (~300 pCi/g dry weight) of both plutonium isotopes were observed in sediments. The concentrations of plutonium in sediment core segments to sampling depths of 30 cm were relatively uniform from the effluent outfall to 1280 m post-outfall, where surface water exists at least part of the year. The plutonium in sediment samples beyond 1280 m, where the stream channel is dry, was increasingly concentrated in the top 2.5 cm.

There was an apparent distribution relationship between the cesium-137 concentrations in the 0-2.5 cm layer of post-outfall sediments and the corresponding data for plutonium-238 and plutonium-239. A linear regression of the log-transformed data provided correlation coefficients (*r*) of 0.97, *n* = 10 (¹³⁷Cs vs ²³⁸Pu), and 0.88, *n* = 10 (¹³⁷Cs vs ²³⁹Pu). Interpretation of this observation is not clear at this time but may indicate that distributive mechanisms for these two radionuclides may be similar in Mortandad Canyon sediments.

Maxima of 0.03 pCi ²³⁸Pu/ml and about 0.001 pCi ²³⁹Pu/ml were observed in surface water at the effluent outfall. Levels steadily decreased with distance downstream and approached analytical detection limits (0.05 pCi) for plutonium at the 5120-m post-outfall sampling station. The plutonium levels

in water averaged about 10⁻³ of the corresponding levels in sediment.

The concentrations of plutonium in vegetation followed a pattern with distance which was similar to that described for sediment and water. Highest concentrations (5 pCi/g wet weight) were observed in grasses near the effluent outfall, whereas shrubs and tree maxima were 1 pCi/g and 0.1 pCi/g, respectively. Concentrations in all plant types had decreased to pre-outfall levels (< 0.04 pCi/g) at the 5120-m post-outfall sampling station. In general, the lower growth forms (grasses) contained the highest plutonium concentrations. Grass-to-sediment activity ratios ($\frac{\text{pCi/g dry plant}}{\text{pCi/g dry sediment}}$) of 8×10^{-2} (plutonium-238) and 3×10^{-2} (plutonium-239) were relatively high compared to values of 10⁻⁶-10⁻³ reported for the root uptake of plutonium from plant-soil systems.⁴⁻⁷ However, in Mortandad Canyon it is possible that the plutonium was deposited externally on the grass samples and did not represent root uptake of the material.

Plutonium concentrations in the liver, lungs, hide, and carcass of rodents from Mortandad Canyon varied by as much as 10³ in samples from the same collection location. Mean plutonium concentrations were highest in the lung and hide samples, which suggested that resuspension of plutonium may have been an important mechanism of concentration. Post-outfall concentrations varied from about 8 pCi ²³⁸Pu/g wet weight in the lungs of one rodent to about 0.01 pCi/g in other tissues. Concentrations of plutonium in control rodent samples measured 0.01 pCi/g or less. Liver and carcass samples from the contaminated area averaged 10⁻¹-10⁻² of the plutonium concentrations in hide and lungs. There was no apparent relationship between plutonium concentrations in tissues of rodents and distance from the effluent outfall.

The ²³⁸Pu/²³⁹Pu activity ratios for the various sample types demonstrated that nearly all samples contained a preponderance of plutonium-238. Throughout most of the period since 1969, when significant quantities of plutonium-238 first appeared in Mortandad effluent, the activity ratio (²³⁸Pu/²³⁹Pu) in discharged effluent was about 4. Prior to 1969, nearly all plutonium discharged to Mortandad Canyon was plutonium-239. An activity ratio of about 4 was also observed in most sediment core segments, indicating that the relatively complete vertical mixing

of plutonium-238 has occurred within a three-yr period.

Plutonium data for alluvium samples from DP-Los Alamos and Acid-Pueblo Canyons are incomplete at this time, pending analyses of center and side channel samples. These and additional data for Mortandad Canyon will be presented in a later report.

In summary, stream channel sediments clearly are the major reservoir of waste plutonium. The degree of mixing of plutonium in the alluvial sediments of the canyon appears to be associated with the presence or absence of surface water. Grass-to-sediment plutonium concentration ratios of 0.03-0.08 were from 1-3 orders of magnitude higher than some values reported for the root uptake of plutonium in experimental plant-soil systems. The nature of the association of plutonium with vegetation is yet to be determined. Concentrations (pCi/g wet weight) of plutonium in rodents generally measured less than in vegetation; however, some lung and hide samples exceeded the levels observed in plants.

Cesium-137 in Soils of Waste Discharge Areas

(J. W. Nyhan and K. V. Bostick)

Cesium-137 determinations were performed on soil samples from various depths (0-2.5, 2.5-7.5, 7.5-12.5, and 12.5-30 cm) taken at the outfall station and at distances of 20, 40, 80, 160, 320, 640, 1280, 2560, and 5120 m downstream in all three waste discharge study areas (May-July 1973). In general, the maximum concentrations of cesium-137 occurred in the upper canyon areas, most of the cesium inventory having been deposited from 0-2560 m from the point of waste discharge. Maximum cesium-137 concentrations of 3258 and 1128 pCi/g dry soil occurred at 160 m in Mortandad Canyon (0-2.5 cm depth) and at 20 m in DP-Los Alamos Canyon (2.5-5.0 cm depth), respectively. Minimum concentrations of about 10 pCi/g were found in both canyons 5120 m from the outfall station. Very little cesium-137 was detected in Acid-Pueblo Canyon, most of the samples containing less than 2 pCi ¹³⁷Cs/g. A residual pocket of cesium-137 contamination was found 80 m below the outfall station, and the content of this soil sample was about 21 pCi ¹³⁷Cs/g (12.5-30 cm depth). Background levels in the general area were about 1 pCi ¹³⁷Cs/g.

Several interesting trends were observed in the distribution of cesium-137 with soil depth and in seasonal changes of soil cesium, as related to distance from the outfall stations in Mortandad and DP-Los Alamos Canyons. No significant changes in cesium concentrations as a function of soil depth were found at the outfall stations. However, in the upper portions of Mortandad (0-320 m) and DP-Los Alamos (0-80 m) Canyons, higher levels of cesium occurred near the soil surface (0-2.5 or 2.5-7.5 cm) than deeper in the soil profile. In the lower portions of Mortandad Canyon (1280-5120 m), there were significantly higher levels of cesium-137 at the 2.5-7.5 cm depth than elsewhere in the profile. Most soil samples from 160-5120 m below the outfall in DP-Los Alamos Canyon exhibited a general increase in cesium concentration with depth. The cesium-137 contents of soil samples from the study areas at two sampling times (October-November 1972 and May-June 1973) were compared with temporal changes in soil cesium. Increased levels were found in Mortandad (20, 80, 160, and 5120 m post-outfall) and in DP-Los Alamos (40, 80, and 5120 m post-outfall) Canyons, reflecting relocation and laboratory waste additions of cesium-137 in these canyons.

Cesium concentrations in soils were correlated with soil pH and cation exchange capacity in several sampling stations in Mortandad and DP-Los Alamos Canyons. As the pH of soil samples taken in lower Mortandad Canyon gradually dropped from 9.3 (at 640 m) to 7.9 (at 5120 m), the cesium-137 concentrations decreased from 833-1.1 pCi/g. The cesium-137 content of DP-Los Alamos Canyon soil samples (2.5-7.5 cm depth) decreased dramatically from 617-17 pCi/g, between 80-160 m below the outfall, as cation exchange capacity dropped from a maximum of 21-6 milliequivalents/100 g soil. Thus, the decreased cesium concentrations were related to relatively acidic conditions and a low cation-holding capacity which, in turn, was partially related to relatively low concentrations of soil particles less than 53 μ m in diameter.

Cesium-137 determinations were performed on all 10 soil samples taken at every depth at eight stations in Mortandad and DP-Los Alamos Canyons (0, 40, 640, and 2560 m post-outfall) to allow for

estimates of statistical variation in soil cesium. The results of a preliminary statistical analysis of these data indicated (1) significant differences in cesium levels as a function of soil depth at some sampling stations; (2) most of the cesium-137 data were either normally or log-normally distributed in the upper soil depths but probably log-normally distributed at the lower soil depths; and (3) an increased variation in cesium concentrations with increased sampling depth but not with distance from the outfall station. Two- to three-fold differences in cesium-137 concentrations were the most common, corresponding to coefficients of variation (CV) of 0.20-0.30. Increases in CV to a maximum of 1.18 (DP-Los Alamos Canyon, 40 m post-outfall, 12.5-30 cm depth) were observed frequently and are related to natural variations in the penetration depth of cesium into the soil profile.

Radiation Ecology Studies at Trinity Site

(T. E. Hakonson, W. C. Hanson, J. W. Nyhan, and K. V. Bostick)

A survey of the plutonium concentrations in soils, vegetation, and rodents was conducted during September 1972 and 1973 along the fallout pathway of the atomic bomb detonated at Trinity Site. Samples were collected from ground zero (GZ) and at 8-km increments out to a distance of about 56 km during 1972. Plutonium concentrations in samples along the fallout pathway were generally highest in the GZ area, were lowest about 24 km from GZ, and then generally increased in all sample types from 24-56 km. Plutonium levels in GZ soils were as much as 10^4 times higher than the 0.01-0.1 pCi $^{239}\text{Pu/g}$ which has been reported for several New Mexico area soils.⁸ A maximum of about 260 pCi $^{239}\text{Pu/g}$ was observed at GZ in both the 0-2.5 and 2.5-7.5 cm core segments. The maximum concentration in non-GZ soil (1.4 pCi $^{239}\text{Pu/g}$) was measured in the 0-2.5 cm segment from the 56.4-km sampling station. Olafson *et al.*⁹ also noted that the highest plutonium concentration in non-GZ soils, vegetation, and small mammals occurred about 45 km from GZ.

The vertical distribution of plutonium was relatively uniform to sampling depths of 7.5 cm in 30-cm core samples from GZ to the 24-km station. This suggested that plutonium which was initially deposited on the soil surface as much as 27 yr ago had

migrated at least 7.5 cm into the soil profile. On the other hand, plutonium in soils from distances greater than 24 km was increasingly concentrated in the upper 2.5 cm. Olafson *et al.*⁹ and Olafson and Larson¹⁰ reported that the plutonium deposition in Trinity area soils about 20 yr ago was almost exclusively confined to the top 2.5 cm of soil. These differences were elucidated during September 1973 by the hydrological experiments described below.

The plutonium concentrations in grasses were consistently higher than similar measurements in other areas of New Mexico,^{1,8} although plutonium concentrations in forb and shrub-tree samples were generally indistinguishable from worldwide fallout levels in New Mexico vegetation which measured about 0.001-0.005 pCi $^{239}\text{Pu/g}$ wet sample. The plutonium data for grasses as a function of distance from GZ generally followed the pattern which was observed for the 0-2.5 cm soil core segment. The maximum concentration in grasses (0.77 pCi $^{239}\text{Pu/g}$ wet sample) was observed at GZ and decreased to a minimum of 0.005 pCi $^{239}\text{Pu/g}$ at the 24-km station. Plutonium concentrations in grasses then generally increased with distance to the 56-km sampling station.

In general, rodent lungs had the highest mean plutonium concentrations of all small mammals sampled and exhibited a pattern with distance from GZ that was similar to the 0-2.5 cm layer of soil and grass. Lung deposition of plutonium suggested that resuspension of soil may be an important mechanism in the biological redistribution of plutonium. Other investigators have noted high lung concentrations in small free-roaming mammals.^{11,12}

The activity ratios ($^{239}\text{Pu}/^{238}\text{Pu}$) for the 0-2.5 and 2.5-7.5 cm core segments averaged 19 and 18, respectively, while the 7.5-30 cm segment averaged 9. Mean values were about 8-12 for vegetation and 0.5-2.0 for rodent tissues. The significance of the decreasing $^{239}\text{Pu}/^{238}\text{Pu}$ ratio from soils-to-vegetation-to-rodent tissues is not clear at this time but may indicate that plutonium-238 in the Trinity environs is more mobile than plutonium-239.

The results of this preliminary investigation indicated that the general pattern of plutonium distribution in soils, vegetation, and rodents as a function of distance from GZ was similar to findings of Leitch¹³ and Olafson.^{9,10} However, there has been an increased migration of plutonium into the

soils since the last measurements were made about 20 yr ago.

In September 1973, four permanent study sites were established in the Trinity Site environs to define plutonium contamination patterns in soils and biota and to identify plutonium transport pathways and mechanisms. Sites were chosen at 5.6 km south of GZ (control area); 1 km northeast of GZ, an area of high-level contamination; 16 km northeast of GZ, where an enhanced vertical migration of plutonium was observed during 1972; and at 50 km northeast of GZ, where the maximum concentrations in all sample types beyond GZ had been observed. The sampling techniques were designed to provide estimates of (1) distribution of plutonium within various soil size fractions and with depth and distance from GZ; (2) variability of plutonium concentrations in soils and vegetation; (3) variability in plant biomass; (4) water infiltration rates in various sites and soils; and (5) plutonium concentrations of ground-dwelling small mammals and passeriform birds.

Hydrological Characterization of Soils at the Trinity Site

(J. W. Nyhan)

The rate of water uptake by soils is one of the more important processes in the soil phase of the hydrologic cycle which is important in determining the distribution of radionuclides in the soil profile. The amount of water that will enter a soil in a given time depends upon the amount of water that the soil can store and the rate at which the excess water is transmitted through moist soil to the wetting front in contact with the dry soil below. Soil properties governing water uptake rates are inherent in the genetic composition of the soil profile such as the existence of soil layers with various particle size distributions, surface crusts, and soil structure.

Four determinations of water uptake rates were performed at each of the three intensive study areas located about 1, 16, and 48 km from GZ of the Trinity Site. The average water uptake rates of these three sites were found to be 1.83, 3.20, and 1.53 mm/sec with CV of 0.19, 0.19, and 0.08, respectively. A comparison was made of the plutonium concentrations (plutonium-239 plus plutonium-238) in soil samples taken close to these sites in 1972 with these data.

The soils of the 16-km site, which had a rapid water uptake rate (3.2 mm/sec), exhibited a 50% increase in concentration of plutonium at the 0-2.5 cm depth (0.31 pCi/g) over that found at the 7.5-30 cm depth (0.21 pCi/g). Plutonium concentrations at the 0-2.5 cm depth (0.28 pCi/g) were 28 times higher than those at the 7.5-30 cm depth (0.01 pCi/g) in the 48-km station soils, where the lowest infiltration rate was observed.

Four soil cores to 60-cm depth were collected at each study area for field capacity determination of the soils at various depth. Each soil profile received a measured addition of water, and the amount of water held in the soil after excess gravitational water had drained away and the rate at which downward movement of water decreased were determined. These data will be included in a future report.

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INTRODUCTION

The objective of this program is to observe and interrelate radiation effects on biologically important systems by progressing systematically from models assembled from molecular species of known structure with the closest possible control of conditions to corresponding systems of increasing complexity represented by subcellular fractions, intact cells in culture, and finally whole animals. These studies represent the cooperative efforts of investigators with diverse scientific interests and backgrounds. As a consequence, the attack on problems is at times unconventional and on occasion unique. Although progress in this direction has developed over a period of years, in February 1973 the Cellular and Molecular Radiobiology Sections were re-organized to form a group which has extended further the formal associations, with some changes in program emphasis.

Within the framework of radiation effects studies on DNA model systems, identification of products of radiolysis is being pursued more vigorously, while organic synthesis and enzymatic amplification continue as do studies of radiation effects on transcription and translation fidelity and bacterial virus induction. Cycle-dependent, radiation-

induced, metabolic effects have been augmented by techniques for mass synchrony and rapid cell-cycle analysis. Clear effects on small RNA metabolism, histone phosphorylation but not methylation and acetylation, and deoxynucleotide triphosphate pool kinetics have been shown to occur following exposure to biologically significant radiation doses. These are subtle but significant effects not readily demonstrable by conventional analyses. We anticipate that these studies will be expanded appreciably as the availability of a dedicated biomedical channel producing an intense beam of negative pions approaches at the LAMPF.

Interaction with theorists is reflected by contribution to the studies of radiation effects on DNA-like polymers and precursors in solution and to problems of immune response. The latter studies currently do not have an experimental counterpart within the group, but the intention is to initiate a program of radiation effects on the immune response in the near future.

Collaborative studies between biologists and biophysicists continue to exploit the enormous advantages afforded by unique flow microfluorometric (FMF) instrumentation developed by the Biophysics Group (H-10). Interspersed through the detailed

reports which follow are numerous examples of the remarkable precision with which cycle-dependent phenomena can be characterized by combining FMF techniques with biochemical and cytogenetic procedures. Although DNA content has been the general parameter of interest, the cell surface as well as the nucleus is of interest both from the standpoint of chemical composition and receptor properties.

In the past year, the Laboratory participated in a tutorial on cellular aging under the sponsorship of the National Institute of Child Health and Development. Basic cell culture and a variety of specialized techniques were offered to a class of 22 participants. The First Annual Life Sciences Symposium on "The Mammalian Cell: Probes and Problems" was concerned with modern instrumentation for cell analysis and identification of contemporary problems in cell biology amenable to investigation by new techniques. Summaries of participants at the tutorial and the symposium appear in the appendix.

STUDIES WITH MODEL DNA POLYMERS

Chemical Synthesis of Oligodeoxyribonucleotides

(D. L. Williams and E. L. Martinez)

As part of our continuing program, homooligodeoxyribonucleotides of any specific size are synthesized from mononucleotides and made available for radiation studies and fundamental biological investigations. Some of these polymers are isotopically labeled, and many are kept in stock at low temperature. Oligodeoxynucleotides of known base sequence are prepared by stepwise chemical condensation reactions¹ using functional blocking groups, where applicable. Protected dinucleotides are synthesized and are either chemically polymerized to obtain polydimers or are used in the synthesis of trimers¹ or longer oligonucleotides. Di- and trinucleotides of specific structure are useful models for the study of radiation effects at the molecular level in DNA. Chemical polymerization² of the trimers, followed by anion exchange chromatography, affords polytrimers in the size range from hexamer to dodecamer in addition to the larger unresolved polymers. Complementary oligonucleotide strands serve as templates for the generation of large, double-stranded, DNA-like polymers using DNA polymerase from *Escherichia coli*.³

In the stepwise chemical synthesis of oligonucleotides of known base sequence, if purity of sequence is to be maintained, the ideal protective group for phosphate should remain intact during the condensation and isolation steps and should be removable at the desired time without alteration of other parts of the product structure. Since the widely used 2-cyanoethyl group⁴ does not meet these requirements, a number of new protective groups for phosphate have appeared in recent literature. However, in no case has the degree of retention of a suggested group, under reaction conditions, been demonstrated directly. We have evaluated⁵ a number of these groups which appeared to have satisfactory removal conditions, together with a new group of our own design. The latter, 1,1,1-trichloro-2-methyl-2-propyl (trichloro-tert-butyl), has proven to be superior, in terms of retention, to all those evaluated, including a very recent group, the 2-phenylmercaptoethyl group.⁶ Although the trichloro-tert-butyl group is 97-99% retained under our test conditions, with either DCC or the sulfonyl chloride type condensing agents, application to mononucleotides is slow (35-70% yields), and removal from oligonucleotides is incomplete (about 90% from dimers and about 68% from trimers).⁷ In comparison, we have prepared the 2-phenylmercaptoethyl esters of d-pT and d-pA^{Bz} in quantitative yield, and removal of the group from dimers and trimers⁶ appears to be quantitative. Retention of the 2-phenylmercaptoethyl group on d-pT under our test conditions with triisopropylbenzenesulfonyl chloride was only fair, 58.2% as ϕ SEtpT and 12.6% as ϕ SEtpTpT, making the total retention about 71%.

In the synthesis of oligonucleotides, when two different nucleotide bases are used in preparing the initial dimer, loss of the phosphate protecting group may result in contamination of the desired sequence with protected homodimer. In actual practice, this has not proven to be the case; d- ϕ SEtpT-ⁱBu has been prepared in high yield (68%), and some ϕ SEtpT was recovered. Removal of the ϕ SEt group was quantitative, and upon degradation of the dinucleotide with venom diesterase the ratio of d-pT to d-pG was 1.01 [see section on "High Pressure Liquid Chromatography (HPLC) of Nucleic Acid Derivatives"].

Our immediate objectives in chemical synthesis are (1) preparation of ^3H -thymidylate oligonucleotides via polymerization of ^3H -5'-thymidylic acid; (2) preparation of three complementary trinucleotide pairs (namely, d-TGG:d-ACC, d-CCT:d-GGA, and d-TTC:d-GAA); and (3) chemical polymerization of each trinucleotide to afford repeating-trimer oligonucleotides of suitable size to serve as templates for DNA polymerase. To this end, we have prepared

$\text{Cl}_3\text{-}\underline{\text{tert}}\text{-BupT}$	Bz pA OAc
$\text{Cl}_3\text{-}\underline{\text{tert}}\text{-BupC}^{\text{An}}$	Ac pA OAc
$\text{Cl}_3\text{-}\underline{\text{tert}}\text{-Bupg}^{\text{iBu}}$	An pC OAc
ϕSEtpT	pTOAc
$\phi\text{SEtpA}^{\text{Bz}}$	BOC pC OBOC
$\text{Cl}_3\text{-}\underline{\text{tert}}\text{-BupTpG}^{\text{iBu}}$	CCT
$\text{Cl}_3\text{-}\underline{\text{tert}}\text{-BupG}^{\text{iBu Bz pA}}$	(TTC) _n
$\text{Cl}_3\text{-}\underline{\text{tert}}\text{-BupC}^{\text{An An pC}}$	
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High Pressure Liquid Chromatography of Nucleic Acid Derivatives

(G. T. Fritz)

Analyses of enzymatic digests of chemically synthesized oligonucleotides and synthetic polynucleotides have been performed, primarily at the nucleotide level, using high pressure liquid chromatography (HPLC).¹ The instrument used is a Picker Nuclear (now Varian Aerograph) Model LCS-1000 liquid chromatograph equipped with an 8- μ l. cell volume, 1-cm pathlength, and 254-nm ultraviolet flow cell. The 3-m x 1-mm inside diameter column used for nucleotide analyses is packed with a pellicular strong anion exchange resin, AS-Pellionex-SAX (H. Reeve Angel and Company). The column temperature is normally maintained at 50°C.

The following conditions are those usually used for analysis of nucleoside monophosphates. The eluent gradient is linear in concentration of potassium dihydrogen phosphate from 0.01-1.0 M. Both the low concentration and high concentration buffers are brought to pH 3.8 with phosphoric acid. Constant pH is used both to facilitate computation and to achieve greater accuracy. The mixing chamber initially contains 20 ml of starting buffer. Flow rates are 24 ml/h for the column and 12 ml/h for the concentrated buffer. Samples are injected in volumes of 1-10 μ l. at concentrations to give 0.1-10 nmoles of each species. These conditions give elution times of 5 min for d-CMP, 8.5 min for d-TMP, 10 min for d-AMP, and 14 min for d-GMP.

Nongradient elution, with 0.75 M potassium dihydrogen phosphate, pH 3.8 buffer, is used for the

separation of the mono-, di-, and triphosphates of a single nucleoside. For a flow rate of 12 ml/h, elution times for completely resolved peaks of the G series, the series which takes the longest to elute, are 6 min for GMP, 7.5 min for GDP, and 11 min for GTP.

Peak area measurements were made by the height times the width at half-height method. Base ratios were calculated using the peak areas and the molar absorptivities at 254 nm and pH 3.8 ($\epsilon_{254}^{pH 3.8}$). The following values were determined for $\epsilon_{254}^{pH 3.8}$: 4.22 x 10³ for d-CMP; 6.90 x 10³ for d-TMP, 14.2 x 10³ for d-AMP, 13.7 x 10³ for d-GMP, and 10.1 x 10³ for d-IMP.

The reproducibility of the method is shown in Table I. The runs were made on three separate days with the ambient temperature varying from 73°F (run 3) to 88°F (runs 5 and 6). A variation in room temperature would be expected to affect primarily the sampling procedure. For this set of experiments, the sample size was kept constant. Other experiments (Table II) have shown that the volume of sample taken is not a determining factor of the reproducibility.

It is not certain why the results are low for d-AMP and d-GMP in run 5 (Table I). However, the flow rate in this run was observed to be somewhat erratic, and it is possible that there was an undetected change in the rate sufficient to account for the low values.

Whenever possible, the peak for either d-AMP or d-TMP is used as the standard since these peaks are the most reproducible. The d-CMP peak, appearing early in the chromatogram, is a relatively sharp one and thus has an unfavorably small ratio of the width at half-height to the height. The d-GMP peak is frequently less reproducible than the others because changes in the baseline are often nonlinear in this region. It has been found that the EDTA-succinate buffer system used for spleen phosphodiesterase digestions interferes with the d-GMP peak under the usual conditions, thus necessitating a change in the procedure. Possible changes are delaying the start of the gradient, varying the amount of starting buffer in the mixing chamber, or otherwise changing the gradient concentrations. The set of conditions used must be chosen for the particular mixture to be analyzed to

TABLE I
CHROMATOGRAPHY OF DEOXYNUCLEOTIDES

Run Number	d-CMP	Relative Peak Area			d-TMP d-CMP	d-AMP d-CMP	Peak Area Ratios			
		d-TMP	d-AMP	d-GMP			d-GMP d-CMP	d-AMP d-TMP	d-GMP d-TMP	d-GMP d-AMP
1	5.20	8.04	14.17	17.95	1.55	2.73	3.45	1.76	2.23	1.27
2	5.39	7.87	14.12	18.04	1.45	2.62	3.35	1.81	2.31	1.28
3	5.40	7.87	14.15	17.40	1.46	2.62	3.22	1.80	2.21	1.23
4	5.39	7.74	14.16	17.50	1.44	2.63	3.25	1.83	2.26	1.24
5	5.45	7.71	13.61	16.59	1.41	2.50	3.04	1.77	2.15	1.22
6	5.56	8.09	14.20	17.42	1.46	2.55	3.13	1.76	2.15	1.23
Average	5.40	7.88	14.07	17.48	1.46	2.61	3.24	1.79	2.22	1.24
Standard Deviation	0.12	0.16	0.22	0.52	0.05	0.08	0.15	0.03	0.06	0.02
% Standard Deviation	2.2	2.0	1.6	3.0	3.4	3.1	4.6	1.7	2.7	1.6
Average ^a			14.16	17.66						
Standard Deviation ^a			0.03	0.31						
% Standard Deviation ^a			0.2	1.8						

^aWithout run 5.

TABLE II
REPRODUCIBILITY WITH CHANGE OF SAMPLE VOLUME

Run Number	Volume Taken (μl.)	Relative Peak Area	Peak Area (per μl.)
1	5	6.07	1.21
2	5	6.02	1.20
3	4	4.88	1.22
4	3	3.65	1.22
5	2	2.43	1.22

avoid non-Gaussian peaks, a problem of particular importance in chromatograms of d-IMP.

Some of the recent applications of the technique have been the analyses of venom phosphodiesterase digests of the chemically synthesized oligonucleotides d-pTpG and d-pGpA (see section on "Chemical Synthesis of Oligodeoxyribonucleotides"). For d-pTpG, the ratio of d-pG to d-pT was found to be 1.02 ± 0.01 ; for d-pGpA, the ratio of d-pG to d-pA was 1.07 ± 0.02 . Another study in progress is the detection of d-Ip in synthetic polynucleotides of the type d(CG)_nd(CG). Results have been reproducible with 3-10% of d-I relative to d-C in both venom and spleen phosphodiesterase digests.

In the course of analyzing the digests of chemically synthesized oligonucleotides containing d-T

and d-A, it was observed that d-TpT could not be separated from either d-pT or d-Tp. Apparently the effect of increased bulk of d-TpT exactly counteracts the effect of the reduced charge.

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Radiation Chemistry of Models for DNA

(F. N. Hayes, D. E. Hoard, E. C. Wilmoth, and V. E. Mitchell)

To establish the degree to which the various kinds of radiation-produced lesions occur in DNA, we have continued our investigation of the change in absorbance upon exposure to X-radiation produced in solutions of monomeric DNA constituents and of polydeoxynucleotides. A second aim has been to establish to what extent the various species of free radicals produced by the radiolysis of water contribute to damage under different experimental conditions. Most of the studies in the present year have dealt with solutions of thymine, d-TMP, and poly dT; we hope to be able to correlate their results with our earlier work,¹ in which we measured changes produced by irradiation in the T_m of single-stranded polymers

annealed with unirradiated complementary strands and changes produced by irradiation in their gel permeation chromatograms.

We have established that solutions of the free base thymine, a compound which has been employed extensively in the past as a model in investigations of radiation-produced DNA base damage, behave significantly differently when irradiated under oxic or anoxic conditions than do solutions of thymidine, d-TMP, or poly dT. Destruction of the ultraviolet absorption of thymine proceeds more rapidly in the presence of oxygen than in its absence; that of the other compounds mentioned occurs more rapidly when no oxygen is present. Plots of the surviving fraction of the ultraviolet absorption at 264 nm against radiation dose delivered to thymine solutions under aerobic conditions are linear; this implies that each attack of a thymine molecule by a hydroxyl free radical destroys it. Under inert atmospheres (pure argon or nitrogen), chromophore destruction follows an exponential relationship with dose. This latter condition implies that, on the average, more than a single radical attack will have occurred before the chromophore is destroyed. The dose-response for destruction of the chromophore of both thymidine and

d-TMP in solution (λ_{max} 267 nm) appears to be intermediate between purely linear and purely exponential when oxygen is present but is exponential in its absence. The heightened dose-response of thymidine, d-TMP, and poly dT solutions under anoxic conditions implies that reducing free-radical species (hydrogen atoms, hydrated electrons, or both) participate in destruction of the chromophore. Decreased destruction of the absorption of thymine solutions irradiated under anoxic conditions is explicable if, in this case, reducing radical species can give rise to restitution reactions that are more significant quantitatively than the reactions of reducing radicals which lead to chromophore destruction. The similarity in behavior of solutions of nucleoside, nucleotide, and polynucleotide suggested that a structural feature held in common (i.e., substitution of a glycosyl moiety for a hydrogen atom at position-1 of the thymine ring) was responsible. This inference was confirmed by observing the rate of destruction of the ultraviolet absorption of solutions of the model compound ethyl thymine-1-acetate irradiated under air and under inert atmosphere; chromophore destruction proceeded more rapidly with dose under the inert atmosphere.

TABLE I

SUMMARY OF EXPERIMENTAL CONDITIONS

Experiment Number	Solvent	pH	Atmosphere	Scavenger Concentration (μM)
1	Deionized water	6.7-7.2	Inert (Ar)	--
2	0.014 M phosphate	7.0	Inert (N_2)	$8.9 \times 10^3 \text{ H}_2\text{PO}_4^-$
3	$\text{C}_4\text{H}_9\text{OH}$ in deionized water	6.7-7.4	Inert (Ar)	$1 \times 10^6 \text{ C}_4\text{H}_9\text{OH}$
4	$\text{C}_4\text{H}_9\text{OH}$ in 0.014 M phosphate	7.0	Inert (N_2)	$1 \times 10^6 \text{ C}_4\text{H}_9\text{OH}$ $8.9 \times 10^3 \text{ H}_2\text{PO}_4^-$
5	Deionized water	6.4-6.8	Air	227 O_2
6a	0.014 M phosphate	7.0	Air	$8.9 \times 10^3 \text{ H}_2\text{PO}_4^-$ 227 O_2
6b	0.014 M phosphate	7.0	Oxygen	$8.9 \times 10^3 \text{ H}_2\text{PO}_4^-$ $1.1 \times 10^3 \text{ O}_2$
7	0.025 M nitrate in deionized water	6.7-7.2	Inert (Ar)	$2.5 \times 10^4 \text{ NO}_3^-$
8	0.025 M nitrate in 0.014 M phosphate	7.0	Inert (Ar)	$2.5 \times 10^4 \text{ NO}_3^-$ $8.9 \times 10^3 \text{ H}_2\text{PO}_4^-$

From the foregoing results, d-TMP appears to be a better model for assessing radiation-induced damage to DNA than is thymine. Experiments in which solutions of d-TMP at varied concentrations were irradiated with 25 000-rad doses of X rays have been performed, both with and without added free-radical scavengers present. Conditions of the individual experiments are summarized in Table I. Over the range of d-TMP concentrations studied ($C_0 = 18$ – $1800 \mu\text{M}$), plots of the concentration of d-TMP destroyed (C^*) vs initial concentration describe the curves similar in shape. In all cases, a rapid rise in C^* occurs as C_0 is increased from zero to about $400 \mu\text{M}$, after which the rate of increase in C^* with C_0 is sharply decreased. The initial rapid change in C^* is probably due to the damping out of free-radical recombination as solute concentration is increased.² The level of C^* at which the change in C^* with C_0 reaches a quasi plateau varies from experiment to experiment, reflecting the suppression of the contribution of one or more solvent free-radical species to chromophore destruction by the added free-radical scavenging compounds. The data have been subjected to mathematical analysis, taking into account published G values for the free radicals generated in the radiolysis of water³ and published second-order rate constants for the reactions of each radical species with the various scavenging compounds employed.^{4,5} A description of the mathematical analysis is given in the section on "Kinetics of Radiation Damage by Radicals."

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Kinetics of Radiation Damage by Radicals

[W. B. Goad (Theoretical Division)]

Owing to radiation-induced reactions, the composition of a solution under irradiation changes as the irradiation progresses. In particular, the reaction of solution components with radiation-induced free radicals transforms them into new components that react differently, if at all, with the radicals produced by further irradiation. In the experiments at hand, this can be a large effect for, in order to observe accurately the destruction of the thymine chromophore, it is necessary that a finite and often large fraction of the chromophore be destroyed. In such experiments, as the irradiation progresses there is substantial change in the lifetime of the free radicals produced, with resulting changes in the probability per unit dose that a remaining undamaged moiety suffers damage. We wish to account for this in extracting the reaction rate for damage from the experiments.

Let $C(D)$ be the concentration of undamaged chromophore after a dose D has been delivered and C_s be the concentration of radical scavenging compound(s) added in a particular experiment to manipulate the relative importance of the three water radicals. We assume, as is usually the case, that C_s is sufficiently large that it can be taken constant during the irradiation. Then we suppose that the lifetime of each radical species takes the form:

$$\tau_i = \frac{1}{(k_i^s C_s + k_i^* C(D) + k_i^\Delta C_0 + \beta_i)}$$

$$i = 1, 2, 3, (\text{H}, \text{OH}, \text{e}_{\text{aq}}^-).$$

k_i^* is the rate of the reaction in which the damage of interest is suffered (it is the primary objective of this analysis); k_i^Δ is the rate of all other reactions with the chromophore-bearing moiety, which is assumed unchanged by irradiation; k_i^s is the reaction rate to added quencher; and β_i is an intrinsic, or background, reaction rate. $C_0 = C(0)$ is the initial concentration. Then the destruction of the chromophore per unit dose is:

$$-dC/dt = C(g_1 k_1^* \tau_1 + g_2 k_2^* \tau_2 + g_3 k_3^* \tau_3)$$

where g_i is the number of radicals produced per unit dose per unit volume. Inserting the lifetimes (τ_i),

the differential equation can be integrated to give an equation for the concentration of undamaged material $C = C(D)$ at the end of the experiment:

$$C_0 - C = D(g_1 + g_2 + g_3) + A_1 \ln C/C_0 + A_2 \ln \left(\frac{C + B_1}{C_0 + B_1} \right) + A_3 \ln \left(\frac{C + B_2}{C_0 + B_2} \right) \quad (1)$$

The coefficients A_1 , A_2 , A_3 , B_1 , and B_2 are algebraic functions of the reaction rates and the values of g_1 . Equation (1) is a transcendental equation for C , which must be solved numerically for each case of interest. Its transcendental character stems essentially from the necessity of spanning continuously the range from linear to exponential kinetics.

Total reaction rates, k_1^S and $k_1^* + k_1^A$, are measured in pulse radiolysis experiments by observing the effect of added materials on the decay rate of bursts of radicals. Values for all of the needed total reaction rates are reported in the literature. Adopting them, we determine values of β_1 and k_1^* that fit the solutions of equation (1) to many experiments simultaneously (up to 70 or so) -- experiments with various values of C_0 and with various added scavengers. This is done utilizing a nonlinear least-squares search program that deals competently with limits on the region of search. For example, limiting it to positive values of all reaction rates.

Our experience to date makes it clear that to extract accurately k_1^* and to fully understand the phenomena involved, it is necessary to complete the analysis further, taking account of a number of additional reactions (between water radicals and the thymine-bearing moiety and between transient products of water radical reaction).

Ultraviolet-Induced Cross-Links between RNA Polymerase and DNA

(G. F. Strniste, A. M. Martinez, V. E. Mitchell, and D. A. Smith)

Last year we described the induction by ultraviolet light of cross-links between *Escherichia coli* RNA polymerase and $d(A-T)_n \cdot d(A-T)_n$.¹ These linkages were in stable to 2 M salt solutions, while non-irradiated complexes of RNA polymerase and $d(A-T)_n \cdot d(A-T)_n$ were completely dissociated in salt solution. The complex of enzyme and polymer could be isolated

by centrifugation to equilibrium in cesium chloride. Characterization of these complexes has continued. The stability of the complexes most likely is due to covalent linkages between the enzyme and polymer, as shown by its resistance to detergent, alkali, and heat treatment as well as high ionic strength. Formation of stable complexes was found to be a function of the incident ultraviolet dose, the input molar ratio of enzyme-to-polymer, and the components present in the irradiation solution.^{2,3} In the absence of mercaptoethanol in the irradiation solution, the yield of ultraviolet-induced stable complex was 60-110% greater than in its presence. This increase was also dependent on the molar ratio of enzyme-to-polymer. The reduction in complex formation in the presence of 2-mercaptoethanol might be due to the fact that mercaptoethanol absorbs light at 254 nm, the principal emission line from a germicidal lamp, thus reducing the ultraviolet dose to the sample. Furthermore, mercaptoethanol may compete with the active sites on the polymerase in the cross-linking process, since Smith⁴ showed that cysteine could compete with bovine serum albumin in the ultraviolet-induced cross-linking of this protein to DNA.

With $d(A-T)_n \cdot d(A-T)_n$, stable complex formation as a function of dose was rapid initially and then proceeded much more slowly. This biphasic response is evidently a function of the polymer used, since preliminary studies using $dA_n \cdot dT_n$ show linear, stable complex formation with dose over a range from 0-130 000 ergs/mm².

From density distribution profiles of ultraviolet-induced complexes in cesium chloride equilibrium gradients, an attempt was made to determine which subunit(s) of RNA polymerase was involved in cross-linking. After extensive detergent treatment to denature and dissociate all nonlinked protein, a significant fraction of the complex banded at a buoyant density of 1.58 g/cc (Fig. 1). Most likely this fraction contains only one linked polypeptide subunit of the multisubunit enzyme, and calculations⁵ indicated that this might be the α or σ subunit of the enzyme.

Experiments designed to investigate more directly which subunit(s) of the enzyme is linked to DNA are in progress. Radioactive RNA polymerase was purified⁶ from *E. coli* grown in the presence of

Fig. 1. Cesium chloride equilibrium analysis of detergent-treated, uv-induced stable complexes of *E. coli* RNA polymerase and ^{14}C -labeled $\text{d(A-T)}_n \cdot \text{d(A-T)}_n$.

Fig. 2. Chromatography on agarose A-5m of a mixture of ^3H -RNA polymerase and $\text{d(A-T)}_n \cdot \text{d(A-T)}_n$ which had been irradiated with ultraviolet light.

^3H -leucine. Purity of the enzyme was demonstrated by electrophoresis in sodium dodecyl sulfate polyacrylamide gels. The uv-induced stable complex between ^3H -RNA polymerase and $\text{d(A-T)}_n \cdot \text{d(A-T)}_n$ can be isolated by gel chromatography. Figure 2 shows the results of a preliminary run in which the high-salt stable complex is eluted from an agarose gel column ahead of uncomplexed RNA polymerase. Control runs of irradiated enzyme lack this early peak. In order to identify the subunits which are covalently bound to

Fig. 3. Chromatography on agarose A-5m of ^3H -RNA polymerase which had been dissociated in urea.

the polymerase, it will be necessary to isolate the various subunits. Attempts to separate the subunits are in progress. Figure 3 shows the results of a control run in which ^3H -RNA polymerase has been treated with 7 M urea and chromatographed on an agarose column equilibrated with urea. Subunits of the enzyme are partially resolved. Further resolution can be accomplished by chromatography on ion exchange columns. These experiments should lead to a definitive identification of the subunit(s) involved in covalent links with DNA and thus to a better understanding of the molecular morphology of RNA polymerase:DNA complexes.

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Effects of X Irradiation of DNA on Transcription

(D. A. Smith and A. M. Martinez)

Initiation of RNA synthesis *in vitro* and presumably *in vivo* normally takes place with a purine nucleoside triphosphate as the first nucleotide in the RNA chain.¹ Another nucleotide is added to the 3'-hydroxyl group of the purine nucleoside, and chain growth continues by this mechanism. *In vitro*, at least, oligoribonucleotides complementary to a DNA template can serve to initiate RNA chains and are covalently incorporated onto the 5'-end of the new RNA.² Last year we reported that, with a single-stranded DNA template, complementary oligodeoxyribonucleotides also could initiate RNA synthesis through covalent incorporation of the DNA oligomer onto the RNA chain.^{3,4} We have extended these studies to include double-stranded DNA templates and also have begun investigation of the effects of x irradiation on the mechanism of initiation of RNA synthesis *in vitro*.

The synthetic polymer $d(A-T)_n \cdot d(A-T)_n$ was prepared, and a fraction of 462 nucleotide units average length was isolated by gel chromatography. This fraction was used as a template for RNA polymerase in the presence of the substrates ATP and UTP, one of which was labeled with tritium. The reaction mixture was made 5% in trichloroacetic acid, and acid-insoluble material was collected and washed several times by resuspension. The final pellet was dissolved in 0.5 N NaOH and incubated at 37°C to hydrolyze the RNA product, $(A-U)_n \cdot (A-U)_n$. Any ribonucleotide covalently linked to $d(A-T)_n \cdot d(A-T)_n$ would remain attached after alkaline hydrolysis, while all the rest of the RNA is hydrolyzed under these conditions.

The hydrolysis reaction was then neutralized, and an aliquot was centrifuged to equilibrium in cesium chloride along with a marker of $d(A-T)_n \cdot d(A-T)_n$. Figure 1 shows the results of a reaction run with 3H -UTP. Tritium banded in the cesium chloride gradient at the same density as the

Fig. 1. Cesium chloride sedimentation equilibrium gradient analysis of $d(A-T)_n \cdot d(A-T)_n$ which had been incubated with ATP, 3H -UTP, and RNA polymerase.

$d(A-T)_n \cdot d(A-T)_n$ marker. However, in several reactions run with 3H -ATP as the labeled substrate, very little, if any, tritium was found associated with $d(A-T)_n \cdot d(A-T)_n$. These results show that RNA synthesis can be initiated *in vitro* by covalent attachment of the RNA to a double-stranded DNA template. Furthermore, they seem to indicate that initiation of $(A-U)_n \cdot (A-U)_n$ synthesis on $d(A-T)_n \cdot d(A-T)_n$ preferentially or entirely begins with addition of UMP to the template. The reason for this apparent specificity is not known.

X irradiation damages DNA in several ways, including the production of breaks in the DNA chain. Such damaged DNA is less able to direct RNA synthesis catalyzed by RNA polymerase. To extend the above experiments, we investigated the effect of x irradiation upon the mechanism of RNA chain initiation *in vitro*. High molecular-weight $d(A-T)_n \cdot d(A-T)_n$ was isolated and irradiated. The effect of x irradiation on the ability of the template to direct $(A-U)_n \cdot (A-U)_n$ synthesis was measured. The amount of RNA initiated (with 3H -UMP) on the DNA chain was also measured by determining the amount of tritium which was acid-insoluble after alkaline hydrolysis. Results of these experiments are shown in Fig. 2. Over the dose range of 0-80 krad, the total amount of RNA synthesized decreased to less than 30% of the unirradiated control. On the other hand, there was nearly a 50% increase in the amount of 3H -UMP

Fig. 2. Comparison of inhibition of total $d(A-U)_n$ - $d(A-U)_n$ synthesis and RNA chain initiation by template extension as a function of the x-irradiation dose given to $d(A-T)_n$ - $d(A-T)_n$.

covalently linked to $d(A-T)_n$ - $d(A-T)_n$. This indicates that at least some of the x-ray-induced DNA strand breaks can serve to initiate RNA synthesis. Thus, we have much less overall RNA synthesis on irradiated DNA but an increase in RNA chain initiation on the DNA. It is not yet known whether x irradiation leads to RNA initiation on DNA *in vivo*.

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MICROBIAL STUDIES

Derepression and Inactivation of a Bacterial-Virus Chromosome in the Lysogenic State

(B. J. Barnhart and S. H. Cox)

A bacterial virus which has become integrated into the chromosome of its bacterial host exists in a state of lysogeny and is termed a "prophage." Prophages are repressed from replicating autonomously but are replicated in harmony with the bacterial DNA-like neighboring genes of bacterial origin. Prophages may be derepressed or induced by physical and chemical agents which alter DNA structure or replication.

Relatively low doses of ultraviolet radiation (254 nm) bring about induction of prophages of *Haemophilus influenzae*¹ and *Escherichia coli*.² Our ongoing research on induction and replication³ of the prophage HPlcl of *H. influenzae* has led us to a consideration of the mechanism of derepression by ultraviolet radiation. We have correlated the frequency of induction in irradiated lysogenic populations of DNA-repair deficient (uvr^-) and repair efficient (uvr^+) bacteria with the frequency of pyrimidine dimers (i.e., ultraviolet photoproducts in the DNA) per prophage size segment of the bacterial chromosome (Fig. 1a). The Poisson probability distribution has been used similarly to correlate the inactivation of induced uvr^- lysogens with the dimer frequency (Figs. 1a and 1b). In Fig. 2 the inactivation slopes for ultraviolet-irradiated cell-free phage particles are compared with the inactivation of induced lysogens. Similarities between curves for phage assayed on uvr^- and uvr^+ bacteria and inactivation of the respective lysogens suggest that loss of the derepression response is due to lethal damage incurred by the prophage.

We conclude from these studies that, when one dimer occurs per prophage, induction results; two or more dimers within a prophage genome inactivate if the host bacterium lacks the excision-repair capability. If this DNA-repair capability is functional, then dimer frequencies of greater than one are not lethal. Nevertheless, one dimer per prophage brings about the derepression response. While DNA-repair neither prevents nor is necessary for prophage induction, it clearly rescues prophage from inactivation at elevated ultraviolet doses, resulting in up

Fig. 1. (a) Fraction of induced or derepressed lyso-genic *H. influenzae* in populations of repair-deficient BC100 (HPlcl) [$uvr^-(\phi)$] strains irradiated with various doses of ultraviolet light. (b) Data from the inactivation component of the $uvr^-(\phi)$ curve normalized to the maximum frequency of induced centers. The probability points are taken from a Poisson distribution table and are based on the average number of photodimers ($\hat{TT}$) per prophage size segment (20×10^6 daltons) of DNA in the bacterial chromosome.

Fig. 2. Comparison of inactivation of *Haemophilus* phage HPlcl irradiated with ultraviolet light and assayed for plaque-forming ability on BC200 uvr^+ and BC100 uvr^- cultures with the inactivation of the ability of irradiated lysogens, $uvr^+(\phi)$ and $uvr^-(\phi)$, to be induced and form plaques.

to 15 pyrimidine dimers within the prophage segment of DNA. Inactivation of the derepression response in either repair-deficient or repair-efficient lysogens apparently occurs when a second photodimer remains permanently fixed in the prophage genome.

Whether the molecular events triggering induction involve (a) the direct effect of ultraviolet photoproducts rendering ineffectual the repressor-operator complex on the prophage operon or (b) the production as a result of DNA damage of an inducing protein⁴ which either deactivates the repressor protein or competes with it are open to speculation. Inactivation of the derepression response perhaps can be attributed directly to the presence of an additional photodimer which precludes the necessary transcription and/or replication of the prophage complement of genes.

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The Development of Bacterial Virus HPlcl after Ultraviolet Induction

(B. J. Barnhart, R. T. Okinaka, and S. H. Cox)

The repair of ultraviolet-induced damage to DNA has been studied extensively in prokaryotic systems. The bacterium *Haemophilus influenzae* has been particularly suited to such investigations because it relies almost entirely on excision mechanisms to repair DNA.¹ A temperate phage, HPlcl, has facilitated these studies by providing a convenient assay system to measure repair of damaged DNA. Attempts to define the role that phage DNA synthesis may play in these repair processes have led to a more careful characterization of phage development itself.² The following experiments attempt to

describe some of the morphological events that occur following ultraviolet irradiation of *H. influenzae* cultures harboring the temperate bacteriophage HP1c1.

The intracellular production of phage and phage-like products produced in strain BC200 of *H. influenzae* lysogenic for HP1c1 was determined using three different techniques. Premature lysis growth curves indicated that the first phage appear between 40 and 45 min after ultraviolet treatment. Similar cultures of BC200 were fixed and embedded using standard electron microscopic techniques at appropriate times following ultraviolet irradiation. Thin-sections of cells fixed 60 min after ultraviolet treatment revealed the presence of significant

quantities of incomplete phage particles which lacked the intense staining property of DNA-filled phage heads (Fig. 1).

These results are also reflected in a small burst of viable phage and imply that the DNA packaging mechanism in HP1c1 is inefficient or aberrant. Phage growth and cell lysis data indicate that phage development precedes cell lysis by only a few minutes; this may provide a reasonable explanation for the presence of incomplete particles (i.e., the cells lyse before maturation is complete).

Isotopic labeling of phage-producing cells and analysis of the cell contents on sucrose gradients also confirm the presence of these incomplete

Fig. 1. Thin-sections of *H. influenzae* lysogenic for HP1c1 60 min after ultraviolet treatment. Strain BC200 cells were fixed in 2% OsO₄ 60 min after ultraviolet induction. Cells were then embedded in Vestopal W, sectioned, stained, and examined in a Phillips 200 electron microscope. Note that cell A is intact and contains one dense DNA-filled phage, while cell B is lysing and has lost much of its internal contents.

particles. Figure 2 represents a sucrose gradient profile of an experiment designed to determine if these incomplete particles could serve as precursors to viable phage. Phage-producing cultures were pulsed with ^{14}C -leucine and chased for different periods (as indicated in Fig. 2). These profiles indicate that the incomplete phage fraction (top of gradient) remains significantly larger than the phage peak even after the chase, indicating that the majority of the incomplete fraction remains either aberrant or unfinished. It can be noted also that some label is chased into the viable phage peak

Fig. 2. Distribution of label of phage structures accumulated in ultraviolet-induced cultures of BC200 (HPlcl). Cells were labeled with ^{14}C -leucine for 5 min beginning at 50 min after ultraviolet treatment. Samples were removed at 55 min (control, no chase), 60 min (5-min chase), and 65 min (10-min chase). The samples were lysed, DNase'd, and layered onto 5-25% sucrose gradients before centrifugation. The gradient profiles show two peaks. The top contains empty or incomplete particles, while the bottom contains viable phage; these were examined in an electron microscope.

(bottom of gradient) and suggests (inconclusively) that some incomplete particles may be converted to viable phage.³

The data presented indicate that, during normal HPlcl development, many incomplete phage particles are produced. This is in direct contrast to other phage systems (T4, lambda, P22) where normal phage development does not result in large quantities of incomplete particles. The possibility that this accumulation of structures is due to an inefficient timing mechanism between maturation and lysis is being investigated.

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CELLULAR METABOLISM

Modification of Deoxyribonucleic Acid

(A. G. Saponara and C. E. Hildebrand)

A substantive body of work from many laboratories suggests the possibility that DNA exists *in vivo* in intimate association with a hydrophobic material, perhaps membrane or protein. The contention is based, in part, on the isolation of DNA-containing fractions which have abnormal physical properties when compared with analytically pure DNA. These include a low buoyant density, an ability to complex with ionic detergents, a high affinity for nitrocellulose and methylated albumin kieselguhr, and a tendency to accumulate at organic-aqueous interphases such as phenol-water and chloroform-water. These properties have been variously interpreted as indicative of biologically important *in vivo* associations or as artifacts. Proponents of functional association have best stated their case in bacterial systems. Thus, DNA isolated from certain genetically defective induced lysogens, which are unable to replicate, behaves like pure DNA; however, the

induction of wild type lysogens results in phage DNA having the physical properties of an associated form.¹ There exists an implied disagreement among laboratories concerning the structural implications of their findings. Genetic markers mapping near the origin and terminus of the *Bacillus subtilis* chromosome are differentially isolated as complexes.² In this case, the ability to associate appears to be restricted to specified and fixed parts of the DNA. However, other studies have shown a preferential association of newly replicated DNA, a finding which suggests that all parts of the DNA are associated at one time or another.

Our studies with the mammalian cell line CHO have concentrated on the chemical characterization of associated DNA. GL-³H-Deoxycytidine has been used to label the DNA, since incorporation into both deoxycytidine and its modified derivative 5-methyldeoxycytidine can be followed simultaneously. Free and associated DNA were separated by precipitation of the sodium laurylsarcosinate binding fraction with Mg⁺⁺. By this criterion 30% of the DNA is associated. Prolonged DNase I treatment of the Mg⁺⁺ SPS precipitate solubilizes 90% of the radioactivity but leaves a resistant fraction which retains over 60% of the labeled 5-methyldeoxycytidine. This fraction contains less than 4% of the ³H-deoxycytidine incorporated into total DNA but approximately 40% of the ³H-5-methyldeoxycytidine. If we assume that protection from DNase I is mediated by association, then the data argue against random association of DNA at least for the entity we have isolated. It is possible, of course, that a family of complexes with varying dissociation constants exists and that we have isolated the more stable of these. In any event, our preliminary findings of enrichment in 5-methyldeoxycytidine implies that a substantial proportion of the modified bases of DNA is clustered. We are currently examining other methods of isolation of associated DNA to determine whether or not a high proportion of 5-methyldeoxycytidine is a general compositional feature of such DNA.

The deoxynucleoside analysis employed in these studies consists of phosphocellulose chromatography of enzymatic hydrolysates obtained with crude snake venom and alkaline phosphatase. The hydrolysis conditions have consistently yielded quantitative conversion to nucleosides or deoxynucleosides for all

preparations of purified RNA and DNA we have examined. However, the DNase I-resistant fraction was partially resistant to the venom digestion, as evidenced by the appearance of a small fraction of ³H-deoxycytidine label preceding thymidine which, by virtue of its lack of charge, is normally the first deoxynucleoside to elute. We are hopeful that further examination of this resistant component will lead to a better understanding of the requirements for DNA association.

Although there is general agreement for the presence of 5-methyldeoxycytidine in mammalian DNA, scattered reports of additional modified bases have not been widely accepted.³ Our ³H-deoxycytidine-labeled DNA preparations have consistently contained components chromatographing with and slightly following thymidine on phosphocellulose at pH 3.85. Paper chromatography of this material resolves four components which are clearly distinguishable from thymidine. A standard technique for DNA base compositional analysis involves degradation with strong acid at elevated temperature and resolution of the isolated free base. Since the sugar component is destroyed in the process, there is no guarantee that the original substance was, in fact, a deoxynucleoside. We are developing a sensitive technique which will allow the sugar component of a nucleoside or a deoxynucleoside to be identified and quantitated. A promising method for pyrimidine (deoxy)nucleosides involves degradation with anhydrous hydrazine.⁴ This liberates the sugar component(s) as a hydrazone which can be reacted with isotopically labeled 1-fluoro-2,4-dinitrobenzene to yield the labeled 2,4-dinitrophenylhydrazone of the sugar. Chromatographic solvents for the separation of the 2,4-dinitrophenylhydrazones of ribose, 2'-O-methylribose, and deoxyribose from one another and from unreacted 1-fluoro-2,4-dinitrobenzene are being screened.

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Modification of Ribonucleic Acids

(A. G. Saponara)

We have reported the presence of the rare nucleoside 3- γ -(α -amino- α -carboxypropyl)-uridine (ABA-4) in the transfer RNA of a mammalian cell line (CHO) growing in culture. The α -amino- α -carboxypropyl (or α -aminobutyrate) moiety is derived from the amino acid methionine as evidenced by incorporation of label from (1- 14 C), (2- 14 C), and (1,2,3,4- 14 C)-methionine. The nucleoside has properties which suggest that it may be the unidentified uridine derivative X found at position-47 or 48 in the variable loop of certain aminoacyl-tRNAs.¹ Particularly noteworthy is the finding of Friedman^{2,3} that *Escherichia coli* tRNAs known to contain X₄₈ are acylated by phenoxyacetoxy-succinimide. ABA-4 is also acylated by esters of N-hydroxysuccinimide. We attribute the reactivity to the presence of an aliphatic amino group, since unmodified polynucleotides are unaffected by the reagent. In those cases where the nucleotide sequences of X₄₈ containing tRNAs are known, its immediate neighbors are a 5' 7-methylguanosine (7 MeG) and a 3' cytidine. Since 7-methylguanosine bears a positive charge at physiological pH, the possible presence of an amphoteric amino acid side chain on an adjacent nucleoside takes on added interest. A molecular model of ABA-4 can be positioned near the quaternary nitrogen of 7 MeG, a favorable position for the formation of a salt bond between the two. Such ionic interaction would be favored by acylation of the amino of ABA-4, a condition which we find in about half the α -aminobutyrate uridines of CHO cells. Interactions of the variable loop may make an important contribution to the overall confirmation of tRNA. An invariant feature of all tRNAs sequenced to date is a base at position-15 (in the dihydrouridine loop) which is

the complement of a base in the variable loop (at position-48 or 49 in the class of tRNAs we are discussing). We further note that, when the variable loop contains the sequence 7 MeGpU (or X) pCp, the complementarity is more extensive, since the sequence terminating at position-15 is CpApGp in those cases. Crystallographic analysis of yeast phenylalanyl-tRNA⁴ shows that the variable loop sequence 7 MeGpUpCp lies near the CpApGp of the dihydrouridine loop. Further, Igo-Kemenes and Zachau⁵ have shown that the 7 MeG of yeast phenylalanyl-tRNA does not exhibit the reactivity toward NaBH₄ expected of free 7 MeG. Interaction with the dihydrouridine loop can be invoked in the protection mechanism, since the isolated 3' half of the molecule is reduced by NaBH₄.

We are currently attempting to determine whether X and ABA-4 are identical. If they are, we will make use of the specificity of (2- 14 C)-methionine labeling to determine if ABA-4 always resides adjacent to a 7 MeG. Our additional finding of an α -aminobutyrate derivative of pseudouridine as an exclusive constituent of 18S ribosomal RNA, which is the only cellular RNA aside from tRNA to contain 7 MeG, leaves open the possibility that α -aminobutyration always occurs on a nucleoside adjacent to 7 MeG.

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Sequential Biochemical Events in Preparation for DNA Replication and Mitosis

(L. R. Gurley, C. E. Hildebrand, P. M. Kraemer, R. L. Ratliff, R. A. Tobey, R. A. Walters, J. G. Valdez, and E. W. Campbell)

One of the primary effects of ionizing radiation on living cells is the arrest of cell proliferation.¹⁻⁷ However, the biochemical nature of this radiation-induced inhibition of cell division is unknown. If a rational basis is to be established for the radiation treatment of malignant diseases, an understanding of radiation effects on the regulatory mechanisms of cell division must be acquired. Since the regulation of mammalian cell proliferation is itself an ill-defined complex process consisting of a multiplicity of interacting biochemical reactions, it was necessary to characterize these regulatory mechanisms in unirradiated cells before attempting to elucidate the biochemical nature of radiation-induced division delay. Therefore, we have examined a number of events involved in cell proliferation utilizing a single cell line (line CHO Chinese hamster cells). This has allowed us to make direct comparisons between various processes involved in cell proliferation and to construct a temporal map of some of the important cell-cycle events thought to be involved in radiation damage.

Cell Synchronization Procedures. Studies of cell-cycle-specific processes were facilitated by use of synchronized cell populations. A variety of synchronizing techniques were employed to obtain maximum cell synchrony in each cell-cycle phase under study. Such cells were obtained by arresting cells in G_1 by transferring them to medium containing suboptimal quantities of isoleucine.⁸⁻¹⁰ Following restoration of isoleucine, the cells resumed cell-cycle traverse in synchrony, as illustrated in Fig. 1A. Biochemical events in S and G_2 were studied in cells synchronized at the G_1/S boundary. This was accomplished by releasing cells synchronized by isoleucine deprivation into medium containing hydroxyurea.¹¹ Following removal of hydroxyurea, these cells entered S phase immediately and synchronously commenced cell division 6 h later (Fig. 1B). In another procedure, cells released from G_1 -arrest by restoration of isoleucine were synchronized in mid-S phase by treatment with 20 mM

Fig. 1. Techniques employed to synchronize CHO cells: (A) fraction of cells synthesizing DNA (incorporating 3H -thymidine), continuous exposure) is represented by triangles and diamonds, while cell number increase is represented by circles and squares in cultures synchronized by isoleucine-deficiency and mitotic-selection techniques, respectively; and (B) fraction of cells synthesizing DNA (15-min pulse-labeling periods with 3H -thymidine) is represented by triangles and diamonds, and the fraction of cells dividing is represented by circles and squares in cultures synchronized by isoleucine-deficiency/hydroxyurea or mitotic-selection/hydroxyurea techniques, respectively. The divided fraction represents $N/N_0 - 1$.

thymidine.¹² Following removal of the excess thymidine, the cells traversed the S and G_2 phases in synchrony. The biochemical events in mitosis were studied in cells arrested in metaphase by Colcemid. Following removal of metaphase cells from monolayer by mitotic selection, these cells were resuspended in normal medium free of Colcemid. Such cells completed mitosis, divided, and entered G_1 in synchrony.¹³

Histone Phosphorylation. A direct correlation between the rate of cell proliferation and the extent of phosphorylation of histone f1 has recently been demonstrated in a variety of tumors.¹⁴ Our Laboratory has shown that x irradiation inhibits histone f1 phosphorylation at the same time that it inhibits cell division.¹⁵ Furthermore, we have shown that caffeine reduces both the x-ray-induced inhibition of cell division as well as the x-ray-induced inhibition of f1 phosphorylation.¹⁶ These

observations strongly suggest a role for histone phosphorylation in the x-ray inhibition of cell proliferation and has led us to investigate the details of histone phosphorylation in the CHO cell cycle.

CHO cultures were synchronized by isoleucine deprivation to obtain cultures in G_1 -arrest. Following restoration of isoleucine, cells were obtained traversing G_1 (Fig. 1A). Cells were also resynchronized by hydroxyurea treatment to produce cultures rich in S and G_2 cells (Fig. 1B). Histones were isolated from these four cultures,¹⁷⁻²² and histone phosphorylation was measured by preparative electrophoresis¹⁵ as shown in Fig. 2. Two histones

were found to be phosphorylated in these cells, f1 and f2a2. Histone f2a2 was phosphorylated in all stages of proliferating cells as well as in non-proliferating G_1 -arrested cells (Fig. 2). However, histone f1 phosphorylation was completely absent in nonproliferating G_1 -arrested cells and in cells traversing the early part of G_1 (Fig. 2). To determine the time at which f1 phosphorylation began during the transition from the nonproliferating to the proliferating state, the rate of f1 phosphorylation

Fig. 2. Preparative electrophoresis of histones isolated from cells arrested in G_1 by isoleucine deprivation, from cells traversing G_1 2 h after release from G_1 -arrest, from cells in S 2.5 h after release from hydroxyurea blockade, and from cells in G_2 6 h after release from hydroxyurea blockade. Cells were prelabeled with ^3H -lysine for three generations and pulse-labeled with ^{32}P for 1 h. (A) The lysine-rich histones and (B) the arginine-rich histones were subjected to electrophoresis in separate gels.

and synthesis was measured along with the rate of entry into S phase in synchronized cells following restoration of isoleucine (Fig. 3). Although the synthesis of f1 commenced at the time that DNA replication began (Fig. 3B), f1 phosphorylation preceded entry into the S phase by 2 h (Fig. 3A). Thus, phosphorylation of old, preexistent f1 begins significantly before the cells start to replicate DNA. The phosphorylation of both f1 and f2a2 increased in G_2 over the rates observed in S phase (Fig. 2),

Fig. 3. Rates of phosphorylation (A) and synthesis (B) of histone f1 in CHO cells following reversal of G_1 -arrest. The fraction of cells synthesizing DNA (incorporating 3H -thymidine during 1-h pulse-labeling period) is indicated by circles. Rates of phosphorylation of f1 (squares) and synthesis of f1 (triangles) were determined by measuring the amounts of ^{32}P and 3H -lysine incorporated into electrophoretically pure f1 during a 1-h pulse-labeling period.

indicating that histone phosphorylation is not strictly an S-phase phenomenon.

Interesting changes in histone phosphorylation were also observed as cells enter and exit from mitosis (Fig. 4). In these studies, monolayer cultures were exposed to Colcemid and ^{32}P for 2 h, after which the accumulated metaphase cells were dislodged by mitotic selection to isolate only those cells which were labeled with ^{32}P during the transition from G_2 to M ($G_2 \rightarrow M$, Fig. 4). It was found that, in addition to the phosphorylated f1 and f2a2 normally seen in S and G_2 cells, there were two additional phosphorylated histones, f3 and a slowly migrating subfraction of f1 (which we shall call f1_m). No phosphorylation of f1 or f1_m was observed in the interphase cells left behind in the monolayer following mitotic selection (interphase, Fig. 4), indicating that the f3 and f1_m phosphorylation events were not Colcemid artifacts. When cells were labeled with ^{32}P after they were already in metaphase, the phosphorylation of f3 and f1_m was greatly diminished (M-rich, Fig. 4). When cells labeled with ^{32}P during the $G_2 \rightarrow M$ transition were released from Colcemid arrest and allowed to traverse from metaphase into G_1 ($M \rightarrow G_1$, Fig. 4), the ^{32}P incorporated into f3 and f1_m was rapidly lost. These results are interpreted as strong evidence that the phosphorylation of f3 and f1_m plays a role in mitosis.

From these data we may obtain a view of the relationship between histone phosphorylation and the CHO cell cycle (Fig. 5). The phosphorylation of old f1 prior to initiation of DNA synthesis may represent one of the earliest biochemical events indicating that a cell has embarked upon a program of proliferation.

There are reasons to suspect that the phosphorylation of f1 occurring from late G_1 through mitosis may result in continuous structural alterations in chromatin throughout interphase, culminating in chromosome condensation as cells enter mitosis. In the section that follows it will be shown that an enhanced association of DNA with a lipoprotein complex commences approximately 2 h prior to entry into the S phase and persists at a high level into G_2 . The timing of this enhanced association between DNA and lipoprotein is remarkably similar to the pattern of histone f1

Fig. 4. Preparative electrophoresis of histones isolated from cells traversing from G_2 into mitosis ($G_2 \rightarrow M$), from cells in metaphase (M-rich), from cells traversing from mitosis into G_1 ($M \rightarrow G_1$), and in interphase cells attached to glass in monolayer (interphase). Cells were prelabeled with 3H -lysine for three generations and pulse-labeled with ^{32}P for 2 h. (A) Lysine-rich histones and (B) arginine-rich histones were subjected to electrophoresis in separate gels.

phosphorylation, suggesting that the two processes may be linked. The involvement of histone f1 and f3 phosphorylation in mitosis (Fig. 5) suggests that we investigate next the radiosensitivity of these particular events.

DNA-Lipoprotein Complexes. Complexes of DNA associated with lipoprotein can be isolated from cells by their interaction with the detergent sodium lauroyl sarcosinate crystallized with $MgCl_2$.²³⁻²⁵ In view of the possible involvement of such DNA-

lipoprotein complexes with some aspect of genome replication, cell-cycle studies were initiated. Cells whose DNA had been prelabeled with ^{14}C -thymidine were resuspended in label-free medium following synchronization by mitotic selection, and the amount of old DNA associated with lipoprotein was determined as the cells traversed early interphase (Fig. 6A). Similar measurements were also made as the cells traversed late interphase following synchronization of cells by a combination of

Fig. 5. Relationship of histone phosphorylation to the cell cycle of line CHO Chinese hamster cells. The 16.5-h generation time of these cells in F-10 medium may be divided into a 9-h G_1 , 4-h S, 3-h G_2 , and 0.5-h M.¹⁰ The periods in which histones f1, f2a2, and f3 were phosphorylated are indicated by the shaded bands. F1_i denotes f1 phosphorylation observed in interphase. F1_m denotes phosphorylation observed in the slower migrating f1 subfraction of cells entering mitosis (Fig. 4).

mitotic selection and hydroxyurea treatment (Fig. 6B). These data indicate that a small amount of DNA is complexed to lipoprotein during mitosis and early G_1 . However, a sudden increased association commenced at approximately 2 h prior to initiation of DNA replication (Fig. 6A). In hydroxyurea blockade, when cells are accumulated at the G_1/S boundary, the amount of old DNA complexed to lipoprotein was already at a high level even though DNA replication had not yet begun (Fig. 6B). After removal of hydroxyurea, there was a further enhancement of DNA associated with lipoprotein which persisted through S into G_2 and then decreased as the cells began to divide.

It is concluded that the relationship between DNA and lipoprotein undergoes two major transitions: an increased interaction at 2 h prior to initiation of DNA replication and a decreased interaction at mitosis. The concomitant occurrence of histone f1 phosphorylation (Fig. 3) and enhanced association between DNA and lipoprotein (Fig. 6) suggests that we investigate the possibility that these two processes may be linked and are, therefore, radio-sensitive.

Fig. 6. DNA-Lipoprotein complexes in synchronized CHO cells: (A) following mitotic selection, the amount of old prelabeled DNA associated with lipoprotein in sarkosyl crystals was determined (circles), and the fraction of cells synthesizing DNA (incorporating 3H -thymidine) (squares) was measured in a non-prelabeled culture synchronized in parallel; and (B) following mitotic-selection/hydroxyurea treatment, the amount of old pre-labeled DNA associated with lipoprotein was determined as in (A) (circles) with the fraction of cells synthesizing DNA (labeled with 3H -thymidine) (squares) and divided fraction (diamonds) determined from a non-prelabeled culture synchronized in parallel. The divided fraction represents $N/N_0 - 1$.

Deoxyribonucleoside Triphosphate Pools. Before cells can initiate DNA synthesis, they must have an adequate supply of deoxyribonucleoside triphosphates. Therefore, the levels of all four of these DNA precursors in the acid-soluble pool were measured in cells synchronized by mitotic selection. The analytical techniques employed were similar to those of Skoog²⁶ and Lindberg and Skoog,²⁷ modified to use DNA polymerase isolated from *Micrococcus luteus* and α - ^{32}P -labeled deoxyribonucleoside triphosphates. Results showed that the levels of dTTP, dATP, and dGTP were all maximal during mitosis but decreased dramatically at the time the cells completed mitosis (Fig. 7A). The level of dCTP was low during mitosis but decreased even more upon entry into G_1 (Fig. 7A). During G_1 , the levels of all four of these nucleotides were extremely low, but 0.5 h prior to S phase all four species began to increase in preparation

Fig. 7. Levels of deoxyribonucleotides in acid-soluble pools in CHO cells during escape from mitosis (A) and during early interphase (B): dTTP (squares), dCTP (inverted triangles), dATP (circles), dGTP (diamonds), and fraction synthesizing DNA (incorporating thymidine) (triangles). Cells were synchronized by mitotic selection.

for DNA replication (Fig. 7B). When synchronized cells traversing G_1 were prevented from entering S phase by hydroxyurea blockade, a similar pattern was observed with one important difference: the level of dATP failed to increase (Fig. 8A). Within 3 min after removal of hydroxyurea, the level of dATP increased nine-fold, and concomitantly DNA replication was initiated (Fig. 8B). Thus, it is concluded that hydroxyurea specifically prevents accumulation of dATP in CHO cells, thereby inhibiting initiation of DNA replication.

Following the initial perturbations of release from hydroxyurea blockade, the levels of dTTP, dGTP, and dATP increased through S, G_2 , and M, whereas the dCTP content increased across S phase and then decreased as cells entered G_2 (Fig. 8B). Thus, it is concluded that only the level of the dCTP pool is roughly correlated with DNA replication.

Cell Surface Heparan Sulfate. Heparan sulfate is an important cell component found in a variety of different cells^{28,29} and appears to be integrated into a metabolically active cell surface fraction. The content of heparan sulfate was consequently examined at different stages of the cell cycle in cultures of CHO cells synchronized by the combined isoleucine-deficiency technique and the reversible inhibition of DNA synthesis with excess thymidine. Heparan sulfate was isolated by a combination of molecular sieve and DEAE-cellulose chromatography,

Fig. 8. Levels of deoxyribonucleotides in acid-soluble pools in CHO cells traversing G_1 in the presence of hydroxyurea (A) and following removal of hydroxyurea (B). The symbols are the same as in Fig. 7.

and the amount present was then determined by assaying the uronic acid residues.³⁰ Results revealed a significant loss of heparan sulfate from the cell surface at approximately the time that cells entered mitosis (Fig. 9). The quantity of cell sap heparan sulfate (not shown) did not decrease during this period of loss from the surface, and the rate of synthesis and transport of heparan sulfate to the cell surface remained unaltered during this portion of the cell cycle. The level of another cell surface constituent, the surface glycopeptides, also did not change significantly during late interphase. Thus, it is concluded that the loss of heparan sulfate from the cell surface is not merely part of a general premitotic loss of cell surface constituents but instead represents a specific cell surface biochemical change as the cells prepare for mitosis.

Temporal Sequence of Biochemical Events in the CHO Cell Cycle. By combining the results presented above with data from previous studies carried out in this Laboratory, it is possible to construct a map illustrating the temporal arrangement of biochemical events occurring during the CHO cell cycle (Fig. 10). The earliest indication that a cell is preparing for DNA replication occurs in G_1 phase, 2 h prior to initiation of DNA synthesis, when phosphorylation

Fig. 9. Mass of surface heparan sulfate in CHO cells synchronized by the isoleucine deficiency/thymidine blockade. The mass surface heparan sulfate is represented by triangles, while the fraction of cells in S phase and the divided cell fraction are represented by circles and squares, respectively.

Fig. 10. Temporal sequence of biochemical events in the CHO cell cycle.

of histone f1 occurs and DNA exhibits an enhanced association with lipoprotein. Approximately 30 min prior to entry into the S phase, the pool of all four deoxyribonucleoside triphosphates increases in size. During the S phase, DNA and histone synthesis occur.¹⁷ With the termination of DNA synthesis, only the pool of dCTP decreases. The pool of dATP, dGTP, and dTTP, as well as the phosphorylation of histone f1, remains elevated.

As cells continue progression through late interphase, preparations are made for mitosis and cell division. Approximately 70 min prior to entry into mitosis, the final species of RNA essential for completion of mitosis is synthesized,³¹ while 15 min prior to prophase the last division-essential protein is synthesized.^{2,32} During the premitotic period, the association of DNA and lipoprotein is reduced. A loss of heparan sulfate from the cell surface also occurs, which may result in an uncovering or activation of other cell surface entities and may represent a mechanism by which expression of other cell surface entities are regulated. At the time cells complete G₂ and traverse prophase, the chromatin of the cell is condensed into chromosomes. At precisely this time the phosphorylation of histones f3 and f1_m occurs. It is speculated that these phosphorylation events "trigger" chromosome condensation and represent the final interphase operation, terminating in prophase. We further suggest that the phosphorylation of histone f3 during this final stage of G₂ may alter f3 conformation, resulting in disulfide bond formation observed in f3 in metaphase chromosomes.³³

Upon cell division and entry into G₁, the levels of the deoxyribonucleoside triphosphates drop essentially to zero. Histones f3 and f1_m are rapidly dephosphorylated, and the chromosomes disperse, thus completing the cell cycle. With this biochemical map of the cell cycle, we may now begin an attempt to elucidate the biochemical nature of radiation-induced division delay.

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Effects of Physical and Chemical Agents upon Chromatin and the Mitotic Apparatus

(G. R. Shepherd, B. J. Noland, and J. M. Hardin)

Many physical and chemical mutagenic and oncogenic agents react, either directly or indirectly, with target molecules within the living cell. These reactions give rise to alterations which, if not repaired, may prove deleterious to the cell. The target of primary interest to most investigators at this time is deoxyribonucleic acid. The greater part of our current understanding of damage inflicted by physical and chemical mutagens at the cellular and molecular levels derives from studies of the effects of these agents upon DNA and upon the repair of DNA damage by the cell. However, DNA does not exist in the isolated state in living systems but is complexed in chromatin with proteins, ribonucleic acid, and lipids. It is to be expected that the presence of these non-DNA components of chromatin could modify the response of DNA to physical and chemical agents, and studies in a variety of model systems have shown this to be the case. An understanding of the roles of these chromatin components in modifying DNA damage and repair is essential to bridging the gap between *in vitro* model systems and the expression of damage in living systems.

On the other hand, the phenomenon of radiation-induced division delay does not appear to relate directly to DNA damage and repair but to alteration of some (protein) component of the division mechanism. The current phase of this investigation deals with the effects of ionizing radiation upon chromatin structure and function, upon the synthesis and assembly of microtubules, and upon intracellular levels of the phosphorylation intermediate, cyclic adenosine monophosphate (cAMP), in cultured mammalian cells.

Chromatin. Histones are believed to be the molecular entities primarily responsible for compaction and structuring of chromatin. Structural alterations of histones by acetylation, methylation, and phosphorylation at specific times during the life cycle are believed to play a role in these processes.^{1,2} If such structural alterations are indeed involved in preparation for cell division, radiation-induced variations in their temporal patterns would give rise to concomitant alterations in the division patterns of irradiated cells. Gurley and Walters³ have demonstrated a reduction of histone H1 phosphorylation in the CHO cell following x irradiation. In contrast, we have shown that histone acetylation and methylation are not specifically affected by x irradiation and are not the cause of radiation-induced division delay.⁴ Inasmuch as histone phosphorylation is effected, in part, through the intermediate cAMP, we are currently investigating the effects of ionizing radiation upon the intracellular level of this substance.

We have determined the basal level of cAMP in cultured Chinese hamster cells from low density to high density, as shown in Figs. 1A and 1B. We have also determined the same for the medium in which the cells were grown. It appears from the data that the cAMP content of the cells remains relatively stable but that, after a certain concentration of cells is reached, there is an increase in the amount of cAMP in the growth medium.

The data in Figs. 2A and 2B indicate that there is apparently little effect of 800 rad x irradiation on intracellular level of cAMP during the radiation-induced division delay period. Although caffeine, an inhibitor of cAMP phosphodiesterase, reduces the division delay period of irradiated cells, such a

Fig. 1. Cyclic AMP measurements in cultured Chinese hamster cells ($\bullet$) and the growth medium ($\circ$). These results were obtained in typical experiments performed in triplicate. The cells (10^7) were removed from cultures at the desired cell concentrations. Cells were pelleted from the medium, quickly frozen, and stored at -80°C until all samples were collected. For the extraction of cAMP from the cells, 0.5 ml of cold 6% TCA was added to each pellet. After the solution was centrifuged, the supernatant was extracted with 10 volumes of water-saturated ether. The eluate was lyophilized, dissolved in 0.05 M Na-acetate buffer, pH 6.2, and its cAMP content was measured as described by Steiner *et al.*⁵ For the determination of cAMP content of the growth medium, 2.5 ml of 20% TCA was added to 5.0 ml of the growth medium after the cells were spun out. After centrifugation, 1.0 ml of the supernatant was treated as described above for the cells. All data were normalized because of the difference in binding of the antigen to antibody.

reduction may not be related in any simple way to cAMP metabolism, since Figs. 2A and 2B indicate that irradiated cells contain a normal complement of c-AMP.

Mitotic Apparatus. X irradiation of exponentially growing mammalian cells at intermediate dose levels induces the phenomenon of division delay in which the irradiated cells accumulate in a G_2 -like state prior to resuming division. This accumulation has led to the suggestion that the delay arises from damage to cellular division mechanisms or to the accumulation of some component necessary for initiation of division. Microtubule protein (tubulin) is a necessary component of the mitotic apparatus which

Fig. 2. Cyclic AMP measurements in cultured Chinese hamster cells: (Δ) control cells and ($\bullet$) x-irradiated cells. The cells were irradiated with 800 rad x irradiation. Control and irradiated cells (10^7) were harvested at the indicated times and treated as described in Fig. 1.

must be synthesized and assembled into macrostructures prior to division. We have shown that soluble microtubule protein levels rose to G_2 levels in irradiated cultures, as did RNA and total soluble protein, and declined as cells resumed division.⁵ This indicates that radiation-induced division delay of mammalian cells does not arise from a radiation-induced defect in synthesis and accumulation of soluble microtubule protein.

Our current investigation is based on the hypothesis that division delay results from the inability of tubulin of irradiated cells to assemble *in vivo* into functional microtubules. Recent findings suggest that tubulin subunit A is phosphorylated, possibly as a precursor to self-assembly. At the same time, *in vitro* tubulin assembly assays are being developed in many laboratories to investigate the phenomenon of association and assembly. We are currently investigating the effects of ionizing radiation on the phosphorylation of tubulin of irradiated cells. We are also surveying current methods for *in vitro* assembly of microtubules to determine which are most suitable for application to

Fig. 3. Gel electrophoretic pattern of a soluble microtubule protein from cultured Chinese hamster cells (line CHO) using acrylamide-SDS-urea gel system.

our cultured cells. Figure 3 is a gel electrophoretic pattern of a soluble microtubule protein from cultured Chinese hamster cells (line CHO) on sodium dodecyl sulfate (SDS)-urea gels, showing splitting into two distinct bands.

Soluble microtubule protein samples were prepared by a modification of the ammonium sulfate precipitation method of Weisenberg.⁷ The 7% acrylamide-SDS-urea gel system used is an adaptation from several systems currently in use for resolution of tubulin isolated from several sources.⁸⁻¹⁰

Microdensitometer tracings of stained gels indicate that the system is quantitative for the amount of protein present (Joyce Loebles densitometer), and techniques have been established for quantitative recovery of labeled material from fractionated gels by scintillation counting.

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THE CELL SURFACE

High Molecular-Weight Heparan Sulfate from the Cell Surface

(P. M. Kraemer and D. A. Smith)

Heparan sulfate appears to be a generally occurring cell-surface molecule on cultured animal cells.¹ Studies with Chinese hamster cells have also demonstrated intracellular varieties of this material; the intracellular heparan sulfates represent precursors and degradation products of the cell surface form that appears to be a physiologically important variety.^{2,3} Figure 1 illustrates the major structural features of cell-surface heparan sulfate.

In general, the biochemical characteristics of the heparan sulfates of the different cellular compartments (cell surface, cell sap, and internal membrane associated) are very similar to that reported by other workers.² However, we have recently found that the tryptic fragment of the heparan sulfate of

Fig. 1. Diagrammatic portrayal of the major structural features of cell-surface heparan sulfate.

Fig. 2. Elution profiles on Bio-Gel A0.5M of heparan sulfate isolated from various cell fractions.

the cell surface includes surprisingly large molecules. Figure 2 illustrates elution profiles of the three varieties of heparan sulfate on agarose A0.5M. Only the heparan sulfate isolated from the trypsin-removable material of whole cells included large amounts of material excluded from such a column.

Figure 3A illustrates the elution of cell-surface heparan sulfate on agarose A5M, again showing the large size of some of these molecules. Figures 3B-E illustrate the effect of various degradative treatments on this elution profile. Collectively, these experiments suggest the presence of significant peptide moiety and that unusual linkages

Fig. 3. Elution profiles on Bio-Gel A5M of the tryptic fragments of cell-surface heparan sulfate, either untreated (A) or after various treatments (B-E).

are scattered throughout the polysaccharide moiety.

Equilibrium sedimentation analyses have now been completed on several of these fractions: the largest and middle third of materials isolated as indicated in Fig. 3A and reisolated sugar chains following treatment of the largest and middle third material with alkaline borohydride. Figure 4 illustrates one of these analyses -- in this case, the largest third of the material eluted from A5M in Fig. 3A. When a partial specific volume of 0.60 was used, the average molecular weight was 135 000 daltons. After alkaline borohydride treatment, the reisolated sugar chains had a molecular weight of 44 000 daltons. Comparable values for the middle third were 40 000 and 27 000 daltons. All these values are much higher than the molecular weight

Fig. 4. Plot of sedimentation equilibrium data of cell-surface heparan sulfate in 1.0 M NaCl. The material used represents the first third of the material eluted in Fig. 3A.

usually reported for heparin.⁴ We suggest that the cell-surface tryptic fragments include one to three long sugar chains bound to a single polypeptide moiety.

We have speculated previously that the function of a generally occurring cell-surface molecular entity such as heparan sulfate might be related to the regulation of exposure of cell-specific entities to the immediate cell environment.³ The large size of the cell-surface heparan sulfate molecule would seem to encourage this speculation on the basis that such a large entity could effectively "overshadow" the smaller, topographically adjacent structures. This concept is diagrammatically portrayed in Fig. 5.

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Fig. 5. Speculative diagram of the role of cell-surface heparan sulfate in regulating the exposure of cell-specific surface sites.

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Mathematical Models for Immunologic Phenomena

[C. DeLisi, G. I. Bell (Theoretical Division), G. Siskind, and B. Goldstein]

The Jerne Plaque Technique. A physical chemical theory of the kinetics of plaque development was formulated. The theory takes account of the dependence of the plaque radius on time, antigen concentration, antibody secretion rate, rate constants governing antigen-antibody interactions, and viscosity of the medium. Methods were suggested for determining the forward rate constant and the secretion rate distribution from data obtainable by time-lapse photography.

Plaque Inhibition Experiments. Plaque formation is inhibited by adding free epitope to the medium, which competes for antibodies with red blood cell-bound epitope. It was found that, under appropriate conditions, a differential plot of the inhibition curve may reflect either the affinity

distribution or secretion rate distribution. However, if experimental conditions are not right, the differential plot may reflect neither. An analysis of some published papers revealed that interpretations of many inhibition experiments were probably erroneous and the biological conclusions unjustified.

Affinity Populations from Serum Antibodies. A method was developed for obtaining the distribution of antibody affinities in serum. Binding isotherms obtained from equilibrium dialysis data can be written as an integral over an unknown affinity distribution. The integral can be inverted exactly under a wide variety of circumstances and the unknown distribution obtained.

Analysis of Plaques Formed by Serum Antibody. Hiramoto *et al.*¹ performed experiments under a wide variety of conditions in which a pool of antiserum rather than a lymphocyte served as the source of antibody. These experiments have been modeled mathematically, and the following conclusions were reached: (a) the antibodies which diffuse to large distances are almost entirely low affinity; (b) at small distances the entire distribution of affinities is present; (c) under the appropriate conditions certain plots of the data will give the average affinity of the population; and (d) the possibility exists for obtaining information related to the distribution of antigen sites on a red blood cell from these experiments.

Aggregation Phenomena in Antigen-Antibody Systems. A theory of precipitation and agglutination phenomena was developed for systems composed of multifunctional antigen reacting with multivalent antibody. The antigen-antibody ratios at which large aggregates are expected to form were derived as a function of the antibody and antigen valence and the free energies of intramolecular and bimolecular reactions. It was found that, when intramolecular reaction is possible, a large number of antibodies must be bound per cell in order to obtain agglutination in the range of experimental cell concentrations. This explains the prediction of erroneously high concentrations by a previous theory, since that theory assumes only one or a few antibodies bound per cell.

Interaction of Antigen with Receptors on Immunocompetent Cells. Simple mathematical models were developed to treat the kinetics of binding of

multifunctional antigen to immune cell receptors when binding may be in competition with nonspecific binding of antigen to cell surfaces and with binding of hapten to receptors. The models were applied to the experiments of Davie and Paul. Many difficulties were found to arise from nonspecific binding, including an apparent reduction in the forward rate constant and equilibrium constant as well as an anomalously temperature-dependent equilibrium constant.

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CYTOGENETICS

Chromosome G-Banding and DNA Constancy in Aneuploid Cell Populations

(L. L. Deaven, P. C. Sanders, J. M. Grilly, and D. F. Petersen)

The evolution of chromosomal aberrations within *in vitro* cell populations is poorly understood. Studies in our Laboratory using improved flow microfluorometry techniques seriously challenge the stem-line concept and rather strongly suggest that, although the DNA content of heteroploid cells is greater than that of the diploid parent, there is virtually no increase in variability of cell-to-cell DNA content, that chromosomal variants are equally viable, and that the traditional mechanisms used to explain intrastem-line variability of chromosome number are inadequate.

Cytologists have known for sometime that tumor cells contain abnormal numbers of chromosomes and that extensive aneuploidy can occur in cultured cell lines derived from normal tissues.¹ These changes in karyotype during *in vitro* cellular evolution have been documented extensively and are considered to be similar, if not identical, to the chromosome rearrangements of tumor cells.² The central dogma which emerged in an attempt to draw numerous observations together was the so-called "stem-line theory" originally derived from observations of chromosome patterns in ascites tumors³ and extended to include evolved *in vitro* cell populations.² The concept originally defined "stem cells" as that portion of

the population having specific karyotype and serving as principal progenitor of tumor growth. Cells possessing different karyotypes, according to this notion, were the products of aberrant mitoses, generally incapable of continued proliferation and, hence, transient components of the tumor cell population. More recent discussions have recognized that tumors and *in vitro* cell lines are in a state of flux with changing forms and modes and that stem-line constancy is not absolute.

Our earliest flow microfluorometric (FMF) measurements demonstrated that, although DNA content increased to approximately 3C, variability of cellular DNA content in classical heteroploid lines was no greater than in euploid strains from which they were derived.⁴ Attention was focused immediately on experiments involving manipulation of chromosome number with subsequent FMF measurements of cellular DNA content. Following treatment of Chinese hamster cells (line CHO) with low doses of Colcemid to induce chromosome nondisjunction, DNA dispersion was clearly shown by FMF measurement. Alternatively, clonal selection of HeLa populations provided subcultures with temporarily small perimodal chromosome distributions of 66 and 68. When compared with the parent HeLa line with a mode of 71 and a perimodal dispersion of 10 chromosomes, FMF distributions were indistinguishable. The comparison clearly demonstrates DNA constancy despite chromosome number variability. Taken together, these results argue convincingly that the variability in aneuploid-heteroploid cells cannot be explained by or ascribed to mitotic nondisjunction. To accommodate these seemingly paradoxical observations, Kraemer *et al.*⁵ have proposed the alternative notion that chromosomes normally are not always separate entities, that during a portion of interphase much of the DNA is continuous, and that heteroploidy represents aberrant packaging which appears at metaphase as rearrangements. The model in its simplest form has been tested by Giemsa band analysis of several aneuploid lines, adopting the working hypothesis that the majority of the cells should contain one sequence of bands assembled in a variety of patterns.

The first G-band analysis of an aneuploid cell line⁶ demonstrated that, although numerous rearrangements occurred in Chinese hamster (line CHO)

cells, essentially all euploid chromatin was present in each cell -- in good agreement with FMF comparisons of DNA content in line CHO and euploid Chinese hamster cells. Moreover, one haploid set of chromosomes remained largely intact -- an interesting observation from a genetic viewpoint because the demonstration lent support to the hemizygosity hypothesis advanced to explain the success in deriving mutant lines from CHO.⁷ Infrequent perimodal dispersion in line CHO has consistently involved the smallest chromosomes. FMF measurements of eight HeLa sublines from different laboratories showed differences in the DNA content of independently passaged lines. None exhibited increased dispersions of cellular DNA consistent with the chromosome number dispersion, but a rough correlation existed between DNA content and modal chromosome number.

G-Banding using the cold trypsin technique permitted the following observations. HeLa S-3 chromosomes could be divided conveniently into three groups: (a) unaltered chromosomes; (b) altered chromosomes common to the population; and (c) altered chromosomes unique to a single cell or a small minority of the population (Fig. 1). At least a haploid set of normal human chromosomes remained intact in all the cells examined. All the HeLa lines were basically triploid; DNA content was approximately 1.5 times the human diploid value and, among the larger chromosomes, triploid complements could be found. Comparison of Figs. 1 and 2 shows that, although HeLa 4 contains the same amount of DNA as S-3 and a similar chromosome number distribution, the actual karyotypes were clearly different. HeLa S-3 cells contained about 40% abnormal chromosomes, while in HeLa 4 only 24% were classified as abnormal, resulting in a marked increase in the number of triploid groups. HeLa 229, which contained 33% more DNA, resembled the HeLa 4 karyotype (Fig. 3). Both lines contained approximately the same normal to abnormal chromosome ratio with some abnormalities common to both lines and the major difference an increase in the number of copies of preexisting chromosomes. When a HeLa 229 cell with 76 chromosomes was compared with one containing 80, the differences involved variable numbers of copies within a given group. There was also an indication that the changes were compensatory and appeared primarily in the small chromosomes. The total arm-length measurements yielded results

Fig. 1. Cold trypsin-induced Giemsa bands of a HeLa S-3 cell ($n = 65$). The first line of abnormal chromosomes represents those found in many cells in the population, while those in the second line are unique to one or a few cells.

Fig. 2. Cold trypsin-induced Giemsa bands of a HeLa 4 cell ($n = 64$).

Fig. 3. Cold trypsin-induced Giemsa bands of a HeLa 229 cell ($n = 76$).

consistent with FIF measurements. The cell-to-cell differences in chromosome number primarily involved groups D, E, F, and G, the small chromosomes, an occasional C, but never the A and B groups. When a C group chromosome was missing, it was replaced by two or more smaller chromosomes which appeared to compensate in size and DNA content for the missing larger element. These observations of size compensation are in contrast to a previous report of Minkler *et al.*⁸

When perimodal cells were compared with modal cells with respect to specific chromosome content, few systematic differences could be found. For example, two modal cells, each containing 65 chromosomes, can have as many differences between them as are found between a cell with 60 and one with 65 chromosomes. These observations all suggest that an infrastem-line is a population interrelated on the basis of DNA content but with less regard to specific chromosomes.

In CHO cells, autoradiographic studies have shown that the normal sequence of chromosomal DNA synthesis is altered. Some of the chromosomal sites which replicate late in euploid cells replicate

earlier in CHO, and several late-replicating chromosomes in CHO cells replicate in early or mid-S in euploid material. There are two and sometimes three X chromosomes in HeLa cells; none of these is late-replicating, nor do they replicate simultaneously.⁹ Do these changes in time of replication have any relationship with genetic expression? An answer to this question is now possible through the use of known genetic markers on human X chromosomes, and such information may serve to elucidate the paradox of genetic stability in the face of karyotypic diversity.

Although banding methods allow a much greater resolution of chromosome rearrangements than previous techniques, a word of caution in interpretation of these data is appropriate. It is fairly easy to band and arrange homologs in euploid cells; however, when each chromosome in a cell may or may not be altered, correct classification is much more difficult. Observations of endoreduplicated chromosomes help to define the limits of banding resolution, but more objective automated scanning systems would be very helpful in solving this problem. Finally, although the exact mechanism for banding is not understood, it seems likely that the bands are somehow related to chromosome supercoiling. Reports of altered supercoiling in abnormal chromosomes have already appeared in the literature,¹⁰ and this might further complicate the correct identification of chromosomes in heteroploid cells.

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INTRODUCTION

In early 1973, the Biophysics and Instrumentation Group (H-10) was formed as part of the reorganization in the Health Division. Group H-10 consists of persons previously assigned to the Biophysics or Physical Radiobiology Sections of the old Biomedical Research Group (H-4). Recent staff additions to the group include G. C. Salzman and H. A. Crissman, previously LASL postdoctoral appointees in Groups P-9 and H-4, respectively. In addition, J. M. Crowell, a recent physics Ph.D. from Johns Hopkins University, has joined the group as a postdoctoral appointee.

The research areas actively pursued by members of the group fall into several categories, including (a) instrumentation development for the rapid analysis and sorting of mammalian cells; (b) development of cell staining and preparation techniques suitable for these flow-systems instruments;

(c) application of these instruments to clinical and basic biological research; (d) radiation biology; (e) dosimetry related to the pion beam at the LAMPF; and (f) a continuing effort in computer and electronic development in support of the LASL biomedical program. Specific activities in these various areas will be discussed below in more detail.

During 1973, the LASL-designed multiparameter cell sorter (MPS), developed under a joint AEC/NCI program, was completed and tested with vaginal and clinical materials. A third unit was fabricated and installed at the National Cancer Institute's Laboratory of Pathology in Bethesda, Maryland. This unit will be used by the NCI in their automated cytology program. A sister unit remains at the LASL and is dedicated to routine biological research. At the present time, four flow instruments are in use at the LASL: (a) FMF, which has been described

elsewhere; (b) MPS-I, which is mainly used for instrumentation development; (c) MPS-II, the companion instrument to the NCI unit; and (d) a combination light-scattering and fluorescence photometer used for continuing light scattering research. In addition, a PDP 11/20 computer has been acquired and is presently being integrated into the cell analysis program to permit multidimensional cell descriptors to be formulated for cell identification and sorting purposes.

Other recent developments in this area include development of a cell volume sensing technique which senses the potential charge generated by a cell as it traverses a Coulter orifice, two parameter detection techniques, and a flow microphotometer which senses wide-angle scattering as well as fluorescence.

In the last year, considerable effort has been applied to the problems of cell preparation and staining. Several enzyme methods have been investigated as a means of cell dispersal for both human and animal tissue materials. Development of staining protocols for several cellular parameters, including nuclear DNA and cytoplasmic protein, have been developed with the dyes ethidium bromide, propidium iodide, and fluorescein isothiocyanate.

The year 1973 saw application of the methods to several varying types of biological problems, including (a) evaluation of fluorescent antibody conjugates; (b) detection of virus-induced diseases such as Newcastle's disease; (c) further studies on life-cycle analysis of cultured mammalian cells and the effects of drugs, radiation, and other agents on these cells; (d) measurement of the changes induced by SV-40 infection; (e) measurement of Concanavalin A and other plant lectin binding; (f) development of a white blood cell differential based on acridine orange staining; (g) DNA measurements on human vaginal and cervical material, as well as other human tumors; and (h) continued development of new stain techniques.

Our cooperative efforts with other investigators initiated sometime ago have continued to flourish. This year saw collaboration with individuals from the University of Houston, University of New Mexico,

University of Colorado, University of Virginia, Salk Institute, Roswell Park Memorial Institute, University of California at Berkeley, M. D. Anderson Hospital and Tumor Institute, and Karolinska Institute in Stockholm, among others.

Our pioneering work in cell analysis continues to draw national and international attention. Our personnel have participated in the Second International Symposium on Cancer Detection and Prevention, held in Bologna, Italy (April 9-13, 1973), and in the Symposium on Rapid Methods and Automation in Microbiology, held in Stockholm, Sweden (June 3-8, 1973). Largely due to LASL expertise in flow microfluorometry, the NIH-sponsored course on aging at the cellular level was held at the LASL in July 1973. Group H-10 personnel also participated in the LASL's First Annual Life Sciences Symposium.

The use of small computers for data acquisition and analysis has become rather widespread within the group. The LASL lung counter now uses a PDP 8/I with both hardware and software improvements made over the last year. HUMCO II also uses a PDP 8/F which is currently being tested side-by-side with the present pulse-height analysis system. A PDP 8/E is in routine use in the LASL instrument for karyology. Finally, a PDP 11/40 has been purchased which will be used as a computer-based terminal.

In addition, our FMF data have been subjected extensively to computer analysis. Advances have been made with areas of life-cycle analysis; it is now possible to extract information such as the rate of DNA synthesis from the FMF data.

In the area of physical radiobiology, experiments have been performed to study cell survival under mixed alpha and x irradiation exposures, as well as separate exposures in preparation for the work at the LAMPF biomedical area. Investigation of the applicability of solid state detectors for pion beam diagnostics has also been started.

Our cooperative efforts with the U. S. Department of Agriculture and the National Cancer Institute continued at approximately the same level as in the previous year.

INSTRUMENTATION AND BIOENGINEERING

(J. A. Steinkamp, J. C. Martin, P. F. Mullaney, W. T. West, J. L. Horney, G. C. Salzman, J. M. Crowell, J. D. Perrings, L. J. Carr, J. H. Larkins, and M. T. Butler)

A Potential Sensing Technique for Cell Volume Measurement

A device in common use for the measurement of biological cell volumes employs a small diameter orifice through which a saline solution containing cells is pumped. A high impedance source is used to drive a constant current through the orifice. The presence of an insulating particle such as a cell in the orifice causes a change in orifice resistance which is approximately proportional to the volume of the particle. This device has been studied in detail both theoretically and experimentally by a number of authors.¹⁻⁵

Several serious problems exist for this system. Since the signal of interest is taken from poorly shielded power electrodes, "antenna" type pickup produces low frequency noise. Furthermore, the current flowing from the electrodes into the saline contributes high frequency noise components. Best pulse-height resolution is obtained with orifices long with respect to their diameters; for a given solution flow rate, this results in relatively long pulses, placing a rather low upper limit on the count rate.

In the potential sensing techniques presented here, thin platinum electrodes are embedded inside the orifice, and a high-gain, high-impedance differential amplifier is used to view the voltage pulse produced when a cell passes through the orifice. This technique gives a high signal-to-noise ratio and produces inherently shorter pulses than is possible with the current sensing method. Thomas and Leif^{6,7} have discussed a potential sensing technique using electrodes at either end of the orifice and requiring a balanced bridge network for potential sensing.

A cross-sectional view of the LASL potential sensing orifice is shown in Fig. 1. The orifice consists of a multilayer sandwich of 1.25-cm diameter plastic (Rexolite) disks interleaved with 0.6-cm wide by 25- μ m thick platinum strips. The sandwich was bonded together with Armstrong A271 epoxy,

Fig. 1. Cross-sectional view of the potential sensing orifice. The exit side of the orifice was glued over the hole in a blank Coulter finger. The 1.25-cm diameter of the disk is not shown to scale.

and the edges were sealed with a silastic RTV compound. Shielded leads were soldered onto the exposed parts of the platinum strips. The overall length of the orifice is 457 μ m, while the inner and outer diameters are 102 and 406 μ m, respectively. The distance between the centers of the sensing electrodes is 125 μ m. All dimensions are ± 5 μ m. The orifice is effectively 102 μ m in diameter by 152 μ m in length.

Typical pulse shapes obtained with the above orifice configuration and 11.5 ± 0.4 - μ m LASL-produced plastic microspheres are shown in Fig. 2. Note the undershoot both preceding and following the positive part of each of the pulses shown. This may be explained using the equivalent circuit of Fig. 3. When an insulating particle enters the orifice, it causes a change (ΔR) in the orifice resistance, yielding a small voltage pulse ($v = I\Delta R$). For a

Fig. 2. Some of the pulse shapes obtained with 11.5- μ m plastic spheres, a 200- μ A orifice current, and a 1.6 ml/min solution flow rate. The vertical scale is 2 V/div and the horizontal scale 10 μ sec/div.

Fig. 3. Electrical equivalent circuit of the potential sensing orifice. Resistance and capacitance measurements are discussed in the text. The values are $R_1 = 9.67 \text{ k}\Omega$, $R_2 = 13.1 \text{ k}\Omega$, $R_3 = 15.2 \text{ k}\Omega$, $C_1 = 45 \text{ pF}$, $C_2 = 21 \text{ pF}$, $C_3 = 26 \text{ pF}$, $C_4 = 45 \text{ pF}$, $C_5 = 31 \text{ pF}$, and $C_6 = 32 \text{ pF}$.

perfect constant-current system (i.e., one without the capacitances shown in Fig. 3), a positive output pulse would be present only while the particle is in R_2 between the electrodes. However, the actual circuit capacitances give transient restraints on the system such that, when the particle is within R_1 or R_3 , point (2) is negative with respect to point (3). The rounding of the pulse is believed to be caused primarily by the broad boundary conditions resulting from the relatively thick electrodes in conjunction with the large orifice. The resistance values given in Fig. 3 were obtained by connecting a high-impedance, alternating-current, Hewlett-Packard HP204C oscillator set at a frequency of 10 kHz and an output of 1 V rms in parallel with the existing direct-current source and measuring the voltage drops across R_1 , R_2 , and R_3 using a Hewlett-Packard HP4033 rms voltmeter. All six possible combinations of capacitive reactances in the equivalent four-terminal network were measured using a Hewlett-Packard HP4815A RF vector impedance meter. A system of simultaneous equations was then solved for the discrete capacitance values given in Fig. 3. Computer generated transient response functions have been obtained for the equivalent circuit in Fig. 3. At present, the positive part of the pulse has been well approximated, and work continues on the two undershoot pulses.

The functional relationship between pulse-height and particle volume was determined by measuring separately the pulse-height distributions of each of a

set of known diameter plastic microspheres under calibrated amplifier gain conditions. The relationship appears to be linear over the range of particle diameters from 6-33.5- μm . A plot showing relative pulse height vs particle volume is presented in Fig. 4, and two typical pulse-height spectra are given in Fig. 5.

One problem which exists for potential sensing cell volume measurements is illustrated in Fig. 6 where the mean channel number of the pulse-height distribution for 23.5- μm diameter microspheres is plotted vs temperature of the normal saline solution in which the spheres are suspended. This temperature dependence occurs because the potential sensing pulse height is proportional to the resistivity of the saline solution which decreases with increasing temperature.

Direct comparisons between current sensing and potential sensing techniques using the potential sensing orifice were not possible because the presence of the potential sensing electrodes in the orifice disturbed the electric field in the orifice sufficiently to cause distortion in the current sensing pulses. Hence, current sensing noise measurements were made using a commercial Coulter orifice 100 μm in diameter by 100 μm in length. Noise in

Fig. 4. Relative pulse height vs particle volume for sets of plastic microspheres with diameters of 6, 9.4, 11.5, 14, 23.5, and 33.5 μm . The error bars represent an estimated 5% error in the amplifier gain calibration.

Fig. 5. Typical pulse-height spectra obtained with the potential sensing orifice. These spectra were used in the linearity measurements shown in Fig. 4.

Fig. 6. Channel centroid for 23.5- μ m diameter spheres vs temperature of the saline solution in which the spheres were suspended.

each system was measured using a Hewlett-Packard HP3400A true rms voltmeter. Both systems were adjusted for amplifier gains of 3000 with 200 μ A orifice current, and noise measurements were made at the outputs of the amplifiers and are referred to the input below.

For the current sensing system, the presence of any amount of orifice current between 10 and 200 μ A produced an increase in noise of 2.6 μ V referred to the input of the amplifier, while for the potential sensing system currents from 10-50 μ A produced no detectable noise increase over that of the amplifier alone. For 100- and 200- μ A orifice currents the potential sensing noise rose 0.3 and 1.6 μ A, respectively, over that with the current off. The potential sensing system seems to be considerably less sensitive to noise produced by the orifice current than is the current sensing system. For the potential sensing system, the worst case signal-to-noise ratio was 30:1 and was for the 6- μ m diameter spheres shown in the lower half of Fig. 5.

The high signal-to-noise ratios obtained with the LASL potential sensing technique and the fact that the noise is nearly independent of the orifice current imply that high amplifier gains may be used with this system. This means that larger orifices than are presently in use may be employed, thus reducing orifice plugging problems and allowing a much larger dynamic range of particle volumes to be measured. The electrical circuit modeling of the potential sensing orifice is important in that it may allow the measurement of cell capacitance, a parameter which bears on cell membrane permeability.

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Light-Scattering Studies

Flow Microphotometer for Single Cell Light-Scattering Detection at Several Angles. The flow microphotometer developed at the LASL¹ was designed to measure simultaneously the light scattered by cells in the forward direction, as well as cellular fluorescence. The main purpose of the scatter detectors was to provide a means of cell sizing and a method of gating the fluorescence signal so as to remove electronically fluorescence noise due to debris, etc. The relationship between particle size and forward light scatter has been investigated further experimentally² and found to be quite sensitive to the geometry of the input beam shaping and the method of light collection.

Experimental work on the scattering of suspensions of cells with well defined nuclei³ has shown that the internal structure of the cell does influence the scattering outside the Fraunhofer diffraction region, as predicted by theory.⁴ This suggests that a combined sensor, responsive to cell size (small-angle scattering) and internal structure (moderate- to large-angle scattering), might provide an additional method of cell identification. With this purpose in mind, the light-scattering flow microphotometer has been redesigned to permit these measurements.

A diagram of the apparatus is shown in Fig. 1. A photodiode detector, similar to that used in the earlier unit,¹ detects the scattered light at angles between 0.6° and 2.0° from the optical axis of the system. A second detector is mounted on an optical bench which can be rotated about the flow chamber, remaining co-planar with the small-angle detector, the point of cell stream/light beam intersection, and the fluorescence detector. The wide-angle detector, with an acceptance angle of $1-2^\circ$, can be located at any desired scattering angle between 8° and 35° from the optical axis of the system. Earlier calculations demonstrated that the scattering intensity from a cell-like object drops off rapidly with increasing angle; at a scattering angle of 30° , $10^{-4} < I_{30^\circ}/I_0 < 10^{-3}$. In order to achieve the required sensitivity, a multiplier phototube was

Fig. 1. Schematic diagram of the flow light-scattering photometer. Focal lengths of the lenses used are $L_0 = 18$ cm; L_1 and $L_2 = 20$ cm; L_3 and $L_4 = 12$ cm; and $L_5 = 5$ cm. Filters used are F_1 (Corning 3-69) and F_2 (a 488-nm pass filter).

selected as the detector. The sensor acceptance angle is defined by the aperture pinhole; the remaining optics in the sensor assembly image this pinhole on the photocathode of the multiplier phototube. After detection, the resulting electrical signal is amplified in a manner similar to that used for the forward scattering detector.

It is well known that there is an inverse relationship between the angular extent of the scattering pattern and the particle size. Light scattered by small debris consequently may compete with that scattered by a larger cell at $20^\circ < \theta < 35^\circ$. In order to rescue the large-angle signal from such noise, the gating electronics developed previously have been employed.¹ The two scattering signals from each cell are then stored in the memory of a two-parameter pulse-height analyzer.

Preliminary experiments have been conducted with human white blood cells, since these show a bimodal forward-angle scattering pattern. The cells were treated with saponin to remove (lyse) the red blood cells, leaving a suspension of white blood cells. These cells were examined with the photometer, and the two-parameter spectra were recorded, showing classification of the cells into three regions. The meaning of these preliminary results is not fully understood at this time.

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Light Scattering by Cellular Suspensions:

Comparison with Theory. As reported in the previous annual report, we have attempted to model some mammalian cells as coated spheres, the core of the sphere being the nucleus and the coating the cytoplasm. The model predicted that scattering at smaller angles was determined mainly by the particle silhouette, while internal details (nucleus) influence the scattering at larger angles. Experimental verification of this model was obtained with suspensions of cells (line CHO).¹

More recently, we have challenged this model with a rather different biological object which shows essentially no internal structure and presents essentially a silhouette to the exciting light. This object is a red blood cell ghost. When formed, a ghost consists of a hollow sphere with a very thin membrane.

The cells used in this study were prepared by Dr. Robert Fiel at Roswell Park Memorial Institute, Buffalo, New York. Scattering measurements were made on suspensions of ghosts using a Virtis high resolution light-scattering photometer as described by Fiel.² The data were displayed in the form of an intensity vs scattering angle plot. The data were compared with a theoretical plot of scattering intensity vs angle derived from the coated sphere model developed at Los Alamos. The value chosen for refractive index is that reported by MacRae *et al.*³ It was further assumed that all the refractile material is concentrated in a thin shell; the interior of the sphere is hollow (i.e., filled with a suspending medium). The refractive index of the suspending medium was measured as 1.33. The model proposed for the ghosts is shown in Fig. 1.

It has been shown, even in the case of low relative refractive index, that diffraction is the main scattering mechanism at small angles. In order to select a size parameter for the ghosts, the position of the first scattering minima ($\theta_{\min}$) was noted and

Fig. 1. Model of red blood cell ghost: D = cell diameter; d = core diameter; m_1 = relative refractive index of shell; and m_2 = relative refractive index of core.

the size parameter (γ) calculated using the Fraunhofer diffraction relationship:

$$\gamma \sin \theta_{\min} = 3.83,$$

where γ is related to the particle diameter D (in microns), and the wavelength of the exciting light in the medium (λ_0) in microns:

$$\gamma = \frac{\pi D}{\lambda_0 / m_2}$$

where m_2 is the relative refractive index of the shell. A second size parameter (α) must also be constructed for the core (diameter = d) of the sphere:

$$\alpha = \frac{\pi d}{\lambda_0 / m_1}$$

where m_1 is the core relative refractive index, in this case $m_1 = 1$. The shell thickness is then $(D - d)/2 = t$.

In the present case, the ghosts were illuminated with unpolarized light of wavelength $\lambda_0 = 0.540 \mu$. The total scattering intensity function to be calculated (I_T) is then:

$$I_T = I_T(\gamma, \alpha, m_1, m_2, \theta).$$

The only variable for which a value is not known is

α . Thus, I_T was calculated for various α values and matched with experiment. The best match, shown in Fig. 2, yielded the following optical characteristics for the ghosts: $D = 7.45 \mu$, $t = 150 \text{ \AA}$, and $m_2 = 1.05$.

We also assumed the ghosts to be purely diffracting objects with $D = 7.45 \mu$. The main features of agreement between the coated sphere (shell) model and the experimental data is in the positions of the maxima and minima. The amplitude of the two curves varies somewhat in the sense that the calculated peak-to-valley ratios are greater than that observed. Previous studies of suspensions of particles indicated similar effects. This washout effect can be due to the presence of debris as well as the size distribution of the particles. The suspension was modeled as a single particle with the above characteristics. However, we have shown that for suspensions with a volume coefficient of variation $< 20\%$ the main features of the scattering pattern will not be lost and that such a model as the present one is valid.

This study verifies that cells cannot be considered as simply diffracting objects and that light transmission characteristics must be taken into account.⁴ Fraunhofer diffraction is applicable only in the extreme forward lobe. The fact that the

Fig. 2. Comparison of experimental scattering distribution (-----) with a hollow sphere model for red blood cell ghosts.

scattering is sensitive to a membrane approximately $100 \text{ \AA}$ thick suggests that scattering may be used to reveal subtle changes in the conformation of large mammalian cells. The thickness value ($D0 \text{ \AA}$) is consistent with the values reported elsewhere.⁵

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Cell Sorter Advances

An improved multisensor cell analysis and sorting instrument has been developed for cell biology research involving measurement of physical and biochemical properties of single cells. This system is presently being used for numerous biological experiments plus an instrumentation development test bed. A second cell sorter was placed into operation late in the fall and is to be dedicated to biological experimentation. This progress report describes the basic features of multiparameter analysis methodology applied to the measurement of fluorescein-conjugated Concanavalin A (Con A) lectin bound to cultured mammalian cell surfaces and the analysis and sorting of two discrete classes of human peripheral lymphocytes stained with the metachromatic fluorochrome acridine orange.

A comprehensive description of the instrumentation has been described elsewhere.¹ Cells stained with fluorescent dyes that label specific biochemical components are introduced into a flow chamber (Fig. 1) at approximately 500/sec. Cells first pass centrally through a volume sensing orifice (Coulter principle) and then intersect an argon laser beam (488 nm wavelength), causing light scatter and fluorescence. Cell volume signals can be raised to the two-thirds power, producing signals proportional to cell surface area, assuming sphericity. Total or two-color fluorescence and light scatter are electro-optically measured. After measurement, the emerging liquid stream carrying suspended cells is

Fig. 1. Multiparameter cell separator illustrating the flow chamber, laser illumination, droplet generation, charging and deflection scheme, and sample collection.

broken into uniform liquid droplets (45 000/sec) with approximately 1% of the droplets containing a cell.

Signals from the cell sensors are processed on a cell-by-cell basis as single parameters, ratios, and gated single parameters. A multichannel pulse-height analyzer accumulates and displays processed signals as frequency distribution histograms. Complete two parameter analysis is also available by selecting two processed signals as inputs to a dual parameter multichannel pulse-height analyzer. Processed signals also activate cell sorting according to conformance or nonconformance to preselected standards by causing a group of droplets to be electrically charged and electrostatically deflected into collection vessels. Those cells not satisfying the sorting criteria pass undeflected into a separate vessel. Sorted cells are counterstained and microscopically evaluated.

Cell volume, surface area, total surface fluorescence, and surface fluorescence-to-area ratio distributions recorded from exponentially grown CHO

Fig. 2. Frequency distribution histograms of CHO cells reacted with Con A-F: (A) cell volume and surface area; (B) gated and ungated surface fluorescence; and (C) gated surface fluorescence-to-area ratio distributions.

cells reacted with fluorescein-conjugated Con A-F² are shown in Fig. 2. Figure 2A shows the respective single parameter cell volume and surface area distributions which are broad, unimodal, and typical of a cell population in exponential growth. Dispersion of the cell surface area distribution is less than that of the cell volume plot because surface area is proportional to cell diameter squared, whereas volume is proportional to diameter cubed. Uniform plastic microspheres also have been used to calibrate the two-thirds function generator showing linear measurement of surface area.³ Figure 3B vividly illustrates the use of gated single parameter analysis to cancel interference from small fluorescing debris in the cell surface fluorescence measurement. The small fluorescent debris is

Fig. 3. Frequency distribution histograms of L5178Y, P815, and CHO cultured cells reacted with Con A-F: (A) cell surface area; (B) gated and ungated surface fluorescence; and (C) gated surface fluorescence-to-area ratio distributions.

responsible for the high number of interfering counts in the left portion of the ungated fluorescence distribution. These counts are eliminated by recording only those fluorescence signals coincident with cell surface area signals between channels 23-40, shown by the shaded region of Fig. 2A. The resulting gated cell surface fluorescence distribution (Fig. 2B) is essentially free from background interference but contains dispersion due to cell size and other factors. Figure 2C illustrates a method to correlate lectin binding to cell surface area as a surface density of binding sites function using ratio analysis. This methodology is further validated in Fig. 3 which shows cell surface area, surface fluorescence, and fluorescence-to-area ratio distributions for L5178Y, P815, and CHO cultured cells reacted with Con A-F. These distributions, which were recorded at fixed amplifier gain settings, show differences in both surface area and fluorescence. Gated single parameter techniques were used in both cell surface fluorescence (Fig. 3B) and surface fluorescence-to-area ratio (Fig. 3C) measurements to reduce background interference caused by debris. Figure 3C illustrates different site-surface density of lectin binding as expressed in the cell surface fluorescence-to-area ratio distribution modal channel. Similar results³ recorded for two types of animal and human derived cultured cells show large differences in site-density ratios. This technique

has potential application in numerous experiments involving lectin binding.

The green and red fluorescence distributions of normal human peripheral lymphocytes vitally stained with acridine orange⁴ are shown in Fig. 4A. The green fluorescence distribution is unimodal, illustrating similarities in leukocyte nuclear staining, whereas the red fluorescence distribution shows three distinct peaks which characterize leukocytes into three groups based on the red fluorescence of cytoplasmic granules. Peaks 1, 2, and 3 have been reported to consist primarily of lymphocytes, monocytes, and granulocytes, respectively.^{4,5} The green-to-red fluorescence ratio distribution (Fig. 4B) shows four distinct groups, permitting leukocyte characterization as a function of nuclear-to-cytoplasmic staining properties. The small shoulder on the right side of the lymphocyte peak of the red fluorescence distribution accounts for peak 3 in the green-to-red fluorescence ratio distribution. This is further verified using two parameter analysis methods as described below. Leukocytes having green-to-red fluorescence ratio signal amplitudes

Fig. 4. Frequency distribution histograms of acridine-stained normal human peripheral leukocytes: (A) green and red fluorescence and (B) green-to-red fluorescence ratio distributions.

Fig. 5. Photomicrographs of sorted normal human leukocytes counterstained with Wright's Giemsa: (A) enriched leukocyte sort; (B) sorted large lymphocytes; and (C) sorted small lymphocytes. Erythrocytes contained in the photomicrographs were separated along with selected leukocytes.

corresponding to sort regions 1, 2, 3, and 4 were separated, counterstained, and microscopically identified as granulocytes, monocytes, and two types of lymphocytes.⁶

Figure 5A shows a photomicrograph of an enriched leukocyte population obtained by sorting

leukocytes based on green fluorescence signals having amplitudes ranging between channels 5-30 of Fig. 4A. Figures 5B and 5C show photomicrographs of sorted and counterstained lymphocyte fractions corresponding to sort regions 3 and 4 of Fig. 4B. Microscopic examination suggests that sorted fractions 3 and 4 are the two cell types normally classified as large and small lymphocytes, respectively. The nuclear chromatin of the small lymphocytes counterstains more densely than chromatin of the large lymphocytes. Also, the nucleus of the large lymphocytes is kidney-shaped, compared to the round nuclei of small lymphocytes. These results imply two lymphocyte types which are morphologically and functionally distinct. Leukocytes corresponding to sort regions 1 and 2 (Fig. 2B) were identified microscopically as granulocytes (neutrophils) and monocytes, respectively. Cell volume analysis of sorted lymphocyte fractions revealed cell types of different sizes but with considerable overlap in volume.⁶

Figure 6 shows a three-dimensional frequency distribution histogram (isometric display) and a two-dimensional contour view of two parameter red fluorescence and green-to-red fluorescence ratio

Fig. 6. Two parameter green-to-red fluorescence ratio plots of acridine orange-stained normal human leukocytes: (A) three-dimensional frequency distribution histogram (isometric plot) and (B) two-dimensional contour view. Regions 1-4 consist of granulocytes, monocytes, large lymphocytes, and small lymphocytes, respectively.

relationships in human leukocytes. The isometric display provides a method to quantitate the number of leukocytes within a given range to cellular properties. The contour view, which is a base-plane sectional view of the isometric display, vividly illustrates the relationship in the four leukocyte regions. The two lymphocyte types (regions 3 and 4) have nearly overlapping red fluorescence values but well separated green-to-red fluorescence ratios as viewed with respect to each axis. These results correspond well with the individual distributions of Fig. 4.

In summary, multiparameter flow analysis of cells reacted with Con A-F provides new methodology to study lectin binding as a surface density of binding sites function irrespective of cell size variability. Evaluation of the two sorted lymphocyte populations suggests two morphologically and functionally distinct lymphocyte types. When considered by volume measurement alone, the two sorted classes were not distinct but had overlapping volume distributions. However, the fluorescence ratio distribution clearly indicated that these are distinct classes rather than representing an arbitrary continuum.

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CELL STAINING AND PREPARATION

(H. A. Crissman, A. Romero, P. K. Horan, and T. T. Trujillo)

Dispersal of Gynecological Material

When attempting to quantitate the fluorescence intensity of stained squamous cells in a flow system, it is essential that the vast majority of cells to be examined exist as single cells. With the available instrumentation, a single cell stained for DNA, fluorescing with an intensity 2I, cannot always be distinguished from a clump of two cells each fluorescing with intensity I. In the typical Papanicolaou gynecological specimen, approximately 40% of the cells are single cells and the remaining cells are found in aggregates (involving two or more cells). These specimens must be disaggregated not only to prevent clogging of the instrument but, more importantly, to permit a correct quantitative single-cell analysis where false information is not generated by clumps.

Clumps of squamous cells can be removed from a specimen by use of either mechanical or biochemical techniques. Mechanical disruption in the form of ultrasonication or shear is capable of disaggregating the cells; however, one runs the risk of cell rupture. It is also possible to remove the clumps by filtration. However, the method of choice, probably biochemical, would minimize clumping as well as cell loss. The biochemical approach involves dissolution of chemical bonds which are considered responsible for cell adhesion. Some of the chemical bonding agents are:

(1) Mucopolysaccharides (i.e., hyaluronic acid). Hyaluronic acid occurs in both free and salt-like combinations with proteins of tissues and tissue fluids. In tissues, hyaluronic acid is a cementing substance which contributes to tissue barriers. This chemical is broken down by the enzyme hyaluronidase.

(2) Collagen is a major fibrous protein of extracellular connective tissues. It is produced by mesenchymal cells and collects in the intercellular spaces and is subject to digestion by the enzyme collagenase.

(3) Elastin is a yellow refractile material having a fibrillar structure arranged in a three-dimensional meshwork without free ends. The enzyme elastase specifically degrades elastin.

(4) Two other materials are known to form intercellular connections for the purpose of holding cells together. These substances are keratin and reticulin. Both substances are not subject to enzymatic digestion and, since keratin is important in the diagnosis of certain forms of cervical cancer, it should not be destroyed.

During the last year, we have used microscopic slides which have a uniform grid photographically reproduced on the slide. The cell suspension (10 μ l) is placed on a slide and a glass coverslip placed over the suspension. Microscopic evaluation of singlets, doublets, and higher multiplicity of cells is made on each sample before and after the dispersal procedure. For each count, approximately 200 cells are counted in the 10- λ aliquot.

Exposure of the cells to sonication resulted in rupture and subsequent loss of a large percentage of the squamous cells. Sonication treatment was also frequently found to be nonreproducible. These findings suggested that disaggregation of cervical cells by sonication alone may be unacceptable. Subjecting a cervical specimen to the shear forces of a 21-gauge hypodermic needle and syringe has also been explored. The high shear forces do not produce disaggregation of all clumps. With appropriate enzymatic treatment, this procedure may hold some promise.

Because proteases are used in tissue culture to disperse monolayers of growing cells, it was thought that similar protocols could be applied to the dispersal of cervical cells. Pepsin, trypsin, and trypsin/ethylenediaminetetraacetate (EDTA) mixtures were tried. A large increase in fraction of single cells was obtained with pepsin treatment; however, high cell loss, coupled with changes in cellular morphology, occurred as revealed by Papanicolaou staining. Trypsin and trypsin-EDTA techniques did not have any effect on subsequent Papanicolaou staining; however, there was a loss of 25-50% of the initial cell population. Cell loss is attributable to the fact that cells obtained by the usual Papanicolaou technique are dead or degenerate. The

membranes of these cells are no longer resistant to tryptic action, and the enzyme totally digests some of the cells. α -Chymotrypsin treatment was also investigated, and although it yielded 95% singlets cell loss reached 50% on occasions. In summary, protease action results in high cell loss, making these procedures unacceptable. Hyaluronidase in various concentrations was also investigated as a chemical cell dispersal agent. Treatment with this enzyme resulted in 50-60% single cells; however, cell loss $\approx$ 33%, indicating that this approach is unacceptable. It appeared from these experiments that hyaluronidase was not the enzyme of choice.

Crude collagenase was used to test its ability to digest intercellular bonds. Cervical specimens are suspended in saline G after removal from the patient. The cells are resuspended in 0.05 M Tris (pH 7.5) containing calcium chloride (0.36 mM) and collagenase (200 units/ml). The cells are incubated at 37°C for 20 min, and cell counts made after dispersal are compared to those before dispersal.

Collagenase has produced suspensions of 90-95% single cells from a cervical sample. Even more encouraging is the fact that we have very little cell loss. Since collagenase is not generally pure, there was some uncertainty as to whether collagenase or trypsin was the main agent of dispersal. The experiments were repeated using chromatographically purified collagenase, and identical results were obtained. As a result of this work, collagenase is presently the enzyme of choice to disperse typical clumps found in cervical cell specimens at the LASL.

Comparison and Evaluation of Dyes for Staining the DNA and Protein of Cells in Suspension

Efforts have been made during the past year to devise new or to modify existing fluorescent staining techniques for quantitative analysis of cellular DNA and protein. The dyes used in the staining process must meet several criteria to be of use in our FMF system. In addition to being specific for a given biochemical component, the dye of choice must be excited optimally near the available wavelengths of the argon-ion laser system presently in use in the FMF. Only a limited number of fluorescent dyes are available which meet both of these criteria.

The dye acriflavine has been used routinely over the past several years as a Schiff reagent in the Feulgen procedure and has been extremely useful in numerous investigations involving cell-cycle analysis¹⁻⁴ as well as comparisons of the DNA content of diploid and heteroploid cell lines as a function of chromosome number.^{5,6} Computer-fit analysis of the DNA distributions of a number of cell types stained with acriflavine has provided an accurate account of the fraction of cells in the various phases of the cell cycle as evaluated on the basis of results obtained by independent biochemical cell-cycle analysis.⁷ Another dye, flavophosphine N (Benzoflavine, Chroma), has also proven useful as a Feulgen reagent. Results obtained using flavophosphine N are comparable to those obtained when using acriflavine as the DNA-specific reagent.

Two intercalating dyes, ethidium bromide (EB)⁸ and propidium iodide (PI),⁹ have been used to stain cellular DNA after RNase treatment of fixed cells. Both dyes emit a somewhat reddish fluorescence when excited at 488 nm; however, PI has a maximum emission peak 10 nm higher than EB. This feature makes PI especially useful in double-staining techniques for simultaneous analysis of cellular DNA and protein where the protein dye emits in the yellow-green range of the spectrum.

The DNA spectra for WI-38 and HeLa cells obtained by FMF analysis after staining with either acriflavine, flavophosphine N, EB, or PI are shown in Fig. 1. The similarity of these spectra for each cell type indicates that these dyes could be used interchangeably to stain cellular DNA. Computer-fit analysis of the DNA distributions also confirms this observation and further indicates that by either staining procedure the computed fractions of cells in G_1 , S, and $G_2 + M$ are essentially identical.

Preliminary studies have shown that another dye, mithramycin,* might be useful as a rapid DNA staining reagent.¹⁰ Mithramycin appears to be highly specific for DNA so that RNase treatment of fixed cells prior to staining is not necessary. Staining and analysis can be accomplished in about 20 min.

*Mithramycin (NSC 24559) was obtained through the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, Bethesda, Maryland.

Fig. 1. DNA distribution patterns obtained by FMF analysis of WI-38 and HeLa cells stained with either acriflavine, flavophosphine N, ethidium bromide, or propidium iodide. The coefficients of variation (CV; standard deviation divided by the mean $\times 100$) are designated for the G_1 distributions.

Cellular DNA distributions of mithramycin-stained cells compare favorably to those obtained by FMF analysis of acriflavine-stained cells.

During this past summer, a collaborative study was conducted with D. A. Freeman, presently a pre-medical student at the Dallas Medical School, to investigate a number of protein dyes and to evaluate their usefulness as cellular stains in the FMF system.¹¹ Most of the dyes commonly used to stain protein have maximum excitation peaks in the ultraviolet range and, therefore, are suboptimally excited by the wavelengths available in the argon-ion lasers currently available at this Laboratory. However, from a large selection of dyes, several were found to be useful for staining and subsequent analysis with the FMF system.

Ethanol-fixed human diploid cells, WI-38 strain, were stained for 30 min with either ANSA (8-anilino-naphtholene sulfonic acid), dansyl chloride, brilliant sulfaflavine, FITC (fluorescein isothiocyanate), RITC (rhodamine B isothiocyanate), or

fluorescamine (Fluoram). Unfixed cells were also stained with fluorescamine in physiological saline. Brilliant sulfaflavine did not appear to bind tightly to cellular protein and was leached from the cells. Protein distributions obtained by FMF analyses of cells stained by the other dyes are shown in Fig. 2. The spectra are unimodal and resemble cell volume distributions obtained from the Coulter volume spectrometer. Fluorescamine does not appreciably penetrate the membrane of unfixed cells (see Fig. 2F); however, this feature might prove useful for viably staining cells and provide for analysis, sorting, and reculturing cells of interest based on membrane-dye binding characteristics.

We have recently used double-staining techniques and the multiparameter cell analysis system to make rapid, simultaneous determinations of DNA, protein, and cell volume in single cells.¹² Electronic processing of the fluorescence and volume signals from each cell has provided a direct approach for establishing relationships of cellular properties at specific phases of the cell cycle. HeLa, L-929, and CHO cells were fixed in ethanol, treated with RNase, and then sequentially stained for DNA and protein, respectively, with PI (red fluorescence)

Fig. 2. Protein distribution patterns of fixed WI-38 cells stained with RITC (A), FITC (B), dansyl chloride (C), ANSA (D), or fluorescamine (E). Unfixed cells were also stained with fluorescamine (F).

and FITC (green fluorescence). Stained samples were introduced into a flow chamber where cell volume and red and green fluorescence were analyzed. The DNA, protein, and cell volume spectra are shown in Fig. 3. Through the use of electronic gating techniques, the relative protein distributions of cells in G_1 , S, and $G_2 + M$ can also be determined. Analysis of exponentially growing populations by simultaneous two-parameter processing of red and green fluorescence signals (see Fig. 4) permits the evaluation of the relationship between DNA and protein throughout the entire cell cycle. These techniques should prove extremely valuable in irradiation or drug studies where the proliferative capacity and gross biosynthetic capacity of a cell population are of particular interest. Using other double-staining techniques, examination of the interrelationships of other cellular biochemical components (i.e., RNA, histones, antigens, etc.) is also possible.

Fig. 3. DNA, protein, and cell volume distributions for CHO, HeLa, and L-929 cells. Protein-to-cell volume ratios (c) were obtained by utilizing both the green fluorescence and cell volume signals from each cell to produce electronically a ratio with an analog divider system. The coefficients of variation of the ratio distributions were 6.8, 6.5, and 5.3%, respectively, for CHO, HeLa, and L-929 cells. DNA-to-protein ratios were obtained similarly by analysis of both the red and green fluorescence signals for each cell. The abscissal scale (channel number) has a depressed zero but otherwise is proportional to either DNA content, protein content, or cell volume. The constants of proportionality are different for the various cell types.

Fig. 4. Complete two parameter analysis display of DNA and protein content of stained CHO cells. The top figure is an isometric display which shows the cell density in particular regions. The lower figure, which is a contour display of the same distribution, is delineated to show various regions of the cell cycle.

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BIOLOGICAL APPLICATION OF FLOW MICROFLUOROMETRY AND CELL SORTING

(L. S. Cram, H. A. Crissman, P. K. Horan, J. C. Forslund, M. R. Raju, T. T. Trujillo, J. A. Steinkamp, E. H. Clinard, and M. L. Bartlett)

Flow Microfluorometric Analysis and Evaluation of the Effects of Several Chemical Agents on Cell-Cycle Progression

Flow microfluorometry (FMF) analysis provides a unique approach for determining the relative number of cells in the various phases of the cell cycle under a wide variety of experimental conditions. These techniques, coupled with cell enumeration and autoradiographic studies, offer a powerful method for analyzing the effects of various chemical agents on cell-cycle progression.

In a collaborative study conducted with Dr. R. A. Tobey of Group H-9, FMF techniques were used to examine the effects of three nitrosourea analogues, BCNU, CCNU, and Me-CCNU, on cell-cycle traverse in synchronized cultures of line CHO Chinese hamster cells.¹ Cells were treated with the agents following release from G₁-arrest and analyzed at intervals to determine the fraction of cells in each phase of the cell cycle. BCNU-treated cells were delayed by 2.5-4.0 h in entering S phase, entered and exited

from S at a lower rate than controls, and synthesized DNA at a reduced rate. Entry into G_2 was delayed by nearly 8 h (part of the delay due to the delay in entering S), and the rate of progression through G_2 was decreased relative to the control as was the fraction dividing and the rate of cell division. Cells treated with either CCNU or Me-CCNU entered S phase at the same rate and time as the control culture, but progression through S (i.e., rate of DNA synthesis) was slowed to a greater extent than in BCNU-treated cells. Upon entering G_2 after a delay period of 8-10 h, the rate of entry was similar to the control rate, but few cells were able to escape from G_2 . The G_2 -arrested cells were larger than normal, implying a state of biochemical imbalance, and the few dividing cells experienced mitotic nondisjunction. Figure 1 shows the DNA distribution patterns of cell populations treated for 32 h with the various drugs.

In another study conducted with Dr. A. L. Kisch of the University of New Mexico School of Medicine, the effects of 2% dimethyl sulfoxide (DMSO) on the growth of polyoma virus-transformed clonal line (C1-I) of baby hamster kidney fibroblasts (BHK-21) were investigated.² The C1-I cells adapted to replication in the presence of DMSO and exhibited a rapidly reversible phenotypic reversion of a number of properties characteristic of the transformed state. Density-dependent growth inhibition with accumulation of cells in the G_1 phase of the cell cycle occurred

and was associated with restoration of contact-dependent behavior and with reversion of histological and ultrastructural features toward those which characterize untransformed cells. The C1-I cells grown in DMSO also lost the ability to form colonies in semisolid medium. The data suggested that DMSO alters the expression of cellular functions which were altered as a result of viral transformation and which may be involved in cell tumorigenicity.

A preliminary study was initiated, during the latter part of this year, in collaboration with Dr. Harry Eagle of the Albert Einstein College of Medicine, Bronx, New York, to determine the effects of pH on cell-cycle progression. These studies are still in progress.

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Soluble Antigen/Fluorescent Antibody Test for Detection of Antibodies to *Trichinella spiralis* and Hog Cholera Virus in Hog Sera

In January of this year, work was started at the LASL on evaluation of the soluble antigen/fluorescent antibody (SAFA) test for detecting antibodies to *Trichinella spiralis* and hog cholera virus (HCV) in swine serum. This test had been shown by others to be sensitive and reliable in detecting antibodies to a variety of viral, bacterial, and parasitic antigens.¹⁻³ Development of a newer serological test, the enzyme-labeled antibody (ELA) test, was already underway at this Laboratory. We thought the SAFA test would serve as a comparative method in developing ELA, and since the two tests are closely similar in principle and technique, information obtained from a careful study of the SAFA reactions would be readily applicable to the ELA procedure. While we anticipated that ELA might prove to be the more sensitive of the two tests, it seemed that the SAFA, being more thoroughly tested in a number of immunological systems, might prove the more acceptable

Fig. 1. FMF-Generated DNA distribution patterns for the initial culture at $t = 0$ at the time of isoleucine restoration (A) and for the cultures treated for 32 h with 3 $\mu\text{g/ml}$ BCNU (B), 30 $\mu\text{g/ml}$ CCNU (C), or 10 $\mu\text{g/ml}$ Me-CCNU (D). The approximate number of cells in these cultures varied between 25 000 and 50 000.

test for immediate use if shown to be effective in detection of *T. spiralis* and HCV in hogs. The long-range goal of this work is implementation of one of these serological tests for detection of multiple diseases.

The unmodified Sadun and Gore¹ protocol was originally used, as outlined below:

(1) A 6-mm cellulose acetate disc is soaked in antigen and dried overnight.

(2) The antigen-coated disc is incubated in serum suspected of containing antibody to the antigen for 45 min. The disc is then washed for 10 min, three times, to remove unbound serum.

(3) The disc is incubated for 40 min in antiserum against the species of animal whose serum is being tested. This antiserum ("conjugate") has been conjugated previously with a fluorescent dye [fluorescein isothiocyanate (FITC)]. The disc is again washed for 10 min, three times, to remove unbound conjugate.

When the disc is exposed to ultraviolet light, the degree of fluorescence observed will be a function of amount of conjugate bound in step 3 (above) which, in turn, will be a function of the amount of antibody present in the suspected serum and bound to the antigen in step 2.

Several experiments were performed to establish optimal assay conditions (i.e., to assure maximum sensitivity in distinguishing negative from positive sera). These experiments involved independently varying the concentrations of reagents, time of incubation with antiserum and conjugate, and time and number of washing steps. An example of the kind of information obtained is shown in Fig. 1. These data show that, after 10 min of incubation in antiserum, the fluorescence of the discs, after treatment with conjugate, is 77% of that obtained when the discs are incubated for 45 min in the antiserum. Because of experiments of this type, it is possible to reduce the time required for the SAFA test from 2.5 h to 35 min.

Specificity and reproducibility of the SAFA test have been evaluated. Discs were incubated in unconjugated (nonfluorescent) rabbit antiswine serum before incubation with FITC-conjugated rabbit antiswine serum. The 50% reduction in fluorescence indicates that the test is due to an immunological

Fig. 1. Discs coated with *T. spiralis* antigen were incubated for varying times in normal hog serum (○) or hog serum containing antibodies to *T. spiralis* (●). The discs were then washed and incubated for 40 min in rabbit antiswine serum. Fluorometer reading = fluorescence of the discs in percent transmission as measured in a Turner fluorometer.

specificity. Replicate discs used to test reproducibility show a standard deviation of less than 10%.

A modification of a method described by Gould⁴ was used to produce a large supply of *T. spiralis* antigen. Rats were infected with 3000-4000 larvae and sacrificed 6 wk later. After lyophilization of minced rat muscle, the larvae were extracted with absolute ethyl ether and then with buffered saline. Lipid-free saline extract was lyophilized and then tested for antigen activity in the SAFA test. This antigen was compared with antigen given to us by Sadun and Gore, a commercially prepared antigen, and a stichosome granule preparation given to us by Dr. Dickson Despommier, School of Public Health, Columbia University, New York City. Our antigen was shown to be as active (stichosome granule) or more active than the others.

At the time this project was started, no data had been published on the use of the SAFA test for detection of antibodies to *T. spiralis* in experimentally infected hogs. It was of major importance in evaluating the test that we obtain information as to the earliest date after infection when antibodies could be detected by the SAFA test.

Each of the four hogs was infected with approximately 12 000 *T. spiralis* larvae by feeding infected meat. Sera were taken before infection and twice a week thereafter for 7 wk. The sera were titrated using the original protocol of Sadun and Gore.¹ A summary of the results is shown in Table I.

ANTIGENIC RESPONSE OF SWINE TO EXPERIMENTAL
INFECTION WITH *T. SPIRALIS* MEASURED BY SAFA TEST

Hog	Cysts/g ^a	Titer (days post-infection)				
		0	4	7	11	14
A	T 254	-	-	-	-	1:16 (peak days 35-42 1:128)
B	D 100	-	-	-	-	1:64 ^b (peak days 14-42 1:64)
C	D 260 T 187	-	-	+1:2	1:16 ^c	(peak days 25-28 1:256)
D	D 400 T 95C	-	-	+1:2	1:16	(peak days 25-42 1:64)

^aCysts/g of diaphragm and/or tongue were estimated by P. M. Giles (Group H-6) using either the projection and/or digestion method developed by him.

^bPig B was still positive at 1:4 at 22 wk post-infection.

^cPig C was sacrificed on day 28.

Sera were taken twice a week ending on day 42 post-infection. T = tongue, and D = diaphragm.

The data showed that sera were positive by the SAFA test, beginning on the seventh to fourteenth days after infection. Information on the number of cysts per gram of diaphragm or tongue, obtained by P. M. Giles of Group H-6, indicates a moderate level of infection, though not at the minimal level we would hope to be able to detect. Nevertheless, at this level of infection, SAFA detected antibody in these sera well before the seventeenth day when the meat would become infective for a second host. With respect to the duration of positive titers, it is noteworthy that pig B was still positive at 1:4 when it was sacrificed 22 wk after infection.

A shortened protocol for performing the SAFA test has been devised which should be amenable to automation and large-scale screening for multiple diseases. Experience has been gained in preparing an adequate antigen from *T. spiralis* for use in the SAFA test. SAFA procedures can detect moderate *T. spiralis* infections in hogs as early as seven days and not later than 14 days after infection, and titers may persist at least up to 22 wk post-infection. We are now prepared to determine the sensitivity* and specificity** of SAFA by screening

* Sensitivity = number of infected animals giving positive result to number infected.

** Specificity = number of noninfected animals giving negative result to number noninfected.

fairly large numbers of unidentified positive and negative sera and to make a critical comparison with ELA.

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Rapid, Differential Diagnosis of Newcastle Disease Virus

Several strains of Newcastle Disease Virus (NDV) have been isolated from chickens in the United States most strains are relatively harmless and result only in loss of production. Recent outbreaks of a new and virulent strain that produces death in less than 6 days have necessitated a rapid, differential test for identification of this particular strain. The plaque assay designed to differentiate velogenic strains of NDV requires 7 to 10 days for completion. We are developing a rapid, differential assay that requires about 2 to 3 days to complete.

Because only small differences exist among the NDV strains, we are not using immunological techniques to classify the causative agent but are measuring the effect of NDV infection on cellular systems. Highly virulent strains of NDV reportedly produce more cell fusion than do less virulent strains.¹ Quantitation of cell fusion using the microscope is both slow and difficult. Flow microfluorometry (FMF) techniques have the advantage of being able to measure rapidly and quantitatively the extent of cell fusion which results in more DNA peaks than normal in the fluorescence spectrum obtained by FMF measurements. A correlation between degree of cell fusion and virus virulence is being sought.

About 14 well characterized NDV strains have been grown up for these experiments, and their titers were determined by plaque assay. Cell fusion induced by four NDV strains grown on chick embryo fibroblasts (CEF) is illustrated in Fig. 1. CEF cells were infected at a multiplicity of infection (MOI) of 100 and incubated 16 h. The cells were dispersed and fixed with formalin, and their DNA was stained using an acriflavine-Feulgen technique. The DNA distribution of control or noninfected cells is illustrated by the solid line in Fig. 1 and is typical for randomly growing cells. It is clear from Fig. 1 that, of the NDV strains used, Fontanna (the most virulent) produced the greatest amount of cell fusion. The relative degree of fusion of other NDV strains is easily visualized. Preliminary data indicate a good correlation between virus virulence and degree of cell fusion.

To understand the variables involved in making cell fusion measurements, several experiments were conducted to determine the dependence of the degree of cell fusion on parameters such as MOI, time of virus incubation, and cell density at time of virus infection. Multiplicity of infection was found not to be an important parameter for cell fusion measurements, provided a MOI of greater than 50 was used. FL cells were infected with the Texas CB strain of NDV (MOI = 100) and allowed to incubate for 4, 12, 16, and 20 h before dispersal and fixation. Cell fusion was found to reach a maximum at approximately 16 h. In a similar manner, the degree of cell fusion was measured at increasing cell densities. Above a cell density of about 5 000 000 cells per T-75 Falcon

flask, the degree of cell fusion no longer increased with increases in cell density. All experiments consequently utilized confluent cell monolayers.

Some strains of NDV induced different levels of cell fusion relative to one another when allowed to fuse different cell lines. For example, Roakin induced slightly more fusion on FL cells than did Fontanna; the reverse was true on CEF cells. This differential sensitivity of a particular virus to cause cell fusion will be very useful in categorizing the virus and in studying its epidemiology.

Recent experiments have been directed toward distinguishing NDV strains that are essentially identical in all other characteristics. Two very similar lentogenic strains, B₁ and LaSota, have been shown to cause different levels of cell fusion, suggesting that the FMF technique provides much greater sensitivity for studying small changes in viral mechanisms than do other techniques.

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Quantitative Evaluation of Fluorescent Conjugates

This project is a part of the Los Alamos Scientific Laboratory's effort for assisting the U. S. Department of Agriculture (USDA) in the development of low cost, high-speed, automated instrumentation to screen large numbers of animals for signs of hog cholera virus (HCV). Presently accepted procedures require high-quality fluorescent conjugates (either direct or indirect) for identifying the HCV antigen or its antibody. In order to improve the present quality of conjugates, we are developing techniques to quantitate their brightness and specificity. Effort is also being expended toward making the PK-15 cell/HCV system more reliable and stable, which is necessary for routine animal screening operations.

Figure 1 is a typical fluorescence distribution of control and HCV-infected PK-15 cells fixed and treated with fluorescein-labeled hog cholera antibody. Infected cells are typically four to six times brighter than control cells, depending on the degree of cell infection and the conjugate used. Because the relative brightness of the two peaks

Fig. 1. Fluorescence distributions of normal and NDV-infected chick embryo fibroblasts.

Fig. 1. Fluorescence distribution of PK-15 cells infected with HCV and noninfected PK-15 cells. Both samples were reacted with fluorescein-labeled hog cholera antibodies.

depends, in part, on the conjugate, FMF provides a means of evaluating conjugate specificity. Non-infected PK-15 cells and PK-15 cells maximally infected with HCV were both fixed in acetone, washed, split into equal amounts, and incubated with diluted conjugate. To date, 36 lots of hog cholera conjugate have been evaluated successfully, and typical results are presented in Table I. The measure of conjugate usefulness is signal (infected cell brightness) -to-noise (noninfected cell brightness) ratio.

The FMF conjugate evaluation technique can detect small improvements made in conjugate production, thereby permitting exploitation of those changes resulting in a better signal-to-noise ratio. Such a procedure has been followed to improve the dialysis labeling technique (binding of fluorescein isothiocyanate to the IgG serum fraction) further. Figure 2 illustrates the results of this project. It can be seen from the graph that a dialysis time of 60 to 120 min gives the best result (i.e., the best signal-to-noise ratio).

Background experiments were conducted to investigate the importance of the variables involved in our conjugate evaluation protocol. It was found that the signal-to-noise ratio did not depend on conjugate dilution (up to 1:256), nor was it affected by the length of time the cells and conjugate was incubated at 37°C. The absolute brightness of the cells does increase with time but has essentially reached a maximum at 20 min (37°C) incubation time used in our standard procedure. The time parameter was studied at only one conjugate dilution

TABLE I
RESULTS OF HOG CHOLERA CONJUGATE EVALUATION

Conjugate Lot Number	Average Cell Brightness Control	Brightness Infected	Signal-to-Noise Ratio
<u>Experiment No. 1</u>			
62302	16	82	5.1
62305	82	163	2.0
62305p	15	24	1.6
<u>Experiment No. 2</u>			
62302	20	80	4.0
62306	43	126	3.0
62307	40	128	3.2
<u>Experiment No. 3</u>			
62302	16	59	3.7
62306	34	99	2.9
62309	15	78	5.2
<u>Experiment No. 4</u>			
62302	17	70	4.1
IMN2 ^a	462	1358	2.9
IMN3 ^a	362	1023	2.8

^a Indirect or antispecies conjugates. HCV antiserum (lot number 60202) was used at a 1:5 dilution. All values have been normalized to a photomultiplier high voltage of 852 volts and a gain of 15. Conjugate concentration for all conjugates was 0.02 (1:48).

Fig. 2. Effect of dialysis labeling time on conjugate performance. The relative brightness was derived from FMF data.

(1:32) for conjugate lot number 55. Using conjugate lot number 62302 at final dilutions of 1:8, 1:24, 1:48, 1:72, 1:132, and 1:144, the increase in cell fluorescence as a function of incubation time (5 to 30 min) at 37°C was measured. The shape of the resulting curves for all dilutions remains essentially unchanged from lot number 55 results at a dilution of 1:32. Thus, for the dilution range studied after an incubation of 20 min, the conjugation reaction has reached a level where small variations in time are not important.

The FMF conjugate evaluation technique has been used to compare 36 conjugates, thus providing guidance for improving methods of conjugate production. Within the limits of current conjugation procedures two parameters, conjugate incubation time and conjugate dilution, have been shown not to affect the signal-to-noise ratio used to evaluate conjugates.

Sensitivity of Tumor Cell Detection

Demonstration of the capability of the Los Alamos multiparameter cell sorter to distinguish heteroploid tumor cells from normal cells has been reported elsewhere.^{1,2} However, if these techniques are to be used as a diagnostic aid in the detection of cancer, it is important to know the limits of detection. To define those limits, predetermined mixtures were made of tumor cells and spleen cells (normal). Whole spleen and cultured MCA-1 cells were dispersed and stained separately as described previously.² Cell counts were made with a Coulter counter to determine the number of cells in each sample. Mixtures of tumor cells and spleen cells were made in the ratios of 1:99, 2.5:97.5, 5:95, and 10:90. These mixtures, as well as the samples which were 100% spleen and 100% MCA-1 cells, were analyzed using the high-speed, flow-analysis system.

In all four pairs of curves shown in Fig. 1, the curve with the solid dots represents the DNA distributions of a specimen which is 100% spleen cells. The distributions are plotted on a semilog plot to enable differences between the mixtures and spleen cells to be observed when nearly 10^5 cells are accumulated in the G_1 spleen peak. The G_1 diploid spleen cells are in channel 10. Since the MCA-1 cells are nearly tetraploid, no MCA-1 cells should be found in channel 10. Therefore, when analyzing the mixed specimens, sample was accumulated

Fig. 1. DNA distributions for spleen cells (—•—) and mixtures of n% tumor cells with spleen cells (— —). In all four pairs of graphs, the specimen containing the mixture of tumor and spleen cells was accumulated until the number of cells in the G_1 peak was identical to the number of cells in the specimen containing only spleen cells.

until the number of cells in channel 10 of the spleen plus n% MCA-1 specimen was identical to the number of cells found in the spleen specimen. As can be seen in Fig. 1, this type of G_1 diploid normalization permits the heteroploid tumor cells to be displayed above the diploid spleen curve. The $G_2 + M$ tumor cells and the tumor cells of higher ploidy are seen in channel 40 and above. The peak in channel 30 is a result of clumps of G_1 spleen cells with $G_2 + M$ spleen cells.

To ensure that the surplus above the spleen specimen is indeed MCA-1 tumor cells, the difference spectrum was plotted and compared to the DNA spectrum of pure MCA-1 tumor cells as measured by FMF.³ As seen in Fig. 2, the point-by-point subtraction of the two curves [(spleen plus 5% MCA-1) - spleen] yields a DNA distribution nearly identical to the one obtained on the FMF for pure MCA-1 tumor cells.

Fig. 2. Comparison of DNA spectrum for 100% MCA-1 tumor cells with difference spectrum [(spleen plus 5% MCA-1) - spleen]. This demonstrates that the cells being detected as a difference between the two spectra are MCA-1 tumor cells.

Therefore, we can conclude that the area between the curves labeled "spleen" and the curve labeled "spleen plus n% MCA-1 tumor cells" is a measure of the number of tumor cells in the specimen. It is evident from the curves in Fig. 1 that near-tetraploid tumor cells can be detected in a sample which has as few as 1% tumor cells in a mixture of 99% normal cells.

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Application of FMF Instrumentation and Cell Sorting to Problems in Radiation Biology Related to Radiotherapy

The distribution in the cell cycle of KHT sarcoma tumor cells in mice and the changes in distribution as a function of dose and time after exposure to 250-KVP x-rays have been reported in our previous annual report. These studies clearly demonstrated that a dose of 300 rad could induce significant changes in the distribution of KHT tumor cells in their cell cycle, reverting back to normal distribution 24 h after exposure. These results have been accepted for publication in the *British Journal of Radiology*.

Encouraged by these results, similar studies have been initiated on tumor samples from cancer patients undergoing radiotherapy in Albuquerque. Preliminary studies by Horan and Romero¹ using FMF techniques indicated that some breast carcinomas, Burkett lymphoma, and rhabdomyosarcomas had DNA content very nearly the same as normal diploid cells. However, a significant fraction of tumors, such as squamous cell carcinoma of the cervix, have elevated DNA. Since normal diploid cells are quite often present in the tumor samples, FMF instrumentation is useful to detect changes in distribution in the tumor cell cycle as well as the appearance of tumor cells during radiotherapy for tumors with elevated DNA. Hence, tumors that have elevated DNA alone are followed during radiotherapy treatment.

We have looked so far at about 25 cervical samples obtained by the use of dacron swabs from patients undergoing radiotherapy treatment. The DNA distribution of most samples was just as good as those obtained from experimental mouse tumors. So far we have been able to obtain samples from only one patient undergoing radiotherapy. This patient had squamous cell carcinoma of the cervix. All

Fig. 1. DNA profiles of squamous cells after acriflavine-Feulgen staining. (A) A normal patient. The specimen contained superficial squamous cells and leukocytes. (B-F) DNA profiles of specimens taken from an 85-year-old Indian woman with carcinoma of the cervix. Dates of specimen collection and total accumulated dose the patient received are given in each panel. The smooth curves are not computer-fit but drawn by the illustrator.

samples were stained with the DNA-specific acriflavine-Feulgen technique. These stained cells were analyzed in the microfluorometer with cell separation capability. Figure 1A shows the DNA distribution of exfoliated cells from a normal patient, and Figs. 1B-1F are from the patient with squamous cell carcinoma during various stages of radiotherapy treatment. The x axis in all figures is the channel numbers proportional to DNA content, and the y axis is frequency. Two peaks were observed in Fig. 1A: one small peak corresponding to channel 11 and another large peak corresponding to channel 19. By separating the cells in these peaks using the cell separator, it was confirmed that the cells in the first peak were white cells and that the cells in the large peak were normal diploid cells. Strictly speaking, both white cells and normal cells should have the same DNA content. White cells seem to stain less intensely than normal exfoliated cells. Hence, the peak due to white cells occurred at a lower channel number than normal cells; this is a staining artifact.

The samples from the patient undergoing radiotherapy treatment were analyzed with slightly different settings; therefore, the channel numbers

corresponding to normal cells were indicated by arrows in these figures. Figure 1A is after two daily treatments of 300 rad each. The peak in channel 28 corresponds to the G_1 phase of tumor cells, and the DNA content of tumor cells in this phase is nearly 1.5 times that of normal cells. There is also another peak in channel 58, corresponding to tumor cells in the $G_2 + M$ phase. The DNA content is nearly twice that of the G_1 phase. The cells in between these two peaks are in S phase. Hence, there is a small but significant proliferating component. There are very few normal cells in this sample. After 7 treatments of 300 rad each, the distinct peak due to tumor cells disappears and there is a continuous DNA distribution corresponding to normal and tumor cells. However, there was a large, distinct peak corresponding to white cells. At time of treatment, the patient had severe infection which continued during the major part of her treatment. The increased number of counts at low channel numbers is due to debris, probably as a result from lysis of dead cells. The DNA distribution of samples after integrated doses of 2700 and 3450 rad is similar to that in Fig. 1C, with decreasing number of tumor cells with increasing dose. After an integrated dose of 4200 rad, there was a distinct peak due to normal cells and a small but significant fraction of tumor cells and practically no white cells. At this time, the patient had no infection.

Figure 2 shows the DNA distribution of cells

Fig. 2. DNA profiles after computer normalization to total cell number between channels 17 and 21. The normalized curves are re-plotted in a semi-log fashion.

from the patient after an integrated dose of 4200 rad, along with a DNA distribution of normal cells in Fig. 1A with the number of normal cells in both samples normalized. There are nearly 8% of the tumor cells present at this stage of the treatment. We cannot tell from these results whether the tumor cells are sterilized.

These results clearly indicate that, in tumors with elevated DNA, FMF instrumentation can be very useful in monitoring changes in distribution of tumor and normal cells during the course of radiotherapy. Nearly optimum fractionation schedules and integrated doses in radiotherapy using conventional radiations were derived empirically with 50 years of experience. By monitoring the changes quantitatively with FMF instrumentation along with histopathological observations during radiotherapy with conventional radiations, one can probably use these data to decide the course of radiotherapy treatment with unconventional radiations such as fast neutrons, negative pions, and heavy ions.

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Age Distribution Studies of EMT6 Tumor Cells *in vivo* and *in vitro* Using FMF Instrumentation

We reported in last year's annual report the age distribution studies of KHT sarcoma tumor cells in mice and the changes that take place after radiation exposure. In KHT sarcoma, nearly 75% of the cells were found to be in G_1 , and the rest (25%) were in the S and $G_2 + M$ phases.

Figure 1 shows the age distribution of EMT6 tumor cells *in vivo*. The first large peak was due to normal diploid cells and the second peak to tumor cells in G_1 . The fraction of tumor cells in S and $G_2 + M$ was very small. Figure 2 shows the age distribution of cells *in vitro* at 12 and 36 h after plating. It can be seen that the fraction of cells in S and $G_2 + M$ under cultured conditions is much higher than *in vivo*. Also, there are some significant changes in this fraction (S and $G_2 + M$) with time after trypsinization, and these changes could be due to partial synchrony caused by the trypsinization process.

Fig. 1. DNA distribution of EMT6 tumor cells *in vivo*.

Fig. 2. DNA distribution of EMT6 tumor cells *in vitro* at 12 h (a) and at 36 h (b) after plating.

In spite of these changes in age distribution *in vivo* and *in vitro*, remarkably similar x-ray cell survival was obtained by Rockwell.¹ Age response studies of this cell line by Rockwell also indicate

that the variation with cell cycle for x-rays is considerably less than for some of the other cell lines. This could explain the similarity in radiation sensitivity of the EMT6 tumor cell line *in vivo* and *in vitro*.

We have also made some preliminary measurements of age distribution of ascites tumor cells using FMF instrumentation in collaboration with Professor Yosh Maruyama, University of Lexington, Kentucky. We have found that the fraction of cells in S and $G_2 + M$ decreases with age of the tumor in accordance with the previous measurements made by Maruyama.

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Quantitative Measurement of SV-40 T-Antigen

After monkey or hamster cells are infected with Simian virus (SV-40), a new antigen (T-antigen) appears in the nucleus of the cell.¹ This antigen is a protein of approximately 70 000 daltons,² but at the present time the functional role it plays in the lytic or transformed state is unknown. Using indirect immunofluorescence³ and flow microfluorometry,⁴ it is possible to measure quantitatively the fluorescence intensity of each cell.

Uninfected CV-1 cells (African green monkey kidney cells) were fixed and immunofluorescently stained as described previously.⁵ Cells were analyzed one at a time, yielding the fluorescence intensity distribution shown in Fig. 1A. As can be seen, the peak is obscured by the presence of a large amount of fluorescent debris. The debris consists of ruptured cells due to the many manipulations required to stain the cells. Since it was inevitable that debris would be present, it was necessary to clean up the signals electronically using a method developed at the LASL.⁶ Light-scatter intensity was recorded as described above. As seen in Fig. 1B, there are a large number of particles which scatter light with an intensity near channel 30. These are intact cells. Clumps and debris scatter light with intensities in the crosshatched areas. Thus, the electronics were set up to interrogate simultaneously

Fig. 1. (A) Fluorescence profile of uninfected CV-1 cells. The cells were stained using the indirect immunofluorescence technique for SV-40 induced T-antigen. (B) Low-angle light-scatter profile for cells whose fluorescence profile is shown in (A). Cross-hatched areas are scatter intensity region representing debris of less than cell size and clumps of cells. Nonhatched region represents scatter intensities of whole cell size. (C) Gated fluorescence profile of uninfected CV-1 cells. The electronics were set up to ignore fluorescence of particles whose light-scatter intensities are represented by the crosshatched regions of (B). These are the fluorescence intensities of particles whose light-scatter intensities are within the nonhatched region of (B).

both the light-scatter signal and the fluorescence. If the particles showed signal characteristics of cells, then the fluorescence was recorded. If the particle scattered light with an intensity equal to whole cells, the fluorescence was recorded. This gated fluorescence distribution is shown in Fig. 1C. Thus, the data of interest were extracted out of the background noise.

The same procedure was performed for both SV-40 infected cells and noninfected CV-1 cells. As seen in Fig. 2, both assays yielded discrete peaks. When hamster anti-T antiserum was used at a dilution of 1:64 and fluoresceinated goat antihamster antiserum was used at a dilution of 1:4, the modal peak of noninfected CV-1 cells at 36 h post-infection (multiplicity of infection of 50) was channel 23, while the modal peak of infected CV-1 cells was channel 171 (channel 19 x gain factor of 9). This means that the infected cells fluoresce 7.4 times more brightly than control cells.

To determine the region of antibody excess, CV-1 cells infected for 36 h and uninfected control cells were reacted with different concentrations of hamster (anti-T) antisera. Eight reaction tubes were set up with equal numbers of infected cells. Each tube was exposed to a different concentration

Fig. 2. (a) Gated fluorescence profile for uninfected CV-1 cells. A 1:64 dilution of hamster anti-T antisera was used as well as a 1:4 dilution of fluorescein conjugated goat antihamster antisera. The modal fluorescence intensity was 23. (b) Gated fluorescence profile for SV-40 infected CV-1 cells. The same dilutions of antisera as in (a) were used. Electronic amplifier gain was 1/9 that used in (a). Hence, modal fluorescence intensity was $(19 \times 9) = 171$. The modal fluorescence of SV-40 infected cells was 7.4 times the uninfected cells.

of hamster anti-T antiserum (from undiluted to 1:128). After adsorption and washing, all tubes were reacted with a 1:4 dilution of fluoresceinated goat antihamster antiserum. Each sample was analyzed using the FMF instrument, and the modal channel of each peak was taken as a measure of the fluorescence intensity of each sample. The same procedure was used for the control cells. The relative fluorescence intensities for control CV-1 cells and infected cells are shown in Fig. 3.

The fluorescence intensity (FI) of the control cells is a result of cytophlic nonspecific binding. Part of the nonspecific (NS) binding is contributed by the hamster anti-T antibody (NS_1), and another portion is contributed by the goat antihamster antibody (NS_2). The resulting fluorescence is a linear combination of the two contributions ($Control_{FI} = NS_1 + NS_2$). The fluorescence intensity of infected cells resulted from both components of nonspecific binding and the specific (S) binding to T-antigen ($Infected_{FI} = S + NS_1 + NS_2$).

To determine the effect of concentration of hamster anti-T on the specific component, we merely subtracted $Control_{FI}$ from $Infected_{FI}$ at each dilution. The resulting difference is the fluorescence intensity associated with the specific binding. As shown in Fig. 4, this difference plot increases until it saturates at a relative antibody concentration

Fig. 3. Relative fluorescence intensity of non-infected and SV-40 infected CV-1 cells. A 1:4 dilution of fluorescein conjugated goat antihamster antisera was used at each dilution of hamster anti-T antisera. The modal fluorescence intensity was used as the measure of relative fluorescence.

Fig. 4. Specific (T-antigen) fluorescence intensity as a function of hamster anti-T concentration obtained by subtracting the value for the control fluorescence intensity (nonspecific fluorescence) from the value for the infected fluorescence intensity [specific fluorescence (T-antigen) plus nonspecific fluorescence].

of 0.25 (1:4 dilution). The increasing region of the curve is the region of T-antigen excess, and only at concentrations of 0.25 or greater is antibody excess achieved.

The data presented in Fig. 1 indicate that some electronic gating is necessary in this system. In other studies using direct immunofluorescence and FMF, this electronic gating was unnecessary.⁷ It is also evident from Fig. 3 that, as the antibody concentration is increased, the nonspecific fluorescence increases proportionately. It should also be noted that, at relative concentrations of hamster anti-T of 0.02 unit or less (Fig. 3), the intensity of the control curve does not change. This most likely is due to the relative contributions of NS_1 and NS_2 to total nonspecific fluorescence. Since conjugate 2 is used at the same concentration (1:4 dilution), the nonspecific component (NS_2) that it contributes is constant. However, at a relative concentration of 0.02 for the hamster anti-T antisera, the nonspecific contribution (NS_1) must be less than NS_2 . This would yield a constant fluorescence intensity for all relative concentrations of hamster anti-T antisera below 0.02.

The plot of specific binding in Fig. 4 yields several types of information important to future studies. The region of antibody excess (0.25 relative concentration) is where every T-antigen is

bound by an anti-T antibody. If the same serum were used, specific antigen expression could be compared for several transformed cell lines. The intensity of the saturated region would be a relative measure of T-antigen production in the cell lines studied.

This type of analysis could also be used to compare sera from different animals against a specific antigen. If the same cells were used to adsorb test sera and to produce data as shown in Fig. 4, then the extrapolation value (to relative concentration of 1.0) would be a comparative measure of antibody production against a specific antigen.

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Purification of Viral Antigens

Some of the problems encountered in serological assay systems being developed at the LASL for the detection of hog cholera virus (HCV) antibodies are caused by using lysed HCV-infected PK-15 (porcine kidney) cells as antigen. Problems include variability of the amounts of HCV antigen produced by cells, nonspecific staining resulting from the material in the PK-15 cell/virus system that is not antigenic, and maintenance of the cell/virus system.

It has been found in these investigations that the substance accounting for most antigenicity is not the virus but a soluble noninfective antigen, and attempts were made for its purification.

At the beginning of this work, it was thought that HCV was the antigen eliciting the greatest antibody response; therefore, purification of the virus was pursued. The assay used in development of these purification techniques was an infectivity assay. Two relatively mild purification methods for HCV were developed and are shown in Fig. 1.

The enzyme-labeled antibody (ELA) technique was used to test antigenicity of the virus; the test revealed that something other than the virus accounted for most of the antigenicity. Section 1 of Table I shows the results. Infectivity tests revealed that all detectable infectious material could be precipitated by 6% polyethylene glycol (PEG), yet the ELA test showed that most antigenicity was in the supernatant. This indicated that there were antigens present which were not infective. Another experiment which showed the existence of noninfective antigens was to use 6% PEG to precipitate HCV from lysed infected cells that were previously labeled with fluorescent antibody to the virus. The supernatant had three times the fluorescence of the precipitate, indicating that the fluorescent antibody was binding to a fraction other than the precipitated virus. Antigen purification was pursued using the ELA technique as the assay system. The starting

I. CENTRIFUGATION IN SUCROSE GRADIENT

Virus + Cells + Medium, freeze-thaw three times to break up cells
 ↓
 Centrifuge 12 000 x g
 Virus in Supernatant
 ↓
 Centrifuge 48 000 x g
 Virus in Precipitate
 ↓
 Resuspend in phosphate-buffered saline and put on sucrose gradient, discontinuous gradient of densities approximately 1.15, 1.17, 1.19, and 1.21
 ↓
 Centrifuge 100 000 x g 90 min
 Virus in Density = 1.15

II. POLYETHYLENE GLYCOL (PEG) PRECIPITATION

Virus + Cells + Medium
 ↓
 Centrifuge 12 000 x g
 Virus in Supernatant
 ↓
 Bring solution to 6% polyethylene glycol and 0.5 M NaCl final concentration, let sit at 4°C 12 h
 ↓
 Centrifuge 12 000 x g

Most of virus in precipitate concentrated and 50 times as pure as original material

Fig. 1. Purification of hog cholera virus.

TABLE I
ANTIGENICITY OF HCV USING THE ELA TECHNIQUE

Section	Antigen on disk	Difference between HC positive and negative sera
1	Starting material	60
	6% PEG supernatant	42
	6% PEG precipitate reconstituted to initial volume	3
2	Starting material	34
	20% (NH ₄) ₂ SO ₄ supernatant	32
	30% (NH ₄) ₂ SO ₄ supernatant	29
	40% (NH ₄) ₂ SO ₄ supernatant	25
	60% (NH ₄) ₂ SO ₄ supernatant	0
	Starting material	52
	20% (NH ₄) ₂ SO ₄ precipitate	0
	30% (NH ₄) ₂ SO ₄ precipitate	7
	40% (NH ₄) ₂ SO ₄ precipitate	53
	60% (NH ₄) ₂ SO ₄ precipitate	41
3	Starting material	64
	60% (NH ₄) ₂ SO ₄ precipitate diluted to initial volume	33
	KSCN eluted fraction	55

material was lysed HCV-infected PK-15 cells. Section 2 of Table I shows the results of ammonium sulfate precipitation. The 60% (NH₄)₂SO₄ precipitate was further purified by affinity chromatography. Serum containing HCV antibodies was brought to 35% ammonium sulfate to precipitate the globulin. This fraction was then covalently bound to an inert substrate: "Br CN-activated" Sepharose 4B. A column was made of this material, and the 60% ammonium sulfate fraction containing the antigen was put on the column. The HCV antigen bound to the HCV antibody-Sepharose complex and most other proteins washed through. KSCN successfully eluted the antigen.

The 60% ammonium sulfate precipitated antigen has been tried in the indium slide test.¹ This technique for detection of antibodies makes use of an indium-coated slide which will adsorb only a monolayer of protein and then turn dark brown. The only other protein that will bind is an antibody to the original protein. If the antibody binds, the slide turns a darker brown. This test appeared to work, but further work is necessary to make the color difference more obvious.

Yield and extent of purity of the antigen are hard to obtain, since the assay test (ELA) is not quantitative. The Oudin gel diffusion test² has been

developed to give a quantitative estimation of HCV antibodies. To pursue purification of the antigen further, this assay is being developed to be a quantitative measure of the antigen.

A search of the literature was also undertaken. This revealed that bovine viral diarrhea (BVD) and hog cholera virus (HCV) produce a common soluble antigen to which antibodies, produced in response to infection by either virus, cross-react.³ There is also another soluble antigen produced by HCV which does not cross-react with BVD. Attempts are being made to purify such HCV antigens. This might be done by binding BVD antisera to "Br CN-activated" Sepharose 4B and adsorbing out the common antigen.

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Radiation Studies Involving Tumor Cells

There is increasing interest currently in the use of flow microfluorometry (FMF) instrumentation for rapid and quantitative measurement of DNA and protein content of individual cells.¹ Fluorescently stained cells pass in single file through an argon-ion laser beam. The fluorescent light is collected at right angles to the laser beam, and the intensity is measured by a photomultiplier tube coupled through amplifiers to a multichannel analyzer. The fluorescent intensity (corresponding to channel number) is proportional to the DNA content of each cell.

This type of instrumentation has been used to measure the cell-cycle distribution changes in a rat rhabdomyosarcoma (*in vivo* and *in vitro*) after radiation exposure.^{2,3} We have previously reported the changes of age distribution of KHT sarcoma tumor cells with time after radiation treatment,⁴ and we describe herein the changes in normal and tumor cell populations of a patient with carcinoma of the

cervix during the course of radiotherapy. Preliminary studies using FMF⁵ indicated that the Burkett lymphoma and rhabdomyosarcomas have a DNA content nearly the same as normal cells. However, a significant fraction of tumor studies, such as squamous cell carcinoma of the cervix, lung carcinomas, or breast carcinomas, have elevated DNA content. Since normal cells and tumor cells are present simultaneously in specimens, DNA content as measured by FMF instrumentation is useful to detect changes in cell populations resulting from radiotherapy if tumors have elevated DNA content. In other studies, it was shown⁶ that it is possible to detect 1% tumor cells in a sample containing 99% normal cells when the abnormal cell population has more DNA per cell than normal cells.

Previous experience has shown⁷ that exfoliated cervical cells can be disaggregated using the enzyme collagenase. Cells from squamous cell carcinoma also have more DNA per cell as measured by FMF. With this information and the previous results on animal tumors, the effects of radiotherapy on squamous cell carcinoma of the cervix were studied.

All samples were stained with the DNA-specific acriflavine-Feulgen technique.⁸ These were analyzed with the FMF instrument. Figure 1A shows the DNA distribution of exfoliated cells from a normal patient, and Figs. 1B-1F are from a patient with squamous cell carcinoma during stages of radiotherapy. The x axis in all figures is the channel number proportional to DNA content, and the y axis is the cell number. Two peaks are observed in Fig. 1A: one small peak corresponding to channel number 11 and another large peak corresponding to channel number 19. By separating cells in these peaks using the cell separator,⁹ it has been confirmed that the cells in the first peak are white cells and that the cells in the large peak are normal squamous cells. Strictly speaking, both white cells and normal cells should have the same DNA content. White cells seem to stain less intensely than normal exfoliated cells; hence, the peak due to white cells occurred at a lower channel number than squamous cells. This is a staining artifact.¹⁰

Figure 1A depicts data taken after two daily treatments of 300 rad each. The peak in channel number 28 corresponds to the G₁ phase of tumor cells, and the DNA content of tumor cells in this phase was

Fig. 1. DNA profiles of squamous cells after acriflavine-Feulgen staining. (A) A normal patient. The specimen contained superficial squamous cells and leukocytes. (B-F) DNA profiles of specimens taken from an 85-year-old Indian woman with carcinoma of the cervix. Dates of specimen collection and total accumulated dose the patient received are given in each panel. The smooth curves are not computer-fit but were drawn by the illustrator.

nearly 1.5 times that found for normal squamous cells. There was also another peak in channel number 58 (nearly twice the amount of G_1), corresponding to tumor cells in the $G_2 + M$ phase. The cells in between these two peaks were in S phase. A significant fraction of cells was cycling; hence, this was an actively proliferating tumor. It should be noted that very few normal cells were present in this sample.

After 2100 rad of treatment (Fig. 1C), a large leukocyte peak was seen. At this point, a uterine infection was discovered, giving rise to a large number of leukocytes in the vaginal area. It should be noted also that a large amount of fluorescent material was seen in channel numbers 1-5. This was fluorescent debris which may be the result of radiation-induced necrosis. Furthermore, it should be noted that no distinct peaks were evident corresponding to the DNA content of normal or abnormal cells. However, comparison of Fig. 1C with Fig. 1A suggests that a large number of tumor cells are present (Fig. 1C, channel number 50). The cells in this region may be the giant cells often seen in cervical Papanicolaou smears from irradiated patients.

After 2700 rad of radiotherapy (Fig. 1D), debris and leukocytes are present. However, at this point, a peak of abnormal cells is also evident. With continuing treatment (Fig. 1E), it is evident that the abnormal peak is diminishing in intensity. However, no normal cell peak is present.

After 4200 rad of radiotherapy (Fig. 1F), a distinct peak of normal cells is present. It should be noted that no leukocyte peak is present. No signs of the uterine infection were seen at this time. While no peak of abnormal cells was present in this specimen, there was a significant fraction of abnormal cells.

In Fig. 2 the normal peak (channel number 19) was computer-normalized for curves in Figs. 1A and 1F, then replotted.⁶ This analysis makes the assumption that normal cells in Fig. 1F have the same DNA distribution found in Fig. 1A. Hence, abnormal cells are expressed as the difference between the radiotherapy patient and normal in channel numbers 28-100 (Fig. 2). From this analysis, it was found that approximately 8% of the sample in Fig. 1F was abnormal cells. Cytopathological examination also revealed the presence of a few percent of abnormal cells. At termination of treatment on November 27, 1973 (9720 rad), no abnormal cells were detected by FMF or classical Papanicolaou examination.

These results clearly indicate that, in tumors with elevated DNA content, FMF analysis can be very useful in monitoring changes in the distribution of

Fig. 2. DNA profiles shown in Figs. 1A and 1F after computer normalization to total cell number between channel numbers 17 and 21. The normalized curves are replotted in a semi-log fashion.

tumor and normal cells during the course of radiotherapy, although the changes in exfoliated cell populations may not strictly represent changes in the tumor. Nearly optimum fractionation schedules and integrated doses in radiotherapy using conventional radiations were derived empirically with 50 years of experience. By monitoring the changes quantitatively with FMF, along with cytopathological observations during the course of radiotherapy with conventional radiations, one can probably use these data to decide the course of radiotherapy treatment with unconventional radiations such as fast neutrons, negative pions, and heavy ions.

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DATA PROCESSING

(J. H. Jett and P. N. Dean)

Automatic Data Processing

1. Gamma Spectroscopy with a PDP 8/I System.

The phoswich crystals for lung counting and the 9 x 5-in. sodium iodide whole-body counter which are interfaced to the PDP 8/I computer have been in operation during the past year. During that time, several major improvements to the computer program have been made to include system efficiency in the burden calculations and an improved display routine that displays alphanumeric information such as date, count time, etc., along with the spectrum. This system has been used to count 1056 people during the year, and the counting schedule was missed once due to air-conditioning problems.

2. HUMCO II. The interface to the PDP 8/F computer was built, tested, and is in use. It provides start and stop signals, ADC interfacing logic, and numerical inputs for the computer. The computer program for the system was written and is operative. It has the capability to record spectra, calculate count rates and efficiency corrections, and then calculate the potassium and cesium levels present in humans. At the present time, the final checkout of the complete system is in progress to verify that all aspects of the new system are functioning properly.

3. PDP 11/40 General-Purpose Computer. A PDP 11/40 computer, manufactured by Digital Equipment Corporation, was delivered early in FY 1974. This computer, purchased initially as a minimum system, is scheduled to become a computer based terminal (CBT) under the HYDRA network. This will provide the Health Research Laboratory (HRL) with direct access to the central computing facility (CCF) computers, currently three CDC 6600 and two CDC 7600 computers. With this facility all computing requirements of Groups H-4, H-9, H-10, and H-11 can be handled from the HRL building. The PDP 11/40 system is currently being expanded to permit most calculations to be made in HRL on an interactive basis. This will allow immediate analysis of data with the capability of multiple, consecutive runs.

Many problems are being run on the current version of the system. Among them are analysis of the life span of hamsters exposed to plutonium ("hot

particle" project), analysis of data obtained from several scintillation counters, least-squares analysis of data from various experiments, and an initial start on a data base for the "hot particle" project. On the expanded system, we will perform such calculations as multiple exponential radioisotope retention curves by least-squares, theoretical analysis of DNA "melt" curves, life-cycle analysis of cells, karyology, and theoretical modeling of the carcinogenicity of plutonium in the lung. It is expected that this computer system will provide better utilization of personnel, more efficient usage of the CCF, and increased utilization of computer techniques by more personnel through easier access.

Analytical Support

1. Flow Microfluorometry Analysis of the Life Cycle. The annual report for 1972 described a computer program developed to analyze DNA distributions derived from flow microfluorometry (FMF). Several experiments were performed in 1973 to assess the validity of the assumption of a normal curve for the G_1 and $G_2 + M$ peaks and the accuracy of the method of resolving the FMF spectrum into its three components. To determine the shape of the peaks, two populations of CHO cells were used. One batch of cells was maintained in suspension culture until it grew to confluency and the total number of cells stabilized. A sample of these cells was measured by FMF. The spectrum obtained is shown in Fig. 1A. Almost all cells are in G_1 , and the small number of cells in other phases can have little influence on the shape of the peak which is fit very well with the normal curve. Another population of cells was synchronized by mitotic selection and measured about 15 min after selection, at which time about one-third of the cells had divided. The spectrum is shown in Fig. 1B. Virtually all these cells are in either M or G_1 . As shown in Fig. 1A, the few cells in S can have little influence on the shapes of the peaks. In this case, both peaks are fit very well with normal curves.

Autoradiographic techniques were used to validate the calculation of the fraction of cells in the S phase of the life cycle. CHO cells in exponential growth were pulse-labeled with 3H -thymidine prior to staining. In addition, a population of WI-38 cells was also pulse-labeled and analyzed by FMF.

Fig. 1. DNA distributions for two different populations of CHO cells. A population of cells was allowed to grow to confluency such that the total number of cells was not changing with time. A sample of these cells was removed from the culture, and the spectrum shown in (A) was measured with virtually all cells in G_1 . Another population of cells was synchronized by mitotic selection. The spectrum in (B) was measured 15 min after selection when the cells were partly in G_1 (the peak at channel number 30) and partly in M (peak at channel number 60). In both spectra, the solid circles are the data, the solid line is the fit to the data, and the dashed line is the broadened polynomial.

Autoradiographs were then made, and the fraction of cells in S was obtained by visual scoring. The results of these measurements are shown in Table I. The agreement between the methods is clearly excellent. To test reproducibility, a batch of exponentially growing CHO cells was separated into 10 samples, each of which was pulsed-labeled, stained, autoradiographed, and measured by FMF. The average

TABLE I
PERCENT CELLS IN S BY AUTORADIOGRAPHY AND FMF
AND PERCENT CELLS IN G_1 AND $G_2 + M$ BY FMF

S Phase Autoradiograph	FMF	G_1 Phase	$G_2 + M$ Phase
<u>CHO Cell Population (1A)^a</u>			
37.8	38.9	53.6	7.5
<u>L5178Y S-S Cell Population (3)^a</u>			
63.0	64.3	20.1	15.5
<u>WI-38 Cell Population</u>			
36.3	38.9	53.5	7.6
<u>L5178Y Cell Population</u>			
51.8	54.8	38.6	6.6

^aFigure number of spectrum is in parentheses.

percent of the cells in S was 37.4 ± 1.1 by FMF and 36.7 ± 2.1 by autoradiography.

2. General FMF Data Analysis. As a part of the FMF life-cycle analysis, as described in the 1972 annual report, the shape of the S distribution is computed. This shape is determined by the rate of DNA synthesis across the S phase of the life cycle. In principle, the shape of the polynomial used to represent the S phase then should yield information on the rate of DNA synthesis. A method of analysis analogous to the Collins-Richmond method for determining rate of volume growth is now being attempted. Calculations are also being attempted using the residual spectrum obtained by subtracting the two normal curves from the gross spectrum. This method makes no assumption about the shape of the S distribution but requires unbroadening of the spectrum. Results of these two approaches will be reported at a later date.

Karyology: Semi-Automatic Chromosome Analysis

Development of the technique described in the 1972 annual report has continued. A classification scheme has been added to the computer program, permitting the karyotyping of human chromosomes. It uses the Denver-London system of classifying chromosomes on the basis of long-to-short arm ratio. Initial tests using very good chromosome preparations were very successful. A program is now proceeding to test the system with clinical material through the help of Professor T. T. Puck at the University of Colorado Medical School in Denver.

PION RADIOBIOLOGY AND ASSOCIATED DOSIMETRY

(M. R. Raju, C. Richman, J. H. Jett, and J. M. Blossom)

Relative Biological Effectiveness and Oxygen Enhancement Ratio Measurements for Mixed Alpha-Particle and X-Ray Exposures

Cell survival measurements using human kidney cells (T_1) were made for several mixtures of high LET alpha particles from plutonium-239 and x rays under aerobic and hypoxic conditions. The survival curves were then used to calculate the relative biological effectiveness (RBE) and oxygen enhancement ratio (OER) values at 10% survival for the various mixtures of alpha particles and x rays. The results of the experiments, shown in Fig. 1, indicate that when only a small fraction of the dose is due to high LET radiation the OER is decreased significantly, while the RBE is increased only slightly. The results are consistent with RBE and OER measurements for fast neutrons and for the broadened Bragg peak of a helium ion beam.

Stage Sensitivity of CHO Cells to Alpha Particles and X Rays

The sensitivity of CHO cells to alpha particles and x rays at various stages in the life cycle has been measured. The cells were synchronized by

Fig. 1. RBE and OER values as a function of percent of the dose due to alpha particles. The circles are the OER data points and the squares the RBE data points. The solid lines were calculated by combining the alpha-particle and x-ray survival curves in the correct ratios to calculate RBE and OER. The dashed line shows where the linear variation of RBE with percent alpha dose would fall.

mitotic selection and irradiated at hourly intervals, starting 3 h after selection to allow the cells to become firmly attached to the dishes. Preliminary results indicate that, for alpha particles, there is no stage-sensitivity variation while, for x rays, the sensitivity varies by a factor of 5 or more with the cells in late S being the most resistant.

Cell Survival Measurements as a Function of Depth

Because of changes in radiation quality of negative pion beams as a function of depth, it is essential to measure the biological effects such as cell survival for these beams of different momentum spread of therapeutic interest. Palcic and Skarsgard¹ suggested an elegant method for this type of measurement. Cells are suspended in phosphate-buffered normal saline in 25% sterile gel. Such a gel is tissue-equivalent and becomes liquid at 37°C. This technique has been used for preparatory experiments using x rays. Human kidney cells (T_1) in proper concentration are suspended in a sterile gel solution at 37°C, and this is poured into a cylindrical container and kept in a refrigerator to form a gel. They are then exposed to x rays, and the gel cylinder containing the cells is pushed out using a piston. The gel is then sliced and cell survival determined by placing each gel slice in a separate Petri dish.

Figure 1 shows the results of an experiment in which the gel cylinder containing cells was exposed to 250-KVP x rays by placing it perpendicular to the beam and shielding the ends. These results provide the spatial resolution of this system. It can be seen that resolution is of the order of 2 mm, which is the thickness of each slice. It can be even further improved by cutting the gel into 1-mm slices.

Figure 2 shows the results where the gel cylinder containing the cells was placed in a water phantom in the direction of the 250-KVP x-ray beam and given two different doses at two different distances. It can be seen that cell survival for the control remains the same as a function of depth, thereby indicating that the cells were uniformly suspended. When the cells in the gel were exposed to x rays, cell survival increased with depth because of a decrease in dose with depth for 250-KVP x rays.

Fig. 1. Results of an experiment using cells exposed to 250-KVP x rays by placing the gel cylinder perpendicular to the beam and shielding the ends.

Fig. 2. Results of an experiment using cells exposed to 250-KVP x rays by placing the gel cylinder in a water phantom in the direction of the beam and exposing at two different doses at two different distances.

When the doses were measured, corresponding to 10% and 1% survival positions in both cases (as shown in Fig. 2), they were found to be the same within experimental accuracy. Some more refinements are being made, and this system is also ready to be used with negative pions.

We are indebted to Drs. B. Palcic and L. D. Skarsgard for providing us with the details of the gel technique.

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Preliminary Experiments in Radiation Biology Related to the Pretherapeutic Negative Pion Program

1. KHT Sarcoma Tumor Regression with Fractionated X Rays. We have reported the experimental results of KHT tumor regression and skin reactions for single doses of x rays in our previous annual report. The KHT sarcoma mouse tumor system was chosen upon consultation with Dr. R. F. Kallman from Stanford University because this tumor grows rapidly in a perfect spherical shape amenable for reliable tumor regression measurements. This system will also be used in the negative pion program at Stanford.

We have performed experiments for single doses and for three fractions per week (Monday, Wednesday, and Friday). Figure 1 shows the results. We had experienced some problems of geometric misses. In spite of these problems, the results are in agreement with previous results. We have designed and tested a mouse holder that will eliminate the problem of geometric miss. We are at present planning a series of experiments with different fractionation schemes relevant to therapeutic application of negative pions.

We are indebted to Dr. Kallman and his associates for their help in getting this system started at the Health Research Laboratory.

2. The EMT Tumor System. There are a few tumor cell systems developed that can be used both *in vivo* as well as *in vitro* in cell-survival experiments. One such practical system was developed by Dr. R. F. Kallman and his associates.¹ It is a mouse tumor system designated as EMT6. The end point for cell

Fig. 1. Tumor growth delay in days to go from 1 to 2 cm plotted as a function of dose for single doses and three fractions per 5 days.

survival *in vitro* is the ability of the cell to divide at least five times and *in vivo* indefinite proliferation. Because of this similarity in end points, the radiation sensitivity of cells *in vivo* is found to be nearly the same when they are assayed *in vitro*. The advantage of a tumor cell system that can grow both *in vivo* (natural environment) and assayed rapidly, quantitatively, and economically in cultured conditions. Each colony in a dish (requiring about 10 days) provides basically the same amount of information as a mouse in which a given inoculum of cells grows into a palpable tumor within about 100 days. This type of system is very useful in studying the complex changes that take place in tumors.

Barendsen and Broerse,² using the rhabdomyosarcoma tumor cell system that is also capable of growing *in vitro* as well as *in vivo*, have shown that unexpected complex changes such as repopulation of surviving tumor cells take place at a high rate in irradiated tumors compared to unirradiated tumors. Hence, systems such as these are of value in

pretherapeutic radiobiology of negative pions. We obtained EMT6 tumor cells from Dr. Kallman nearly a year ago and have been growing them successfully both *in vivo* and *in vitro*. Figure 2 shows results of cell survival when cultured cells are exposed and when the tumors in mice are exposed to x rays. Also shown are two points corresponding to the mice that are exposed to x rays after they are killed by asphyxiation. All cells from such mice are hypoxic. After exposure, the tumors were removed, and single-cell suspensions were made and assayed *in vitro*. The survival curve when tumors in live animals were exposed to x rays has two components, corresponding to oxygenated cells and hypoxic cells. The survival experimental points for tumors from dead mice correspond to hypoxic cells. One can estimate the hypoxic fractions by taking the ratio of survival of tumor cells from dead mice to tumor cells from living mice at doses around 2000 rad.¹ A hypoxic fraction of about 0.35 was obtained from the curves in Fig. 2. These results are in general agreement with those of Rockwell and Kallman.¹ The basic experiments with x rays as a preparation toward using this system for negative pions are in progress.

We are indebted to Dr. Kallman and his associates for getting this work started at the Health Research Laboratory.

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Fig. 2. EMT cell survival *in vivo* and *in vitro*. Closed circles designated "hypoxic" correspond to tumors in mice exposed to x rays 5 min after they are killed by asphyxiation.

2. G. W. Barendsen and J. J. Broerse, "Experimental Radiotherapy of a Rat Rhabdomyosarcoma with 15 MeV Neutrons and 300 KV X-Rays," *Eur. J. Cancer* **5**, 373-391 (1969).

3. The Bean Root System. Bean root growth is very sensitive to ionizing radiations. This system is ideal for measuring the oxygen enhancement ratio (OER) for negative pions. Classical work on oxygen effect was done using this system, and recently it was used extensively to study the biological effects of fast neutrons and heavy ions.^{1,2} In addition, the first determination of OER for negative pions was done using this system at Berkeley.³ We are planning to use this system, along with other mammalian cell systems, to measure RBE and OER at different points of interest for negative pion beams of different momentum spread.

Beans from England are normally used; however, we tried to use beans that are grown locally in the Rio Grande Valley. Figure 3 shows the results of growth changes of these beans for 250-KVP x rays under the presence of air and nitrogen, and these results are in agreement with previously published experiments. This system is ready for use with negative pions.

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Fig. 3. Root growth at 10 days as percent of controls as a function of dose in aerated and nitrogen atmospheres.

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Pion Beam Characteristics of Instrumentation for the Negative Pion Radiotherapy Program

Pion dosimetry necessary for this program is complicated by the mixed radiation fields emitted when negative pions stop and are captured by light nuclei. Not all products emitted are well defined. Further, the effects of muon and electron contamination add their own level of complexity. Several counters are required because no single counter of any kind can perform reliable dosimetry on such a mixture of radiations. Multiple solid-state detectors have been selected because of their high resolution and low noise with moderate cooling. A detector holder has been designed and tested with appropriate cooling. Numerous experiments on individual detectors have been performed. Readout electronics have been obtained, and improvements to these are described. Suitable water and solid (Lucite phantoms) have been designed and are nearly complete. A fast (scintillation) detector system to assist in identification of muon and electron contamination has been designed, and provision for pulse pile-up rejection has been examined. A Monte Carlo intra-nuclear cascade calculation has been performed to guide further modifications of the equipment.

1. The Problem in Pion Dosimetry. In contrast to the use of conventional modalities (i.e., x rays, gamma rays, and electrons), dosimetry of a negative pion beam is characterized by special problems. These arise because pions generate a mixed radiation field when they are stopped and are captured by a nucleus of one of the common elements in tissue (carbon, nitrogen, oxygen, and exceptionally hydrogen, calcium, and phosphorus).

Design of the dosimetric devices used in conventional or low LET modalities is based on the fact that the particle beams employed deposit energy in a diffuse manner -- over perhaps centimeters of tissue for typical megavoltage radiations. Dosimeters as large as 1 or 2 cm consequently can be used successfully. It is also important in case of such

dosimeters that all effects of the photon or electron radiation interaction are completely understood; it is almost exclusively electromagnetic in character with little or no nuclear interaction contributing to the dose. Thus, "tissue equivalence" in materials for dosimeter construction is readily achieved.

The relatively new high LET modality of cyclotron generated neutrons, when used in therapy, can be evaluated by an extension of the methods used for photons and electrons because the nuclear interactions that lead to ejection of heavily ionizing charged particles are now well understood. Neutrons have been studied for a number of years in connection with personnel dosimetry for radiation protection. Thus, commercially available dosimeters are fabricated from special materials that are simultaneously tissue-equivalent for neutrons and gamma rays.¹ Depending on the thickness of the detector, the tissue equivalence can be maintained for either macrodosimetry (measurement of the average energy deposited per mass of tissue) or microdosimetry (in which the LET spectrum is measured).² Even with a "pure" beam of negative pions, one would find many problems in dosimetry that are different from those just described:

- a. At entry into the tissue (or phantom), the pions slow down and lose energy in a predictable way, as do all fast charged particles. Conventional gamma- or x-ray dosimeters would measure the energy thus deposited. However, pions can also create "stars," or disrupted nuclei, while still moving at high velocity; this nuclear interaction may or may not annihilate the pion but will, in any case, lead to high LET deposition in the entry region.

- b. Following slowing down and stopping, the pions are captured by light nuclei. The mechanism by which the captured pions are distributed among the individual elements in the tissue is not clear at this time, but it appears that chemical effects may be important. Preliminary studies of pion and muon capture by several research groups at Dubna led to the conclusion that the pion initially replaces an electron of the molecule geometrically and that subsequent transitions to "orbits" around individual nuclei follow from a combination of electromagnetic and Auger transitions.³ (However, there are contrary opinions as to the interpretation of these data.⁴) As a consequence, it is virtually

impossible to predict what fraction of negative pions will actually cause stars from carbon, nitrogen, and oxygen nuclei. However, it is now clear that the often invoked Fermi-Teller "Z law" is not valid even for the simplest compounds.⁵

c. A deficiency exists in the state of our knowledge about the star products from oxygen, the major constituent (other than hydrogen) in all types of tissue, due to the difficulty of forming oxygen into suitable targets for nuclear physics experiments. However, it is important to note that most "tissue-equivalent" solid materials are oxygen-deficient.¹

d. The pion star products from any light nucleus create the previously mentioned mixed radiation field. A substantial fraction of the approximately 100 MeV available for nuclear disruption is believed to be taken up by neutrons (about 70 MeV), and some 20 MeV goes to singly charged hydrogen isotopes, with the remainder expected to go into heavy ions and various electromagnetic radiations. Several studies have also reported quite energetic alpha particles.⁶⁻⁸ The ranges of the known and suspected charged particles are extremely variable, ranging from perhaps 10 to 15 μm of tissue (in the case of heavy ions) to as much as several centimeters for the most energetic hydrogen isotopes.⁹ Thus, if we consider only the limited category of charged particles from pion stars, the thinnest conventional dosimeter will record microdosimetry for long-range particles but serve as a macrodosimeter for short-range particles. When neutrons and gamma rays are also taken into consideration, it is clear that no single detector or single type of detector may be used for pion dosimetry with any confidence.

The points made above are applicable to dosimetry of a pure beam, as noted. It is known from experience on all pion channels in use that some (negative) muon and electron contamination is to be anticipated, each on the order of 5 to 15% of the measured pions. The longer range of these particles creates a rather diffuse, low LET spectrum of radiation in front of, in, and beyond the pion "star" region. This effect will contribute to the dose received in therapy and must be evaluated. These light particles will penetrate any type of detector, giving rise to typically asymmetric "Landau" distributions whose breadths are such that the

distributions overlap each other at the higher energies.

The above considerations have led us to the choice of solid-state detectors (fully depleted silicon wafers or lithium-drifted silicon wafers) of various thicknesses, to be used in our preliminary studies on pion dosimetry. Such detectors are small, can be fabricated to precise thickness (down to thicknesses of a few microns), and have excellent charge-collection characteristics together with adequately short decay times for the pulses obtained. In this way, pulse-height analysis can be done. The intrinsic dynamic range of solid-state detectors can be extended by using moderate cooling to reduce thermal noise. It has been shown by Turner *et al.*¹⁰ that pulse spectra from planar detectors can be "unfolded" to yield distributions of the LET spectra from penetrating particles. To evaluate beam contamination, it has been decided to augment the solid-state dosimetric system with an array of scintillation detectors.

2. Construction of the Detectors. The thicker silicon detectors (50 μm and up) are composed of lithium-drifted silicon wafers with $\approx 40 \mu\text{g}/\text{cm}^2$ gold plating on one side and $40 \mu\text{g}/\text{cm}^2$ aluminum plating on the other. Gold filament contacts are epoxied onto the edge of the wafer on both sides. An electric field is applied such that the aluminum side is positive and the gold side is at ground potential. The wafers are rectangular, about $5.5 \times 6.5 \text{ mm}$, or $\sim 36 \text{ mm}^2$ in active area. The wafer is mounted in an aluminum ring of square cross section to avoid the effects of thermal contraction when cooling to -20°C . This assembly is then mounted in a Lucite holder 1 cm thick which contains a temperature sensor and a Microdot connector. These latter components are removed several centimeters from the detector (see Fig. 1).

The silicon-lithium wafer is mounted asymmetrically so that the active surface of the detector is about 2 mm from one face of the holder and 6 to 8 mm from the other. Removable plugs of Lucite are fitted over both sides of the detector so that when they are removed, low-energy particles from a long-lived radioactive source can be used for calibration. The holder is then attached with nylon screws to the probe (to be described below).

Fig. 1. Diagram of silicon detector system.

3. Probe Construction. The probe is designed to carry an array of four "tissue-equivalent" detectors mounted in such a way to form a counter telescope 4 cm long. One to four detectors can be arranged inside the probe, with the possibility of reversing them to provide an optimum geometry. The probe is designed to be submersible and to hold a 5-millitorr vacuum. It is also possible to circulate ethyl alcohol coolant in a channel which lies around the periphery of the central axis of the detectors. The probe is also adjustable in angle around an axis perpendicular to the beam direction. This motion proceeds in 10° -increments through a total of 180° . Four preamplifiers are mounted on the probe and located some 30 cm away from the central axis of the detector array. The probe components have been machined and are now awaiting final assembly by Group MP-8.

4. Automatic Scanner. The three-axis scanning system, now nearing completion, is expected to be delivered by January 9, 1974. The axes are powered by separate DC stepping motors which rotate $1/4$ turn per step. This rotary motion is translated to linear motion by high precision metric ball lead screws, with each step being equal to 0.1 mm of travel. Each axis is controlled by a driver module containing logic circuitry and motor drivers.

There are four modes of operation: manual, set, increment, and automatic. In each mode there are three stepping rates of 1, 50, and 100 steps/sec. In the manual mode, manual control of the stepping motors is provided for all three axes, both in direction and in start/stop. In the set mode, keyboard entry of position is provided. The desired final position of the probe is keyed in, and when

"start" is triggered, the probe moves to that position automatically. When the increment mode is selected, any desired increment (in multiples of 0.1 mm) can be set, and every time a "step" pulse is received, the probe moves that increment. During the automatic mode, a universal interface is provided for computer control of the probe position. There are four lines into the interface. A 16-bit serial register receives the digital position code from one line, and $\pm$, start/stop, and rate information are received on the other three. In addition, a position sensor gives a position readout to the computer of the probe position in the x, y, and z axes, in a 16-bit serial word.

The scanner also has zero reference capability, thus allowing the use of any point in the scanning volume as a "zero." For example, this allows a designation of the center axis of the beam as the "x-y zero" and the beam entry point on the phantom surface as the "z" direction zero.

5. Phantoms. Both water and solid phantoms utilizing layered Lucite have been built. An x-y-z scanning mechanism to hold the probe has also been assembled by Group E-5 and is lacking only the precision lead screws to be operational. A table to support the phantom and scanner is being designed by Group MP-8, and a service cart for vacuum, power, cooling, and other peripherals is under construction. A sketch of the solid phantom showing the detector arrangement is shown in Fig. 2.

6. Experiments with the 10- μm Detector. The 10- μm detector enclosed by tissue-equivalent material was built at Berkeley and has been tested extensively with an americium-241 source. The americium alpha loses 1.62 MeV in this detector, and

Fig. 2. Sketch of solid phantom with detectors.

this point in combination with a pulser is used to calibrate the detector. The leakage current is reduced to about 60 nA by cooling. An interesting result observed with this detector is that the energy loss distribution has the tail on the low energy side instead of the high energy side as is common for a Landau distribution. Poskanzer at Berkeley has observed a similar spectrum in thin detectors of about 15 μm thickness. At the present time, no one has an adequate explanation for this apparent reversal in energy loss distribution. The resolution in this detector is 1.80 keV/ μ , and the width of the 5.5-MeV alpha particle energy loss peak is about 272 keV. For different amplifications, the calibration curves of pulse-height vs deposited energy are straight lines, and the different portions of the secondary spectra with pions should be clearly identifiable. It is important to note that pions, muons, and electrons will not give sufficiently energetic pulses in this detector; only star fragments can be observed.

Monte Carlo calculations for this detector have given approximate spectra that will be observed based upon the best knowledge available of the capture process of pions. There is a good separation for different LET particles, especially if the number of particles times their energy loss is plotted as a function of pulse height. In the plateau region of the spectrum, the separation becomes very clean; since the secondary particles go mostly forward, the energy loss observed is a measure of the LET in this region of the beam.

Experiments have been carried out with a 20- μm Ortec detector which show that this is also a good detector for separating low and high LET particles. The calibration with this detector was carried out in a different manner. Carefully calibrated copper foils were used to give different energy alpha particles using americium-241. The energies obtained were 5.5, 5.05, 5.85, 4.55, and 3.45 MeV. The spectra for each alpha energy were measured with a pulse-height analyzer, yielding an energy calibration of the system. Since foils by themselves produce a Landau distribution, a lower energy alpha source has been obtained in the form of gadolinium-148, which gives a single alpha of 3.18 ± 0.01 MeV, range of 13.7 μm in silicon. In the future, this source will be used to calibrate the 20- μm detector.

7. Experiments with 50- μm Detectors. Many experiments have been carried out with the 50- μm detector, as it will see pulses of both low and high LET. For the low LET particles, a bismuth-207 source and a cadmium-109 source were used. The bismuth-207 spectrum is very useful, since it contains four monoenergetic electrons of 480, 550, 975, and 1050 keV. The 975- and 1050-keV electrons are especially useful, since they produce minimum energy loss in the detector and are, therefore, close approximations to high-energy electrons in the beam. The cadmium-109 spectrum contains two conversion electrons of 62.2 and 82.2 keV, providing low-energy calibration points.

The detector was cooled to 5.4°F, and calibration curves were obtained. The high-energy electrons lost approximately 28 keV in the detector, detectable only with cooling. The minimum ionizing particles were observed by mounting a 3000- μm detector behind the 50- μm detector. A gate was produced from the high-energy lines of the bismuth-207 spectrum, and the energy loss in the 50- μm detector was observed in the pulse-height analyzer with this gate, and the energy loss peak was very sharp and clear. The FWHM was about 14 keV. The calibration was 0.26 keV/channel. The final value of energy loss was 22.5 keV.

A second study with the 50- μm detector was to observe if the electronics could handle both lightly and heavily ionizing particles simultaneously. For this purpose, a cadmium source was mounted above the detector, and an americium-241 source was mounted below the detector. The dynamic range covered was from 80 keV to 5 MeV.

8. Experiments with 500-, 1000-, and 3000- μm Detectors. In a detector of 500 μm thickness, the high-energy electrons from bismuth-207 are mostly transmitted. Because of multiple scattering, a few are stopped in the detector, and these can be used for calibration. A 500- μm detector will stop an electron of 420 keV nominal energy. With a bismuth-207 source, a second peak is expected, corresponding to the transmitted electrons. This peak has been observed and occurs at 150 keV, which is the correct energy for minimum ionizing particles.

The second peak has also been observed with a 1000- μm detector. This detector has been calibrated with the cesium-137 conversion line at 624 keV and

will see passing pions well and will also see the pion stars. The detector has been calibrated for the stars with americium-241. The experimental mapping of the stars is of special importance for both radiobiology and treatment because this is the best way to obtain the initial energy distribution of the pion beam.

A study has also been made of a 3000- μm detector. A beta spectrum from protactinium-234 was used and a peak observed at about 550 keV. Thick detectors of 3000 and 5000 μm will be used to separate electrons, muons, and pions. This technique will supplement the separation of these particles with the scintillation detectors.

9. Data Collection and Handling. The data readout and processing system is now under construction, and several viable ideas have been investigated. At present, we have a Hewlett-Packard 7004 plotter and a teletype for readout from the Canberra 8100 pulse-height analyzer. Spectra from four channels at each intersection on a 20 x 20 x 20-cm grid with 1-cm increments will be measured. Experience indicates that, even with the very low pion beams now available, data quantities will be such that a magnetic tape or other bulk storage device will be required.

Another problem area is the proper storage of four independent channels. One way to do this would be to manually switch inputs to the ADC from one channel to another and to take four separate spectra at each intersection. However, this is time-consuming and, moreover, it is confusing to keep track of which spectrum came from which detector at which point. Therefore, it has been decided to internally route each of the four spectra from a single detector to a separate 1024-channel section (A/4) of the CI 8100 pulse-height analyzer and to use a tag word generator to write the following information: the relative position of the probe data point from which detector the spectrum was obtained and the actual position of the detector in the probe. This will require very little time and will greatly simplify data processing (e.g., the computer-generated isodose curve plots for the entire treatment volume).

10. Calculations of Particle Distributions.

Two groups at the Oak Ridge National Laboratory have made extensive calculations^{10,11} of what happens to a pion beam when it enters a phantom. All secondary particles in the star region have been tabulated with their frequency of occurrence. The code, known as the HETC, assumes no cross sections but calculates the interactions from first principles. The agreement between available experimental data and the calculations is excellent. An extension of this work has produced LET distributions as observed by our silicon detectors. A fairly good separation of spectra for 10- and 50- μm detectors is predicted. Although one cannot be certain of these calculations, they serve as an excellent guide for instrumentation development.

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INTRODUCTION

Stable isotopes, because of their unique properties and nonradioactive nature, have great potential for many fields of science and technology. In particular, isotopes of carbon, nitrogen, oxygen, and sulfur (the basic building blocks of all biological molecules) would be widely used in biomedical and environmental research if they were economically available in sufficient quantities and in the required chemical forms. The major objective of our program is to hasten the widespread utilization of stable isotopes and commercial involvement through development and demonstration of applications which have potential requirements for large quantities of isotopes. Thus, the demand will be created which is necessary for large-scale production of stable isotopes and labeled compounds and concomitant low unit costs. The program continues to produce a variety of labeled materials (following the necessary research and development of organic and biosynthetic methods) required for clinical, biomedical, chemical, and environmental applications which serve as effective demonstrations of unique and advantageous utilization of stable isotopes.¹⁻³

Many of these projects are carried out through collaboration with investigators in other laboratories, institutions, and industries.

Production of the enriched isotopes is carried out in the Los Alamos Scientific Laboratory's Chemistry and Nuclear Chemistry Group (CNC-4);¹⁻⁵ further major expansion of these facilities is in progress to meet anticipated needs and objectives. The necessity to convert these basic starting materials from the enrichment process (carbon monoxide and nitric oxide) into the wide variety of labeled compounds required for the various applications has resulted in our unique synthesis capability which effectively utilizes and combines the many techniques of organic and biochemical synthesis. Additionally, in accordance with major program objectives (although not reflected in its new title), the group actively initiates and participates in many of the applications of these materials in various research and development projects which include clinical diagnostic trials, biochemical and chemical research, and metabolic studies.

No personnel changes have occurred. Participation in the LASL Co-Op Program continues to be

regarded as most worthwhile -- both from the standpoint of the group and from that of the students. Dr. C. T. Gregg is on LASL Professional Research and Teaching Leave (July 1973-July 1974) at the Pharmakologisches Institut (Embryonal-Pharmakologie) der Freien Universitat, West Berlin, as a Visiting Professor and is conducting research in collaboration with Professor Diether Neubert involving applications of carbon-13 to pharmacology, clinical diagnosis, and molecular biology and use of embryonic systems to study their possible biological isotopic effects.

During the past year, some progress has been made in establishing a better balance between the two products of our efforts (i.e., publications and labeled compounds). Both are essential to the objectives of the program; however, synthesis and applications have generally received priority over submission of manuscripts. Contributions have been made by all members of the group to various conferences and symposia.⁶⁻¹² Emphasis will now be placed on reporting our synthetic methods (primarily in the Journal of Labelled Compounds; also, a compilation giving experimental procedures in greater detail, with periodic additions and updating, is planned in the form of a LASL report). The lead article which appeared in Science³ appears to be accomplishing its primary purpose of making the scientific community more aware of possible applications of stable isotopes. The first issue of a newsletter¹³ has been distributed on a mailing list of several hundred names of persons known to be interested in stable isotopes (conference and symposium attendees, etc.), and additions are solicited. Subsequent issues are planned for publication at 2- or 3-month intervals. Effort has resumed on the long delayed publication of the proceedings of the symposium held in Los Alamos in 1971¹⁴ and will be completed in early 1974.

This report summarizes the methods and results of the past year's activities and collaborations. Similar reports in this series for the previous two years^{1,2} contain additional information concerning the group's interests and capabilities (particularly with respect to synthetic products and intermediates).

PREPARATION OF COMPOUNDS LABELED WITH STABLE ISOTOPES

[E. G. Adame, M. Gonzales, C. T. Gregg, J. L. Hanners, J. Y. Hutson, V. N. Kerr, V. H. Kollman, D. W. Montoya, M. A. Nevarez, D. G. Ott, T. G. Sanchez, T. W. Whaley, G. H. Daub (University of New Mexico), and E. S. Olson (South Dakota State University)]

The repertoire of developed methods for efficient production of labeled compounds increases continuously. Each new synthesis has the additional benefit of simplification of future problems. Particularly valuable are proven procedures for producing basic compounds and key synthetic intermediates which can then be used to introduce specific labels into a wide variety of more complex molecules. The last annual report² included tables listing many of the products and intermediates which can now be prepared, chemical and biosynthetic reactions involved, and pertinent references. Descriptions and illustrations of apparatus and systems which are in use, as well as various considerations concerning syntheses with isotopes, were also given. These will not be repeated in this report, which aims primarily to indicate modifications, improvements, and additions made this past year.

Miscellaneous Products and Intermediates

Ethanol-1-¹³C, Ethanol-2-¹³C, and Ethanol-¹³C₂.

The appropriately labeled acetic acid, water (ca. 150 cm³/mol of acid), and rhenium heptoxide (2 g/mol of acid) were placed in a stainless steel or Hastelloy pressure vessel (ca. 1000 cm³ volume/mol of acid), and hydrogen was added to ca. 3.5 MPa (500 psi). The vessel was heated at 450 K with magnetic stirring, and hydrogen was added as required until uptake ceased (ca. 10 d). After chilling and venting the excess hydrogen, the product was isolated by distillation (ca. 90% yield). Further removal of water from the product can be effected by bulb-to-bulb vacuum transfer from molecular sieves.

Sodium Propanoate-1-¹³C. Ethanol, water (ca. 1-2 mol/mol of ethanol), 57% hydriodic acid (20 cm³/mol of ethanol), and rhodium trichloride (1.5 g/mol of ethanol) were placed in a magnetically stirred Hastelloy C pressure vessel (volume ca.

1000 cm³/mol). Carbon-¹³C monoxide (0.5 mol/mol of ethanol) was added, and the vessel was heated at ca. 470 K until the reaction was complete (as indicated by the pressure change). After cooling and venting the vessel, the crude product was distilled at reduced pressure [ca. 20 kPa (150 torr)] and then redistilled from phosphorous acid (1 cm³/mol) and silver sulfate (1 g/mol). To the fraction containing ethyl propanoate-1-¹³C (in addition to propanoic acid, water, and ethanol), excess (but limited) solid sodium hydroxide was added to hydrolyze the ester. The propionic acid-water fraction was then added, and neutralization was completed with 50% sodium hydroxide solution. The product was isolated in ca. 90% yield by evaporation and recrystallization from ethanol-water.

Propenoic-2,3-¹³C₂ Acid. Ethanol-¹³C₂, water (ca. 1-2 mol/mol ethanol), hydriodic acid (20 cm³/mol), and rhodium trichloride (1.5 g/mol) were placed in a magnetically stirred Hastelloy C pressure vessel (ca. 1000 cm³/mol). Carbon monoxide (ca. 2-fold excess, 5 MPa) was added, and the vessel was heated at ca. 470 K until the reaction was complete. After cooling and venting the vessel, the product (containing water) was isolated in ca. 90% yield as described above for the carboxyl-labeled isomer. Anhydrous product could be obtained by drying with magnesium sulfate (1.2 g/g of water, dichloromethane solvent), azeotropic distillation with carbon tetrachloride, or via the sodium salt.

2-Bromopropanoic-2,3-¹³C₂ Acid. The bromopropanoic acid was obtained in 94% yield by reacting bromine with propanoic-2,3-¹³C₂ acid which was dissolved in a refluxing carbon tetrachloride solution containing phosphorus tribromide as a catalyst. Purification was by distillation under reduced pressure.

Ethyne-¹³C₂ (Acetylene-¹³C₂) and Ethyne-¹²C₂ (Acetylene-¹²C₂). Lithium carbide has been prepared in a specially designed reaction chamber by allowing carbon dioxide (ca. 1 mol) to react with excess molten lithium at 900 K. The rate and temperature of the reaction were controlled by the rate of carbon dioxide addition. Hydrolysis of the lithium-lithium carbide mixture with water liberated acetylene which was purified and cryogenically trapped in a vacuum line connected to the reaction chamber. The acetylene was then cryogenically transferred to storage

vessels. The yield was typically 90-95%; during the year 3 moles of acetylene-¹³C₂ and 0.4 mole of acetylene-¹²C₂ have been prepared by this method.

Benzene-¹³C₆ and Benzene-¹²C₆. A vanadium oxide on alumina catalyst column has been constructed into the acetylene synthesis apparatus described above. Preliminary experiments have shown that the trimerization of acetylene to benzene proceeds smoothly at room temperature by allowing acetylene to sublime into the catalyst column.

Ethynyllithium-¹³C₂ (Lithium Acetylidyde-¹³C₂) and Ethynyllithium-¹²C₂ (Lithium Acetylidyde-¹²C₂) as Ethanediamine Complex. The reaction has been carried out by allowing acetylene (ca. 0.58 mol) to react with N-lithioethanediamine (ca. 0.55 mol) in an anhydrous dioxane slurry. By allowing the acetylene to sublime into the reaction mixture at a controlled rate, it has been possible to perform the reaction at atmospheric pressure using standard laboratory glassware. The air-stable ethanediamine (ethylene-diamine) complex, obtained in typical yields of 85% based on acetylene, is isolated by filtering the solid complex from the reaction mixture. During the past year, 224 g of lithium acetylidyde-¹³C₂ and 24 g of lithium acetylidyde-¹²C₂ have been prepared as the stable ethylenediamine complex.

2-Propanone-1,2-¹³C₂ (Acetone-1,2-¹³C₂) and 1-Methylethanamine-¹³C₂ (Isopropylamine-1,2-¹³C₂). Preliminary experiments on a several step synthesis for converting acetic-¹³C₂ acid to the desired products have been carried out. The direct conversion of acetic acid or lithium acetate to acetone by reaction with methyllithium¹⁵ has been investigated and been found to be unsatisfactory. Two other methods are currently being studied in order to maximize yields. The first of these involves the reaction between acetyl chloride (prepared from acetic acid and benzoyl chloride¹⁶) and dimethylcopper-lithium^{17,18} to give acetone. The second method is based on the reaction of an acetate ester and the sodium salt of dimethyl sulfoxide to give a β-keto-sulfoxide which is reduced to acetone by aluminum amalgam.¹⁹ The isolation of acetone from the above reaction mixtures as its bisulfite addition product has been developed, as have procedures for converting acetone to the corresponding azine by treatment with hydrazine and catalytic hydrogenation of acetone azine to isopropylamine.

Pyridine-¹⁵N. The acid-catalyzed condensation of ammonium-¹⁵N chloride with 2-ethoxy-3,4-dihydro-2H-pyran in the presence of stoichiometric amounts of methylene blue in aqueous solution has been used to prepare pyridine-¹⁵N. After removal of steam volatile materials, the reaction mixture was made basic, and the pyridine was removed by azeotropic distillation. Purification through pyridinium chloride and vacuum distillation of aqueous pyridine from molecular sieve were used to prepare 18 g of anhydrous pyridine-¹⁵N.

Urea-¹³C-¹⁵N₂ and Methyl Carbamimidate-¹³C-¹⁵N₂
4-Methylbenzenesulfonic Acid Salt (O-Methylisourea-¹³C-¹⁵N₂ p-Toluenesulfonate). Urea labeled with carbon-¹³ and carbon-¹² has been prepared previously by allowing a mixture of carbon monoxide, ammonia, and sulfur to react in a suitable pressure vessel employing methanol as a solvent. The crude urea obtained from the reaction mixture has been purified by extraction and crystallization. This method has been extended to the preparation of 8 g of urea-¹³C-¹⁵N₂ from carbon-¹³C monoxide and ammonia-¹⁵N. Synthesis of the O-methylisourea p-toluenesulfonate was carried out by heating a mixture of the urea and methyl p-toluenesulfonate. A total of 11 g of O-methylisourea-¹³C-¹⁵N₂ p-toluenesulfonate has been prepared and converted into arginine-guanidino-¹³C-¹⁵N₂.

1-Methylethyl Formate-¹³C (Isopropyl Formate-¹³C). This ester has been prepared in yields of 80-85% on a 1.5-mole scale by the reaction of carbon-¹³C monoxide and isopropyl alcohol in the presence of sodium isopropoxide. The reaction has been carried out in a stirred autoclave containing the reactants at room temperature and reaches equilibrium in ca. 48 h. After deionization, the reaction mixture has been fractionally distilled to give an azeotropic mixture containing ca. 90% isopropyl formate-¹³C and 10% isopropyl alcohol. Approximately 200 g of this mixture was prepared during the past year.

Formamide-¹³C-¹⁵N and Sodium Cyanide-¹³C-¹⁵N. Treatment of the crude reaction mixture from the isopropyl formate-¹³C preparation with ammonia-¹⁵N followed by deionization and distillation has been used to prepare formamide-¹³C-¹⁵N. A single preparation gave ca. 6 g of product. This yield (ca.

35%) was much lower than expected, since the analogous formamide-¹³C synthesis, which is identical except for the use of excess ammonia, is essentially quantitative. The stoichiometric reaction will be reinvestigated prior to repetition of the doubly labeled preparation. Dehydration of formamide with triphenylphosphine and carbon tetrachloride will be employed to convert the formamide into sodium cyanide-¹³C-¹⁵N.

3-Hydroxy-3-methylpentanedioic Acid (3-Hydroxy-3-methylglutaric Acid). The reaction between bromo-2-propenylmagnesium (allylmagnesium bromide) and an acetate ester has been used to prepare 4-methyl-1,6-heptadien-4-ol. Methods for oxidation of this latter compound are being studied. By using the appropriately labeled acetate ester, 3-hydroxy-3-methylglutaric acid labeled in the methyl group and/or at carbon-3 will be prepared.

Cyclopentadienylthallium-x-¹³C. A four-step synthesis of this compound from isopropyl formate-¹³C has been developed and carried out in conjunction with Group CNC-4. The utility of this method, in contrast to methods for carbon-14 preparations which rely on the cyclization of hexanedioic-1,6-¹⁴C₂ (adipic-1,6-¹⁴C₂ acid), is the incorporation of isotopic carbon into a five-membered ring without loss of half of the carbon isotope. The condensation of isopropyl formate-¹³C with the di-Grignard reagent of 1,4-dibromobutane was used to prepare cyclopentanol-1-¹³C which was converted to 1,2-dibromocyclopentane-1-¹³C via cyclopentene-1-¹³C by dehydration and bromine addition. The final dehydrohalogenation step, in which the label is scrambled, was studied employing a variety of conditions to convert 1,2-dibromocyclopentane-1-¹³C to cyclopentadiene-x-¹³C which was isolated as the thallium salt. Approximately 25 g of the cyclopentadienylthallium has been prepared by this method.

Potassium Cyanate-¹³C. Oxidation of sodium cyanide-¹³C by potassium permanganate-cupric hydroxide was carried out on a ca. 0.2 mole scale. The product, isolated through regeneration of the silver salt, was found to be contaminated with several percent of carbonate. A procedure is being developed to separate this by-product.

Methane- ^{12}C and Methane- $^{13}\text{C-d}_4$. These isotope isomers have been prepared utilizing the same apparatus and procedure (ruthenium oxide catalyzed reduction of carbon dioxide) developed for methane- ^{13}C , following suitable flushing and small-scale reactions to eliminate any holdup in the apparatus. Approximately 5 moles of the mass-21 isomer and 0.5 mole of the mass-16 compound were synthesized.

Carbohydrates and Derivatives

Glucose- $^{13}\text{C}_6$ and Glucose- $^{12}\text{C}_6$. The leaves of three varieties of green plants have been used as a source of uniformly labeled carbon-13 glucose. *Nicotiana glauca* L., variety Coker 319; Swiss chard, variety Burpee's Fordhook Giant; and *Canna indica*, variety Burpee's Dwarf Primrose Yellow, were grown using soilless culture methods and artificial lighting techniques. Leaves having a total weight of about 1.5 kg were cut from the middle portion of young mature plants and incubated in the dark for 24 h to reduce carbohydrate reserves. They were then allowed to photosynthesize in an atmosphere of carbon- ^{13}C dioxide in photosynthetic chambers. Photosynthetic incubation periods were continued for 40 h with tobacco and 24 h with Swiss chard and cannas. At the end of that period, the leaves were removed from the chambers, and the soluble carbohydrates were extracted with ethanol, purified, and separated chromatographically. About 125 g of crystalline glucose- $^{13}\text{C}_6$ have been obtained from the alcohol extract of 20 kg of tobacco, 2.5 kg of cannas, and 3.5 kg of Swiss chard leaves.

Two species of algae, *Anacystis nidulans* (blue-green) and *Chlorella pyrenoidosa* (Sorokin strain, green), are being investigated as potential producers of significant quantities of glucose- $^{13}\text{C}_6$ and other monosaccharides and disaccharides of interest. Both species are grown from slants and mass cultured in an oscillating environmental growth chamber designed and constructed at the LASL. The apparatus can be used to mass culture three separate species of algae simultaneously in 25-l. culture volumes using semi-batch culture processes. Optimal carbon- ^{13}C dioxide levels are maintained automatically by pH demand.^{20,21} Water-soluble monosaccharides, disaccharides, and polysaccharides are removed from the algal cells by sonication of a water-cell suspension of the residue remaining after

lipid extraction. Preliminary results indicate that substantial amounts of glucose- $^{13}\text{C}_6$ can be obtained via the photosynthesis of carbon-13 by algae.

Preparation of glucose- $^{12}\text{C}_6$ has been primarily from tobacco leaves, although substantial amounts of green and blue-green algae containing enriched carbon-12 have been grown. Procedures for the dark incubation of tobacco leaves were designed to reduce the carbohydrate reserves to a minimum and still maintain chloroplast viability. The dark incubation period was extended to 36 h, and the photosynthetic chambers were designed to prevent introduction of atmospheric carbon during photosynthesis in the carbon- ^{12}C dioxide atmosphere. Ethanol extraction of 4.6 kg of incubated leaves has produced approximately 23 g of glucose- $^{12}\text{C}_6$. The extent of carbon-13 depletion is being determined by cmr and mass spectrometric analyses.

Fructose- $^{13}\text{C}_6$ and Fructose- $^{12}\text{C}_6$. The aqueous alcohol plant extract from which glucose- $^{13}\text{C}_6$ is obtained has also been a primary source of fructose- $^{13}\text{C}_6$. Purification is by treatment with ion exchange resins and separation from other sugar components by column chromatography on a 5 x 100-cm column of Dowex 50W X 8 (200-400 mesh, Ba^{++}) resin. The previously specific amounts of tobacco, Swiss chard, and canna leaves used for preparation of glucose- $^{13}\text{C}_6$ have yielded about 125 g of fructose- $^{13}\text{C}_6$. This sugar is also found in algae; however, under the conditions of culture, *Anacystis nidulans* and *Chlorella pyrenoidosa* do not yield significant quantities of this product.

The isolation and purification of fructose- $^{12}\text{C}_6$ from tobacco leaves are previously described in the glucose preparative section. Carbon isotope ratio analyses are in progress on the 27 g of fructose- $^{12}\text{C}_6$ obtained from 4.6 kg of carbon-12 incubated tobacco leaves.

Sucrose- $^{13}\text{C}_{12}$ and Sucrose- $^{12}\text{C}_{12}$. Preparation of this nonreducing disaccharide has been from the previously described green leaf systems. It is extracted from the leaf along with glucose and fructose with aqueous ethanol and is purified and separated from them similarly. Tobacco (20 kg), canna (2.5 kg), and Swiss chard (3.5 kg) leaves incubated in a carbon- ^{13}C dioxide atmosphere have yielded about 120 g of sucrose- $^{13}\text{C}_{12}$. Additional glucose- $^{13}\text{C}_6$ and fructose- $^{13}\text{C}_6$ can be obtained by

acid hydrolysis [0.1 N trifluoroacetic acid (TFA), 3 h under reflux] of sucrose- $^{13}\text{C}_{12}$. TFA is removed from the monosaccharides by ion exchange resins, and the sugars are chromatographically separated. Small quantities of sucrose have been obtained from algae. These organisms, when compared with green leaf systems, are not considered to be primary sources.

Preparation of sucrose- $^{12}\text{C}_{12}$ from tobacco leaves is as described above; about 20 g has been prepared from 4.6 kg of incubated leaves.

Carbon-13 and Carbon-12 Labeled Starch. The principal carbohydrate formed during photosynthetic incubation of tobacco leaves is starch. The leaves are subjected to a 40-h photosynthetic period to ensure uniformity of label within the starch fraction. The leaf residue remaining after alcohol extraction was lyophilized, and the starch was extracted with perchloric acid. Hydrolysis of the labeled starch to glucose- $^{13}\text{C}_6$ was accomplished by dissolving the starch in 0.25 N trifluoroacetic acid (2.5% starch solution) and refluxing the mixture for 8-10 h. When the maximum glucose concentration was reached, as determined by enzymatic assay and optical rotation, hydrolysis was terminated. Trifluoroacetic acid was removed from the hydrolysis mixture by evaporation under reduced pressure and subsequent treatment with ion exchange resins. The resulting neutral solution was concentrated to a thick syrup, and the glucose was crystallized from methanol-isopropyl alcohol. The 20 kg of tobacco leaves used provided 440 g (dry) of carbon-13 labeled starch from which 400 g (91% recovery) of glucose- $^{13}\text{C}_6$ was obtained.

Procedures for the preparation of carbon-12 starch from tobacco leaves are the same as those described for carbon-13 labeled starch except that carbon- ^{12}C dioxide was used during photosynthetic incubation. The incubated leaves (4.6 kg) gave 98 g (dry) of starch from which 90 g of glucose- $^{12}\text{C}_6$ was obtained by acid hydrolysis.

Mannose- $^{13}\text{C}_6$. Algae labeled with carbon-13 serve as a source of mannose, which is extracted from the cell residue remaining after lipid removal and subsequently separated from accompanying monosaccharides and disaccharides (i.e., glucose, galactose, and sucrose) by column chromatography. The crystallization is from methanol-isopropyl alcohol.

Galactose- $^{13}\text{C}_6$. Three species of the marine red algae *Gigartina* (*Gigartina corymbifera*, *Gigartina Californica*, and *Gigartina harveyana*) have been used to prepare the galactoside 2-hydroxy-1-(hydroxymethyl)ethyl- α -D-galactopyranoside. The algae were obtained fresh in 4-6 kg lots and, upon arrival, were dark incubated in an aerated ocean salts medium at 12°C. They were then allowed to photosynthesize in an atmosphere of carbon- ^{13}C dioxide in photosynthetic chambers. Photosynthesis was continued for 11 h, and at completion the thalli were removed from the chambers, washed with distilled water to remove external salts, frozen in liquid nitrogen, and ground to small pieces. A quantity of about 1.5 kg of frozen thalli was extracted three times with 6-1. portions of 80% ethanol under reflux conditions. The cellular residue was discarded, and the combined extracts containing the crude galactoside were concentrated under reduced pressure to a syrup. The galactoside was acetylated with acetic anhydride in pyridine, and the hexaacetyl derivative was separated from solution by the addition of several volumes of cold water and purified by crystallization. The pure galactoside was obtained by deacetylation with sodium methoxide in methanol, followed by treatment with Dowex 50W and recrystallization from alcohol.

Acid hydrolysis of the pure galactoside in 0.1 N trifluoroacetic acid (8 h under reflux) gave equimolar amounts of galactose- $^{13}\text{C}_6$ and glycerol- $^{13}\text{C}_3$. The rate and extent of hydrolysis were determined by optical rotation and enzymatic analyses of the galactose and glycerol concentrations. The hydrolysis solution was treated with ion exchange resins, and the neutral solution was concentrated under reduced pressure to a thick syrup. Separation of the two sugars was by column chromatography and also by direct crystallization of the galactose from the mixture. The wet weight of thalli used was generally 4.5 kg per photosynthetic incubation, from which about 18 g of galactose- $^{13}\text{C}_6$ was obtained.

1,2,3-Propanetriol- $^{13}\text{C}_3$ (Glycerol- $^{13}\text{C}_3$). The method of preparation is described above. About 9 g of glycerol was obtained routinely from 4.5 kg of thalli. The glycerol solution from the chromatographic separation was concentrated under reduced pressure to a thick syrup and stored at -20°C.

D-Glucose-1-¹³C and D-Mannose-1-¹³C. The Kiliani-Fischer sugar synthesis has been applied for these compounds. The condensation of sodium cyanide-¹³C with D-arabinose has given, after hydrolysis of the epimeric aldononitriles, a mixture of D-gluconic-1-¹³C acid and D-mannonic-1-¹³C acid. These compounds have been isolated by crystallization of the barium salt and γ -lactone, respectively. Lactonization of the barium gluconate and subsequent sodium amalgam reduction of the lactone have been used to produce D-glucose-1-¹³C. Separation of D-gluconic-1-¹³C acid and D-mannonic-1-¹³C acid by crystallization techniques has proven to be troublesome, and the possibility of separation of these two compounds by large-scale column chromatography is currently being investigated.

4-O- β -D-Galactopyranosyl-D-glucose-1-¹³C (Lactose-1-¹³C). The preparation of this compound by the Kiliani-Fischer synthesis with sodium cyanide-¹³C is being investigated. The eleven-carbon disaccharide 3-O- β -galactosylarabinose required for the synthesis has been prepared by oxidative degradation of lactose employing sodium hypochlorite.²²

L-2-Hydroxypropanoic-¹³C₃ Acid (L-Lactic-¹³C₃ Acid). A large-scale (ca. 50 g) modification of the bacterial fermentation of D-glucose-¹³C to L-lactic-¹³C₃ acid by *Lactobacillus delbrueckii*²³ has been studied. The L-lactic acid produced by the organism was isolated, after acidification, lyophilization, and extraction, as zinc L-lactate. While preliminary experiments gave pure samples of zinc L-lactate, a carbon-13 preparation contained both D- and L-lactate-¹³C₃. This material has been converted to pyruvic-¹³C₃ acid. The partial racemization was found to have resulted from inadvertent heating in isolation of the product, and a repetition of the experiment is underway.

L-Lactic-¹³C Acids. To obviate the problem of resolving specifically labeled DL-lactic acids, methods are being investigated for the stereospecific enzymatic reduction of pyruvate to L-lactate. Reconversion of NAD to NADH following the lactic dehydrogenase catalyzed reaction is necessary to make the process economically attractive, and the use of alcohol dehydrogenase (with ethanol as substrate) is being developed for this purpose. At present, homogeneous reactions appear to be more desirable than

applications of resin-supported immobilized enzymes. Initial studies indicate that the enzymatic steps can be carried out sequentially with very good yields without isolation of intermediate products. Procedures are being devised for separation and purification of the NADH and L-lactate.

DL-Lactic-2,3-¹³C₂ Acid. A mixture of 2-bromopropanoic-2,3-¹³C₂ acid and a slurry of zinc oxide in water was refluxed for 9 h, then cooled and filtered. The volume of solution was reduced under vacuum, and a 96% yield of zinc DL-lactate-2,3-¹³C₂ was obtained. The free acid was obtained by treating an aqueous solution of the salt with excess Dowex 50 (H⁺).

Butyl DL-Lactate-2,3-¹³C₂. An aqueous solution of lactic acid in four volumes of butanol was refluxed with an equal volume of benzene and an excess of Dowex 50 (H⁺). Water was removed azeotropically, and after 9 h of refluxing the mixture was cooled and filtered. The butanol and benzene were distilled from the mixture, and the product was purified by distillation under reduced pressure (90% yield).

2-Oxopropanoic-2,3-¹³C₂ Acid (Pyruvic-2,3-¹³C₂ Acid). Potassium permanganate and sulfuric acid sufficient to give a slight molar excess over the amount of butyl DL-lactate-2,3-¹³C₂ in a dilute solution were stirred at room temperature for 24 h. The mixture was extracted with ether to remove the product, and the extract was washed with dilute calcium chloride, followed by dilute sodium bicarbonate and water. Purification was by distillation under reduced pressure.

Carbon-13 and Carbon-12 Labeled Lipids

To obtain a wide variety of uniformly labeled lipids, an organism requiring a simple carbon source and having a rapid growth rate, as well as containing the desired lipid material, is necessary. Two species of algae (*Chlorella pyrenoidosa* and *Anacystis nidulans*) are appealing as a source of uniformly labeled lipids and other compounds of biological importance, which are discussed below, due to the wide diversity of biological molecules within the cell. Their rapid growth rate and high incorporation efficiency of carbon dioxide (about 80%) also make them particularly advantageous with respect to mass culture. Using carbon-¹³C dioxide (90% or

greater carbon-13 enrichment), carbon-¹²C dioxide (containing less than 10 ppm carbon-13), and natural abundance carbon dioxide as the carbon sources, about 500 g each of *Anacyetis nidulans* and *Chlorella pyrenoidosa* have been produced.

Exposure of lipids to oxygen during extraction was minimized by use of a nitrogen atmosphere whenever practical; all extraction solvents were purged with nitrogen. A crude algal lipid extract is obtained by washing the cells with absolute ethanol, absolute ethanol-chloroform (9:1), and benzene. Extracts are concentrated under reduced pressure and then placed on the lyophilizer overnight to ensure complete solvent removal. Present efforts are directed toward the large-scale separation of lipids. A variety of identification and quantization techniques and procedures are being investigated such as high-pressure liquid chromatography, thin layer chromatography, chromatography of deacetylated products, and chemical analyses (determination of phosphorus, sulfur, sugar, and ester groups). Fatty acid analysis is by liquid chromatography and gas chromatography.

Carbon-13 and Carbon-12 Labeled Nucleotides

The insoluble algal residue which remains after the lipids and water-soluble material are removed contains mainly nucleic acids and protein. The deoxynucleotide fraction is obtained by treating a suspension of this residue with ribonuclease-free deoxyribonuclease I which hydrolyzes the water-insoluble high-molecular weight DNA to water-soluble oligonucleotides. After the reaction is complete, the oligonucleotide fraction is isolated by centrifugation and treated with purified snake-venom phosphodiesterase, resulting in the liberation of the constituent deoxyribonucleoside 5'-phosphates. The nucleotides are desalted using a Bio-Gel P-2 column (9 mm x 140 cm) and separated on a 9 mm x 75 cm column of Dowex 50W X 4 (-400 mesh, H⁺) resin.²⁴ Elution is with 0.05 M ammonium formate buffer, pH 3.2.

The ribonucleotide fraction is then isolated from the residue which remains after DNase I treatment by extracting with hot 10% sodium chloride solution. The extract is cooled, and the remaining residue is separated by centrifugation, after which the RNA is precipitated with cold ethanol, washed,

and dried. A mixture of the ribonucleoside 5'-phosphates is formed by treatment with purified snake-venom phosphodiesterase. Purification and separation of the nucleotides are described above.

Carbon-13 and Carbon-12 Labeled Amino Acids

Uniformly Labeled Amino Acids. The protein fraction (insoluble algal residue) remaining after lipid, carbohydrate, and nucleic acid extraction is hydrolyzed with hydrochloric acid, and the resulting mixture of amino acids is separated chromatographically. With regard to separation and purification of the carbon-13 amino acids, our effort (when time permits) will be toward developing chromatographic techniques for the rapid separation of/multigram quantities of amino acids in one procedure. When completed, a scale-up by a factor of 7-10 will have been achieved over that previously reported.²⁵

L-Arginine-guanidino-¹³C-¹⁵N₂. This compound has been prepared by the condensation of O-methylisourea-¹³C-¹⁵N₂ (generated *in situ* from the p-toluenesulfonate salt and sodium methoxide) and the copper (II) complex of L-ornithine. After removal of copper (II) ion, the L-arginine was precipitated as the monofluoroborate salt. Decomposition of this salt was found to proceed smoothly employing cation exchange resins at slightly elevated temperatures. Elution of the arginine from the cation exchange resin and crystallization have given 4 g of L-arginine-guanidino-¹³C-¹⁵N₂.

DL-Alanine-2,3-¹³C₂. 2-Bromopropanoic-2,3-¹³C₂ acid was added slowly to a large excess of concentrated ammonium hydroxide (in the ratio of 50 g of acid/2 l. of ammonium hydroxide). After standing for 24 h at room temperature, the reaction volume was reduced to less than 100 ml, and a 4-fold volume of hot methanol was added. The crystalline product was washed with methanol and dried (70% yield).

N-Acetyl-DL-Alanine-2,3-¹³C₂, D-Alanine-2,3-¹³C₂, and L-Alanine-2,3-¹³C₂. The DL-alanine was dissolved in hot glacial acetic acid, and acetic anhydride in 30% excess was added. Reaction time was limited to 1 min; then the reaction was quenched with cold water. The acetylated product was obtained by removing the solvent under reduced pressure (97% yield). Dissolution of the acetylalanine in dilute ammonium hydroxide (1.5 N) and incubation with

how kidney acylase selectively removed the acetyl group from the L-isomer. The L-alanine-2,3- $^{13}\text{C}_2$ was obtained by removal of the enzyme and concentration of the reaction mixture to a small volume with addition and crystallization from ethanol (65% yield). Treatment of the mother liquor with Dowex 50 (NH_4^+) to remove any residual L-alanine gave N-acetyl-D-alanine-2,3- $^{13}\text{C}_2$. The free amino acid was obtained by hydrolysis with dilute hydrochloric acid, followed by the addition of benzenamine equivalent to the amount of amino acid. Addition of ethanol to the concentrated reaction mixture produced crystalline D-alanine-2,3- $^{13}\text{C}_2$ (75% yield).

Discussion of Methods and Results

Comparison of Tobacco, Swiss Chard, and Cannabis for Biosynthesis of Labeled Carbohydrates. The investigation of green leaf biosynthesis was necessary to find ways of producing large quantities of carbon-13 labeled sugars needed for a variety of clinical, biochemical, and isotope effects studies. Emphasis has been on high conversion of carbon- ^{13}C dioxide into desired carbohydrates and on the relative ease with which the desired compounds can be obtained. The carbon-13 labeled sugars produced by each of the three plant systems (tobacco, Swiss chard, and cannabis) using 1.5 kg of excised leaves per incubation are compared in Table I. About 60% of the carbon fixed by tobacco leaves was isolated as starch, glucose, fructose, and sucrose. The apparent starch enrichment (76 atom % ^{13}C) was less than that of crystalline glucose (81 atom % ^{13}C) obtained from it by hydrolysis due to the presence of nonstarch, nonlabeled impurities (cellulose, etc.) which were subsequently eliminated during purification of the hydrolysis mixture. Free glucose, fructose, sucrose, and the glucose obtained from starch were enriched to within 2 atom % of the administered carbon- ^{13}C dioxide (83 atom %). Endogenous carbohydrates, those still remaining after the dark incubation period, were 3% or less for the sugars isolated. For 13 experiments using 20 kg of tobacco leaves, the endogenous material which is dependent on the dark incubation time averaged 10%. Swiss chard and cannabis incorporated 35% and 39% of the fixed carbon into glucose, fructose, and sucrose; isolated products, on the average, were 34% and 59% endogenous in origin, respectively. Hence,

TABLE I
LABELED CARBOHYDRATES FROM TOBACCO,
SWISS CHARD, AND CANNAS

Product	g ^a	Yield		Carbon-13 Concentration (atom %)
		From Leaves (%)	From ¹³ CO ₂ (%)	
<u>Tobacco</u>				
Starch	52.8 ^b	3.0	40.5 ^c	76.0 ^d
Glucose	9.2	0.6	7.1	81.0
Fructose	9.9	0.7	7.1	80.7
Sucrose	7.2	0.5	5.7	81.5
<u>Swiss Chard</u>				
Glucose	10.3	0.7	11.0 ^e	29.5
Fructose	4.6	0.3	10.0	57.0
Sucrose	5.7	0.4	14.0	61.4
<u>Cannas</u>				
Glucose	2.0	0.1	3.3 ^f	37.6
Fructose	1.6	0.1	1.2	17.8
Sucrose	19.3	1.3	34.0	38.6

^aBased on 1.5 kg wet leaf material.

^bStarch moisture content 14%; hydrolysis gave 41 g D-glucose (81 atom % ^{13}C).

^cBased on administered $^{13}\text{CO}_2$ (188 g, 4.2 mol, 83 atom % ^{13}C).

^dLow, owing to nonlabeled impurities present.

^eBased on administered $^{13}\text{CO}_2$ (53 g, 1.18 mol, 74.5 atom % ^{13}C).

^fBased on administered $^{13}\text{CO}_2$ (45 g, 1.01 mol, 74.5 atom % ^{13}C).

there was a significant difference in the level of isotopic enrichment between the carbon- ^{13}C dioxide used and the sugars isolated (Table I).

When the free glucose, glucose obtained from starch, and glucose from hydrolysis of sucrose are considered, the tobacco leaf system converts about half of the carbon dioxide into this compound. In contrast, Swiss chard and cannabis convert considerably less of the fixed carbon to glucose, thereby making tobacco significantly more desirable for labeled glucose production. The ratio of sucrose to reducing sugar is 7.5 for cannabis, 0.7 for Swiss chard, and 0.1 for tobacco, making cannabis the best sucrose production system of the three plants used (under the conditions employed).

All sugars were isolated in pure crystalline form, and the degree of enrichment of carbon-13 in

the sugars (Table I) was determined by mass spectrometric measurement of the $^{13}\text{C}/^{12}\text{C}$ ratios following combustion of the sugars to carbon dioxide.

The green leaf experiments reported here represent a scale-up of 200-fold over similar studies performed using carbon-14 as the labeling isotope.²⁶ The photosynthetic apparatus and large-scale chromatographic procedures which have been developed permit the rapid accumulation of significant quantities of carbohydrates which was not possible utilizing techniques previously used for labeling with carbon-14.

Carbohydrate yield (based on administered carbon- ^{13}C dioxide) from tobacco leaves has been increased about 20% over the example in Table I by using leaves from plants grown to maturity in a greenhouse having an atmosphere of 1000 ppm (rather than the usual 300 ppm) natural abundance carbon dioxide. While producing greater quantities of sugars, initial experiments have shown that the leaves contained higher levels of endogenous material, thus yielding products having lower levels of carbon-13 enrichment. To determine the extent to which the carbon-13 concentration can be increased, an optimum dark incubation schedule needs to be established to minimize endogenous material, and the experiments should be repeated to determine whether or not the higher yield of carbohydrate can be maintained while simultaneously enhancing the level of isotope enrichment.

Reduction of endogenous material to as low a value as possible is extremely important for production of ^{12}C -carbohydrates containing as low a level as possible of carbon-13 contamination. Conditions for the carbon-12 experiments were designed to accomplish this; the analytical results are not yet available.

Use of *Chlorella pyrenoidosa* and *Anacystis nidulans* in Labeling Compounds. Algae are recognized as a valuable source of biologically derived labeled compounds and were the first organisms to be mass cultured using carbon- ^{13}C dioxide having greater than 90 atom % enrichment. The necessary conditions for achieving vigorous algal growth have been described elsewhere,²⁷ and various culture vessels and techniques have been used to meet these conditions in order to ensure a semicontinuous supply of algae. Basic culture units which have been used in

this Laboratory are an oscillating environmental growth apparatus consisting of three 40-l. polycarbonate chambers joined together on a common agitation mechanism, a 12-l. New Brunswick fermentor, and a 200-l. New Brunswick fermentor. The oscillating apparatus and the small fermentor are externally illuminated by high-intensity fluorescent lamps. The 200-l. fermentor is illuminated by three high-intensity sodium vapor lamps contained in a sealed glass tube immersed in the culture medium. Each culture unit is equipped with automatic temperature controls, and carbon dioxide was added to the culture on pH demand. The culture media for *Anacystis nidulans* and *Chlorella pyrenoidosa* were prepared following published methods; additional nutrients were added as needed during the exponential growth phase to reach the desired cell densities.

Growth curves for *Chlorella* and *Anacystis* are shown in Fig. 1. The growth rate for *Chlorella* cultured using 90 atom % carbon- ^{13}C dioxide has repeatedly been observed to be greater than that for *Chlorella* grown on commercial natural abundance

Fig. 1. Growth of *Chlorella pyrenoidosa* (—○—) and *Anacystis nidulans* (—△—) on natural abundance carbon dioxide compared with growth of the algae [*Chlorella pyrenoidosa* (—▽—) and *Anacystis nidulans* (—▲—)] on 91 atom % carbon- ^{13}C dioxide.

carbon dioxide. Growth rates for *Anacystis* cultured on natural abundance carbon dioxide which has been purified by cryogenic distillation and for those grown using 20 and 90 atom % carbon-¹³C dioxide are essentially the same. Both species are produced at rates in excess of 0.1 kg/m³ sec (1 g/l./day). The reason(s) for the higher growth rate of *Chlorella* with carbon-¹³C dioxide is not clear. It is likely that a trace impurity present in the commercial carbon dioxide has a growth-retarding effect. This is substantiated, in part, by a slightly lower growth rate with *Anacystis* using carbon dioxide from the same source. A series of growth studies are planned in which *Chlorella* will be grown using commercial carbon dioxide (purified and nonpurified) with normal isotope abundance. These experiments should eliminate the possibility

of different growth rates arising due to the variable nature of natural abundance carbon dioxide sources.

Algae have been grown in the fermentors with only marginal success. Culture periods varied from 5-10 days, depending on the algal species being cultured, with the yield being about 15% of that obtained per liter of medium with the oscillating apparatus. Nonuniformity of light distribution within the vessel creates clumping of the cells in the region of greatest luminous flux, resulting in abnormally slow growth. Spatial limitations, particularly in the case of the 200-l. fermentor, also do not permit enough lamps to be used to increase the light intensity to the desired levels (i.e., 11 000 lx/l. of medium). Sufficient quantities of algae are being grown in the oscillating growth

Fig. 2. Isolation of labeled cellular components from algae.

chambers to meet present requirements. Harvesting of the algae is accomplished by pumping the algal suspension to an ice-saline solution where it is cooled immediately to 10°C, after which the cells are separated by centrifugation, lyophilized, and stored under vacuum at -20°C. Subcellular components were extracted according to the method shown in Fig. 2. This method utilizes to the maximum extent possible the constituent products of the uniformly labeled organism.

Labeled products from carbon-13 algae are used for a variety of clinical, biochemical, and environmental tracer studies. Sugars obtained from the carbohydrate fraction will be used in conjunction with those from green leaves and kelp; since algae are labeled to the level of enrichment of carbon dioxide used, smaller amounts of carbohydrate will be needed per clinical test. In collaboration with the Cellular and Molecular Radiobiology Group (H-9) and other collaborators, amino acids, which comprise 40% of the extracted material, will be incorporated into a variety of biosynthesis experiments. Fatty acids are used by A. W. Harmon (an AWU graduate student conducting his research in this group under Professor L. Varga of Oklahoma State University) to determine the detection limits of carbon-13 in biochemical and environmental research. Crude lipid extracts are dissolved in anhydrous benzene and transesterified using 2,2-dimethoxypropane, hydrogen chloride, and methanol, and the resulting fatty acid methyl esters are isolated by high-pressure liquid chromatography. The pure methyl esters are analyzed for carbon-13 distribution by GC/MS. Computer analysis of the mass spectrometric data at various levels of enrichment is then used to establish a practical lower limit of enrichment necessary for detection of labeled material. By establishing these limits, it is then possible to evaluate the extent to which enriched compounds can be diluted in an environmental experiment and still be efficiently detected. This work is a necessary prerequisite to large-scale environmental studies using carbon-13. As steroid fractions are obtained, they will be investigated by Dr. D. Hagerman of the University of Colorado Medical School in Denver to determine their suitability as starting materials for applications in research and clinical studies. Nucleotides and nucleosides will be used within the group and in

collaborative efforts. Algae labeled to the 20 atom % ^{13}C level are being used in cmr investigations with N. A. Matwiyoff of LASL Group CNC-4; these include whole cell, crude fraction, and molecular spectra analysis. In conjunction with this effort, Dr. Ellen Weaver of the University of California at San Jose is carrying out live cell electron spin resonance (esr) studies with *Anacystis nidulans* containing 91 atom % ^{13}C .

Large-Scale Preparation of Galactose- $^{13}\text{C}_6$ and Glycerol- $^{13}\text{C}_3$. The large-scale photosynthetic production of generally carbon-13 labeled galactose and glycerol prepared from *Gigartina thalli* has become quite routine. The entire thallus is incubated in the presence of carbon- ^{13}C dioxide (ca. 90 atom % ^{13}C); no attempt was made to remove reproductive structures or nodules. Carbon fixed by the red algae (43%) was isolated as galactose and glycerol (which is about a 2-fold improvement over that previously obtained²⁸). The generally labeled materials were 31 atom % ^{13}C ; thus, the isolated products contained significant amounts of endogenous material (67%).

Two general problems continue with this preparation: (1) the isolated galactose has been shown by cmr spectroscopy to be generally labeled with carbon-13, although not entirely uniformly labeled under the conditions used, and (2) the carbon-13 enrichment of the products was low (31 atom %) with respect to the carbon dioxide (92 atom %) used during photosynthesis. To obtain label uniformity, incubation periods will be extended to 40 h. Attempts to reduce the normal pool of the glycerolgalactoside (and/or precursors) by extending the dark incubation period to 36 h have not resulted in a substantial decrease. Further investigations into the mechanism of the photosynthetic production of carbohydrates by this organism are necessary to reduce the endogenous galactoside.

Large-Scale Chromatographic Separations of Carbohydrates. The purification of multigram quantities of labeled carbohydrates obtained from both biosynthesis and chemical synthesis presents a formidable obstacle to efficient production of these materials. Chemical separations are feasible in some particular cases but are often cumbersome and laborious. Most chromatographic methods are limited in scale and can require the preparation of derivatives. In addition, most chromatographic systems

have been developed for analytical and not preparative purposes. However, one analytical chromatographic system²⁹ seemed attractive because of its simplicity. Some preliminary work showed the feasibility of scaling up the procedure to separate gram quantities of carbohydrate mixtures. A column of Dowex 50W X 8 (200-400 mesh, Ba⁺⁺) is used with water (only) as a developing solvent. It was subsequently found that, by increasing the column to 100 x 5 cm (a 3-fold volume increase), at least 20-g quantities of simple carbohydrate mixtures (a 100-fold increase) could be separated by a single pass through the column.

The Dowex 50 (Ba⁺⁺) column was used originally for the separation of glucose-¹³C₆, fructose-¹³C₆, and sucrose-¹³C₁₂ in soluble carbohydrate extracts from tobacco, Swiss chard, and canna leaves. The eluant from the column is monitored by measuring the refractive index, and a typical chromatogram is shown in Fig. 3. Glycerolgalactoside produced during the photosynthetic incubation of kelp thalli has also been purified on a large scale using the Dowex 50 (Ba⁺⁺) column. Upon hydrolysis, the galactoside yields a mixture of galactose-¹³C₆ and glycerol-¹³C₃ which has also been resolved on the column. Other large-scale separations such as glucose, fructose, and mannose and glucose and galactose have been investigated. Separations of aldonic acid salts and aldolactones have been attempted but have been unsuccessful.

The separation of epimeric aldonic acids is a problem encountered in preparing carbohydrates

Fig. 3. Separation of sucrose (6 g), glucose (8 g), and fructose (8 g) in their order of elution by water (40 ml/h) on a column of Dowex 50W X 8 (200-400 mesh, Ba⁺⁺) resin.

labeled at the 1-position by the Kiliani-Fischer reaction. The possibility of separating multigram quantities of aldonic acids on a Dowex 1 X 8 (acetate) column by elution with 0.5 M acetic acid³⁰ currently is being investigated in conjunction with improving the synthesis of glucose-1-¹³C and lactose-1-¹³C.

Reduction and Degradation of Sugars. In addition to the physical separation of carbohydrates and related compounds, chemical transformations of carbohydrates have been studied. It has been mentioned that the Kiliani-Fischer chain lengthening synthesis produces epimeric aldolnitriles which are hydrolyzed to epimeric aldonic acids and usually separated by crystallization at this point. This separation is time-consuming, and recoveries of material are not good. One alternative to this procedure would be to lactonize the aldonic mixture and to reduce the aldolactones to a mixture of aldoses. This method has not been used previously, presumably because of the difficulty of separating mixtures of aldoses, but these mixtures can now be separated easily employing the Dowex 50 (Ba⁺⁺) chromatographic column. Thus, the reduction of gluconolactone by sodium amalgam³¹ and sodium borohydride³² was studied using both solid crystalline lactone and equilibrated solutions. As reported, both of these reagents gave excellent yields of glucose when solid crystalline lactone was used in the reaction. Water equilibrated gluconolactone solutions, which would be analogous to the synthetic aldolactone mixture, gave much lower yields. Variations in the reaction conditions, such as times between additions of reagents, did not significantly alter the yields. The mutarotation of gluconolactone, which normally takes about 2.5 h, was found to occur in approximately 30 min under the reaction conditions. In the case where crystalline gluconolactone was used, the reduction took place much faster than the mutarotation. On the other hand, the mutarotation mixture must contain a significant amount of nonreducible species and a small amount of reducible lactone. It can be speculated that, since the amount of reducible lactone is small in the mutarotation mixture, reduction of the product is competitive with reduction of the lactone and low overall yields are obtained.

A second area of carbohydrate chemistry which is being investigated is that of degradation methods

for carbohydrates. The starting material necessary for the Kiliani-Fischer synthesis of lactose-1- ^{13}C , 3-O- β -galactosylarabinose, cannot be purchased and, hence, must be prepared by the single-carbon oxidative degradation of lactose. The hypochlorite method²² has been found suitable for this transformation. In anticipation of a future requirement for 2-furan-methanol- $^{13}\text{C}_5$ and 2-furanmethanol- $^{12}\text{C}_5$, several methods for degradation of glucose to arabinose, which can be converted to the furfuryl alcohol, are being evaluated to determine which method gives the best yield.

Some Considerations on Stereospecific Synthesis and Resolution of Racemic Mixtures. For certain compounds containing several asymmetric centers (e.g., glucose- $^{13}\text{C}_6$), biosynthesis is obviously preferred over chemical synthesis. However, for materials with simpler stereochemistry, the choice is not so obvious and depends on various factors such as previously established capabilities and available starting materials, as well as the nature of the possible reaction schemes and products themselves. For example, L-alanine- $^{13}\text{C}_3$ can be obtained from algal protein hydrolysate. It can also be prepared by the series of reactions used above for preparation of L-alanine-2,3- $^{13}\text{C}_2$. In this Laboratory at this time, the latter method would be chosen since the starting materials (ethanol- $^{13}\text{C}_2$ and carbon- ^{13}C monoxide) are readily available and the methodology has already been established for synthesizing DL-alanine and for its subsequent enzymatic resolution. Of course, the maximum theoretical yield is only 50%; however, the other isomer could be of use in itself or converted to other useful products (e.g., lactate- $^{13}\text{C}_3$ or pyruvate- $^{13}\text{C}_3$). In the future, the method of choice may depend on the relative demand for L-alanine compared to other amino acids. Biosynthesis generally produces all of them (as well as other types of compounds), and if there is a use for the compounds produced simultaneously with the specifically desired material and if methods are available for their separation and purification, then it quite likely would be used in preference to chemical synthesis.

At present, a related problem is being studied. L-Lactic-2,3- $^{13}\text{C}_2$ acid is desired for metabolic studies. It cannot be produced biosynthetically as can (and is) L-lactate- $^{13}\text{C}_3$; therefore, chemical

synthesis, which yields a mixture of the D- and L-isomers, must be used. Methods have not been designed previously for resolving this simple mixture (it has not been necessary, since both isomers occur naturally), and the best of several alternatives is not immediately obvious. Perhaps the diastereomeric salts from an optically active amine (e.g., brucine or strychnine) can be fractionally crystallized or a stereospecific (chemical) reduction of pyruvate or hydrolysis of D-alanine can be developed. Or perhaps the stereospecific enzymatic reduction of pyruvate is the best method. These possibilities are being evaluated; the one which gives a reasonable yield with the least amount of effort will be used for requirements at the present level, realizing that demand for a much larger amount in the future may dictate another alternative.

Instrumentation and Analysis. In general, analytical techniques quite standard to synthetic laboratories are being used routinely. However, isotopic synthesis requires certain additional capabilities and, in fact, occasionally "unstandardizes" the standard results. Proton magnetic resonance (pmr) spectroscopy is affected by substitution with stable isotopes having a non-zero nuclear spin (carbon-13, nitrogen-15, oxygen-17, and hydrogen-2). For example, the spin-spin coupling of ^{13}C with ^1H can often be used for analysis of $^{13}\text{C}/^{12}\text{C}$ ratios. This applies particularly well to simple (with respect to the label) compounds (e.g., methanol and the acetic acid isotope isomers as shown in Fig. 4). Also, certain bands in the infrared spectra are

Fig. 4. Proton nuclear magnetic resonance spectra of the four isotope isomers of acetic acid.

shifted by isotopic substitution, but this effect is generally not utilized for information concerning isotopic enrichment, except for deuterium compounds. Although ^{13}C -nmr spectroscopy (cmr) would be extremely useful, this Laboratory does not have a spectrometer, and the research demands on the cmr instrument in Group CNC-4 effectively eliminates its use for routine synthetic problems.

Mass fragmentography, which is rapidly becoming a most important technique in applications of stable isotopes, is not available in this Laboratory, and our mass spectrometric determinations are limited essentially to simple mass ratio analyses (45/44 in carbon dioxide) provided through the facilities and personnel of Group CNC-4. Wet combustion (Van Slyke-Folch) is now being used for carbon isotope analyses; however, other procedures must soon be established to allow for isotopes of hydrogen, nitrogen, and oxygen as well.

When the product of an isotopic synthesis has the same specific isotopic concentration as the starting material, uniformity of labeling is not questioned. However, when the enrichments are considerably different (e.g., in the case of galactose- $^{13}\text{C}_6$ prepared through biosynthesis by kelp), the question arises as to how the isotope dilution took place and if it were or were not random. In principle, cmr spectroscopy is capable of analysis for carbon-13 abundance at each carbon of the galactose; however, the complexity of the spectrum and presently unknown magnitudes of the nuclear Overhauser effect make interpretation difficult. Since metabolic and clinical applications of this carbohydrate depend on knowledge of the relative isotopic concentrations at the molecular positions, degradative procedures are being developed to simplify the analysis by mass or cmr spectrometry.

Carbon-12 Compounds. Carbon-13 experiments conducted in the presence of naturally occurring, normal isotopic abundance carbon (^{12}C) must contend with the background produced by the 1.1% carbon-13 in ^{12}C . This usually does not interfere or can be circumvented; however, carbon-12 (depleted of carbon-13) in many cases should be quite useful [for example, in cmr solvents, in microbiological experiments to reduce the "background" following use of a carbon-13 tracer, or perhaps in volatilization groups (e.g., acetyl or trifluoroacetyl) in mass spectrometry].

Very high purity carbon-12 (> 99.999 atom %) has been produced by Group CNC-4, and methanol- ^{12}C , acetic- $^{12}\text{C}_2$ acid, benzene- $^{12}\text{C}_6$, methane- ^{12}C , urea- ^{12}C , and various ^{12}C -carbohydrates have been synthesized. Some of the methanol- ^{12}C was provided for a collaborative nuclear physics experiment, the methane- ^{12}C in connection with geochemistry, and urea- ^{12}C was used to distinct advantage by N. A. Matwiyoff (Group CNC-4) in a cmr experiment as a protein denaturing agent which did not obscure a region of the carbon-13 spectrum. However, it appears that possible carbon-12 applications have not yet occurred to most investigators; potential applications and demand for this enriched major abundance isotope are being explored.

APPLICATIONS OF STABLE ISOTOPES

[C. T. Gregg, J. Y. Hutson, V. N. Kerr, V. H. Kollman, D. G. Ott, T. G. Sanchez, T. W. Whaley, and W. W. Shreeve (Veterans Administration Hospital, Northport, New York, and State University of New York, Stonybrook, New York)]

To demonstrate various unique and advantageous applications of stable isotopes, a number of projects are in progress most of which involve collaboration with investigators outside the group itself (i.e., at universities, other laboratories and institutions, as well as at LASL). A major objective is development of clinical diagnosis which would ideally be concerned with common diseases which would require a large volume of testing. However, other applications that may or may not require large amounts of isotopes but which rely on the unique capabilities of stable isotopes to benefit various fields of the physical and biological sciences are also important.

Clinical, Pharmacological, Metabolic, and Biochemical Studies

The use of nonradioactive stable isotopes obviates various limitations encountered in the application of radioactive isotopes in humans and would greatly enlarge the scope of isotopic tracer tests for pregnant women and children, for elective diagnostic procedures in large-scale populations, and for many basic physiological and pharmacological studies in healthy subjects. A current objective of

this group is to foster and engage in efforts to demonstrate one or more clinical applications with high probability of widespread adoption and utilization of relatively large amounts of carbon-13. These initial tests have been selected on the basis of being both scientifically and clinically meaningful and technically feasible at the present stage of development of the program. Simplicity and reliability of testing from the standpoints of the nature of the organic compound used, clinical procedure, and technical measurement of the isotope in the metabolic products are other important considerations.

Collaborative relationships are being utilized to carry out the clinical procedures. Dr. W. W. Shreeve was a Visiting Staff Member from January to August 1972, and he established and conducted the first clinical trials. Through his efforts, investigations were begun with Drs. P. Eaton, J. Shoop, and J. Buckman of the University of New Mexico School of Medicine, Albuquerque, which entail extensive interaction with clinical staff members at this nearby institution and the LASL staff. Other clinical applications are being encouraged through collaboration with other investigators: Dr. P. Klein, Argonne National Laboratory, in conjunction with his collaborators, Dr. A. Hoffman of Mayo Clinic (glycine- ^{13}C), and Dr. W. Nyhan, University of California, San Diego (glycine- ^{13}C , propionic- ^{13}C acids), and others; Drs. C. Solomon and E. K. Cotton of the University of Colorado Medical Center, Denver (arginine- ^{13}C - ^{15}N); and Drs. D. Kipnis and K. Arnold, Washington University, St. Louis, with Dr. D. Bier, University of California, San Francisco Medical Center (alanine- ^{13}C and glucose- ^{13}C). In some cases, collaborations involve applications which are limited in clinical volume but are particularly specific for employment of stable isotopes.

In addition to direct diagnostic applications, a broad usage in clinical and pharmacological research would further justify and stimulate production of stable isotopes and development of instrumentation. The latter can involve exploration of the feasibility of new or unique methods of measurement (e.g., nuclear magnetic resonance) adapted for particular biological products in blood, urine, and perhaps tissues. Refinement and adaptation of existing techniques (e.g., isotope ratios by mass spectrometry and infrared gas analysis) are also very

important functions in support of clinical studies.

Through the use of stable isotopes, coupled with gas chromatography-mass spectrometry (mass fragmentography), pharmacologists (in particular) are rapidly pushing detection limits to extremely low levels (currently less than a picogram) for analysis of various metabolites. Some of the compounds prepared in the group primarily for other purposes are also being used as necessary components or synthetic intermediates in selected collaborative projects of this type. Although the amounts of isotopic materials required by a given application are quite small, the technique undoubtedly will become widespread and is an example of utilization of the unique properties of stable isotopes.

Some metabolic diseases are common yet difficult to recognize either clinically or by laboratory tests. Even if recognized, the metabolic categories within and causes for disease entities need better elucidation. Studies with carbon-14 labeled compounds such as glucose and lactic acid have already been aimed toward early diagnosis and metabolic understanding of maturity-onset diabetes. As extensions of these studies, oxidation to expired carbon dioxide of glucose- $^{13}\text{C}_6$ has been studied with the labeled glucose contained in the glucose load of an oral glucose tolerance test.^{7,33} Such a test embodies the principle of simplification of clinical procedures to encourage wide acceptance by patients and physicians. Trials were made for a potential diagnostic application of carbon-13 in which the rate of oxidation of a specific substrate is measured by mass spectrometric determination of the $^{13}\text{C}/^{12}\text{C}$ ratio in carbon dioxide obtained from breath samples of subjects. As expected, severe diabetics showed lower rates of formation of carbon- ^{13}C dioxide than the controls; however, the test did not adequately discriminate mild or moderate diabetics (GTT blood values 200-300 mg % or less) from non-diabetics. This presents additional evidence that the glucose tolerance curve is more a function of regulation of production of glucose than of utilization. Therefore, studies are turning to conversions of glucose-1- ^{13}C , lactate- ^{13}C , pyruvate- ^{13}C , and alanine- ^{13}C to carbon- ^{13}C dioxide and glucose-x- ^{13}C which have the advantage of possibly being able to measure glucose- ^{13}C production in whole blood or plasma by nuclear magnetic resonance.

Another clinical diagnostic trial currently being conducted also utilizes isotopic analysis of breath carbon dioxide as the diagnostic parameter. Galactose- $^{13}\text{C}_6$ has been administered to patients with liver disease (and to control subjects) in an oral dose of 10 g/m^2 of body area; the carbon-13 concentration was adjusted to 1 atom % excess. At subsequent intervals, breath samples were taken in mass spectrometer bulbs for isotope ratio measurements.³⁴ Figure 5 shows the average results for five normal and three abnormal patients. Carbon-14 administrations and measurements which were done at the same time, as well as with 10 other subjects by Dr. Shreeve, produced essentially the same results. These initial trials indicated that this test appears quite promising and that the sensitivity is better than certain other diagnostic procedures presently available (e.g., serum albumin and alkaline phosphatase). Adaptation to a routine procedure would require only one or two breath samples at selected times and, of course, no carbon-14. Further data are being obtained from additional volunteers with a view toward the next larger scale trial. Investigations of the applicability of the test to diagnosis of other disorders (e.g., hypothyroidism and diabetes) will also be made.

Fig. 5. Breath carbon- ^{13}C dioxide concentrations following oral administration of 10 g/m^2 of galactose- $^{13}\text{C}_6$ at 1 atom % excess ^{13}C : (—Δ—) average values for five normal subjects, and (—●—) average values for three patients with liver disorders.

Lactose-1- ^{13}C appears to be well suited for a major clinical application, since conversion of lactose-1- ^{14}C to carbon- ^{14}C dioxide *in vivo* has been shown to reveal readily the condition of intestinal lactase deficiency, which is probably a frequent cause of infant diarrhea as well as milk intolerance in children and adults. A widespread use of lactose-1- ^{13}C in pediatrics may be predicted. A study of the incidence of this genetic deficiency in Southwest Indian groups is indicated in the LASL area, particularly as it may be a cause of their common (and frequently fatal) infant diarrhea. Lactose-1- ^{13}C will soon be available from our synthesis facility, and clinical trials will be initiated in collaboration with Dr. Shreeve and the University of New Mexico School of Medicine staff. It is expected that this test will be simple yet more specific than other (possible) methods (e.g., measurement of gastrointestinal hydrogen production).

Evaluation and applications of the mouse embryo system to studies with stable isotopes¹⁰ have been intensified the last half of this year through Dr. Gregg's Professional Research and Teaching Leave being spent in Dr. Diether Neubert's laboratory at the Free University of Berlin. The system which has been extensively developed and used by Professor Neubert's group in pharmacological and toxicological studies is being developed further through applications of carbon-13 labeled compounds. The uptake of nutrients by embryos in culture at various stages of development from preimplantation through gestation, as well as feeding experiments with pregnant animals, is being studied with the aid of glucose- ^{13}C , algae, and other materials. In addition to providing fundamental metabolic information, the extreme sensitivity of the mouse embryo systems should make apparent any biological isotope effects, if they exist, through perturbation of normal biochemical pathways and observation of progeny, perhaps through several generations.

Since methods, techniques, and instrumentation are not yet available which are capable of obtaining all the potential information produced with this and other biological systems, further developments and adaptations in mass spectroscopy, gas chromatography, and mass fragmentography must be made. Also, isolation techniques are needed to obtain various

desired biological samples, and isotope concentrations that are required for particular applications must be determined (e.g., what degree of labeling must be attained in order to separate DNAs and RNAs by analytical and density gradient ultracentrifugation).

Dr. Gregg has also initiated and is participating in a collaborative clinical study in Berlin of Leigh's syndrome (subacute necrotizing encephalomyelopathy) which appears in children, usually before the age of two years, and is ordinarily fatal. Glucose- $^{13}\text{C}_6$ (and glucose-1- ^{13}C , pyruvate- ^{13}C , lactate- ^{13}C , etc.) will be used in attempts to determine the metabolic lesion which is poorly understood but which appears to be a disorder of carbohydrate metabolism with high levels of pyruvate and lactate occurring in the blood.

The immediate biological precursor of mevalonic acid, which is biosynthetically converted into cholesterol, is 3-hydroxy-3-methylglutaric acid. The enzymatic system which converts it into mevalonic acid has been studied extensively by Dr. T. Scallen and his research group at the University of New Mexico. A series of 3-hydroxy-3-methylglutaric acids labeled at the methyl group, at carbon-3, and at both the methyl group and carbon-3 are being prepared in order to study enzyme-substrate interactions in the 3-hydroxy-3-methylglutaric acid-mevalonic acid conversion using the purified enzyme.

Algae and fish are presently being labeled to the 20 atom % level with ^{13}C for continuing studies of biological membranes. The blue-green alga *Anacystis nidulans* has been grown using 20 atom % carbon- ^{13}C dioxide and, in active collaboration with Professor A. A. Benson of the University of California at La Jolla, *Gymnodinium splendens*, a photosynthetic dinoflagellate, was also grown using enriched carbon dioxide. The dinoflagellate is then fed to fish to obtain the carbon-13 labeled membranes. The algal membranes are prepared for cmr analysis by this group, and the fish membrane preparation is done at La Jolla. Cmr measurements and interpretations are carried out by N. A. Matwiyoff of Group CNC-4; Dr. Matwiyoff is also the overall coordinator. One of the most promising areas for application of cmr will be in the study of whole cells and membranes. Various other research

projects of biological and biochemical nature are being pursued in Group CNC-4, with which Group H-11 naturally shares a mutual interest and varying degrees of collaborative involvement which frequently is through provision of specific compounds (e.g., carbon-13 labeled amino acids, potassium cyanate, and bromoacetic acid for cytochrome c, sickle-cell hemoglobin, and vitamin B_{12} studies, respectively).

Environmental, Chemical, and Other Applications

Isolation, purification, and high resolution mass spectrometric techniques involved in using carbon-13 labeled fatty acids in a multidisciplinary, large-scale ecological study at Oklahoma State University are being developed by Mr. A. W. Harmon. He is a graduate student in the Department of Chemistry (Professor L. Varga, advisor) at the Oklahoma State University and is performing this part of his thesis research at Los Alamos under an Associated Western Universities, Inc., appointment. The two algae systems described in another section of this report are serving as sources of the required materials. Their isolation is carried out with consideration of the need to conserve the other labeled materials present. Capabilities are being developed in gas chromatography and high-pressure liquid chromatography on both analytical and preparative scales.

Methane- $^{13}\text{C}-\text{d}_4$ was prepared for development by the Chemistry and Nuclear Chemistry Division as a universal, nonradioactive atmospheric tracer. The extremely low natural occurrence of this isotope isomer of mass-21 -- approximately 1 part in 10^{17} parts of natural methane (which occurs at around 1 to 2 parts per million in the atmosphere) -- and the probable ability of mass spectrometry to detect 1 part of $^{13}\text{CD}_4$ in 10^{10} parts of $^{12}\text{CH}_4$, will make meteorological studies of transport and dispersion on microscales to macroscales possible following its release in only moderate amounts.

Stable isotopes with non-zero nuclear spins provide a unique nondegradative method for studying molecular rearrangement reactions. Several cooperative projects designed to demonstrate this technique are currently in progress. 2,2-Dimethylpropanoic-1- ^{13}C (pivalic-1- ^{13}C) acid and 2-methylpropanoic-1- ^{13}C (isobutyric-1- ^{13}C) acid have been prepared for mutual interest studies with Dr. A.

Fry of the University of Arkansas. The pivalic acid has been converted to 2,2,4-trimethyl-3-pentanone-3-¹³C, and the acid-catalyzed rearrangement was studied.³⁵ The isobutyric acid is being used to study the mechanistic pathways by which 2-methyl-1-phenyl-1-propanone (isobutyrophenone) may undergo molecular rearrangements. A similar investigation of rearrangement reactions but with a different isotope is being carried out with Group WX-2 at the LASL. Pyridine-¹⁵N and ammonia-¹⁵N have been synthesized and are being used to study possible rearrangement reactions involving 2-aminopyridine.

In attempting to prepare 2-methylpropanoic-1-¹³C acid by carbonylation of 2-propanol, it was observed that the isomeric butanoic-1-¹³C acid was also produced. This scrambling, or rearrangement, of molecular positions (not anticipated from previously published results) was also found to occur with 1-propanol and 2-methyl-2-propanol. Such considerations are very important in the preparation of certain specifically labeled carboxylic acids, and the rearrangement reaction is being studied further. The product from an experiment using ethanol-1-¹³C has been found to be a mixture of the two possible isotope isomers (chemically identical) consisting of about two-thirds propanoic-2-¹³C acid and one-third rearranged product, propanoic-3-¹³C acid. Proton magnetic resonance spectroscopy (which we often find quite useful in studies of carbon-13, in addition to its usual applications) is being used for these analyses.

A number of labeled compounds are made available for research projects in Group CNC-4. Some of these are for fundamental chemical investigations (e.g., metal-ligand interactions and organometallic bonding), although these frequently serve as model compound studies for more complicated structures encountered in biological systems also. Group H-11 welcomes opportunities to provide assistance to other groups throughout the Laboratory where our particular areas of knowledge and capabilities are needed (for example, by synthesizing methanol-¹²C for nuclear physics measurements, ammonia-d₁ and carbon-¹³C disulfide for laser research, and methane-¹²C for a geochemical investigation).

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APPENDIX

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ORGANIC AND BIOCHEMICAL SYNTHESIS GROUP (H-11)

Publications

C. T. Gregg, J. Y. Hutson, J. R. Prine, D. G. Ott, and J. E. Furchner, Substantial Replacement of Mammalian Body Carbon with Carbon-13, *Life Sciences* **13**, 775-782 (1973).

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TALKS PRESENTED AT SEMINARS AND MEETINGS

MAMMALIAN RADIOBIOLOGY GROUP (H-4)

E. O. Goodrich, J. R. Prine, and J. S. Wilson, Iodized Starch Granules as a Cause of Starch Peritonitis, presented at the 1973 Surgical Forum, Chicago, Illinois (October 15-19, 1973).

L. M. Holland and J. R. Prine, Morphologic Techniques, presented at the Respiratory Tract Morphology Conference, The Lovelace Foundation, Albuquerque, New Mexico (May 7-8, 1973).

J. R. Prine, Plutonium Particles in the Lung: Long-Term Biological History, presented at a Science Club Meeting, New Mexico Institute of Mining and Technology, Socorro, New Mexico (November 6, 1973).

C. R. Richmond, Biomedical Research Activities at the Los Alamos Scientific Laboratory, Phi Sigma Biological Honorary Seminar presented at the Biology Department, University of New Mexico, Albuquerque, New Mexico (February 23, 1973).

C. R. Richmond, G. A. Drake, and J. E. London, Enhanced Removal of Radiorubidium from Rodents and Beagles with Ferric Ferrocyanide, presented at the 21st Annual Meeting of the Radiation Research Society, St. Louis, Missouri (April 29-May 3, 1973).

J. F. Spalding and L. M. Holland, Effects of Repeated Acute Gamma-Ray Exposures on Injury and Recovery and on the Long-Term Radiation Lesion in the Monkey, presented at the 21st Annual Meeting of the Radiation Research Society, St. Louis, Missouri (April 29-May 3, 1973).

INDUSTRIAL HYGIENE GROUP (H-5)

J. C. Elder, Chamber Studies of Visibility-Reducing Aerosols, presented at the Rocky Mountain Section meeting of the American Industrial Hygiene Association, Albuquerque, New Mexico (October 11-12, 1973).

P. Lowry, Agglomeration of DOP Test Aerosols, presented at the Rocky Mountain Section meeting of the American Industrial Hygiene Association, Albuquerque, New Mexico (October 11-12, 1973).

J. F. McInroy, E. E. Campbell, and W. D. Moss, Plutonium in Human Autopsy Tissue, presented at the 19th Annual Conference on Bioassay, Environmental and Analytical Chemistry, Jackson Lake, Wyoming (September 4-5, 1973).

J. F. McInroy, Plutonium in Human Autopsy Tissue, presented at the Joint Fall Meeting of the American Industrial Hygiene Association and the Rocky Mountain Section, Rio Grande Chapter, of the Health Physics Society, Albuquerque, New Mexico (October 11-12, 1973).

M. I. Tillery, W. D. Moss, H. J. Ettinger, and G. W. Royer, Effect of Humidity on the Aerodynamic Size Characteristics of Nonhygroscopic Aerosols, presented at the American Industrial Hygiene Association Conference, Boston, Massachusetts (May 20-25, 1973).

M. I. Tillery, A Concentric Aerosol Spectrometer, presented at the American Industrial Hygiene Association Conference, Boston, Massachusetts (May 20-25, 1973).

ENVIRONMENTAL STUDIES GROUP (H-8)

T. E. Hakonson, L. J. Johnson, and W. D. Purtymun, The Applicability of the Honeybee as an Indicator of Environmental Radiocontamination, presented at the 18th Annual Meeting of the Health Physics Society, Miami Beach, Florida (June 17-21, 1973).

T. E. Hakonson and L. J. Johnson, Distribution of Environmental Plutonium in the Trinity Site Environs after 27 Years, presented at the Third International Congress of the International Radiation Protection Association, Washington, D. C. (September 1973).

T. E. Hakonson, Plutonium in the Trinity Site Environs, presented at the 1973 Fall Meeting of the Rio Grande Chapter of the Health Physics Society, Albuquerque, New Mexico (October 11-12, 1973).

W. C. Hanson, Ecological Consequences of the Trans-Alaska Pipeline, presented at a Los Alamos

Scientific Laboratory colloquium (August 7, 1973).

W. C. Hanson, Ecological Consequences of the Trans-Alaska Pipeline, presented at a Sandia Laboratories colloquium, Albuquerque, New Mexico (September 12, 1973).

W. C. Hanson, Refined Estimates of Radiation Exposures of Northern Alaskan Eskimos, presented at the Annual Meeting of the Rio Grande Chapter of the Health Physics Society, Albuquerque, New Mexico (October 19, 1973).

W. C. Hanson, Ecological Consequences of the Trans-Alaska Pipeline, presented at the Hanford Chapter of the Society of the Sigma Xi, Richland, Washington (December 6, 1973).

L. J. Johnson, J. F. McInroy, E. E. Campbell, and W. D. Moss, Estimating the Plutonium Content of the Human Skeletal System from Measured Concentrations in Bone Samples, presented at the 18th Annual Meeting of the Health Physics Society, Miami Beach, Florida (June 17-21, 1973).

J. W. Nyhan, Decomposition of Carbon-14 Labeled Plant Materials in a Grassland Soil under Field Conditions, presented at the 1973 Annual Meeting of the American Society of Agronomy-Soil Science Society of America, Las Vegas, Nevada (November 12, 1973).

CELLULAR AND MOLECULAR RADIOBIOLOGY GROUP (H-9)

B. J. Barnhart and S. H. Cox, Prophage De-repression by Ultraviolet Radiation, presented at the Annual Meeting of the American Society for Microbiology, Miami Beach, Florida (May 6-11, 1973).

B. J. Barnhart, Ultraviolet Induction and Inactivation of Bacterial Prophages, presented at the New Mexico Branch Meeting of the American Society for Microbiology, Las Cruces, New Mexico (October 19-20, 1973).

L. L. Deaven, Analysis of Mammalian Chromosomes by Giemsa Band Staining Techniques, presented at Northern Illinois University, DeKalb, Illinois (March 7, 1973).

L. L. Deaven, Measurements of Mammalian Cellular DNA and Its Localization in Chromosomes, presented at the University of Texas Medical School, Galveston, Texas (May 22, 1973).

L. L. Deaven and D. F. Petersen, G-Band Analysis of Chromosomal Alterations in Aneuploid Cell Lines, presented at the Thirteenth Annual Meeting

of the American Society for Cell Biology, Miami Beach, Florida (November 14-17, 1973).

M. D. Enger, E. W. Campbell, and R. A. Walters, Specific Effects of X-Irradiation on RNA Metabolism of Cultured Chinese Hamster Cells, presented at the 17th Annual Meeting of the Biophysical Society, Columbus, Ohio (February 27-March 2, 1973).

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M. D. Enger, R. A. Walters, and E. W. Campbell, Messenger RNA Metabolism in Cultured Mammalian Cells: Evidence that Informosomal RNA is not a Simple Intermediate in the Conversion of Heterogeneous Nuclear RNA to Polysomal Messenger RNA, presented at the 29th Southwest Regional Meeting of the American Chemical Society, El Paso, Texas (December 5-7, 1973).

L. R. Gurley, R. A. Walters, and R. A. Tobey, The Independence of Histone Phosphorylation from DNA Synthesis, presented at the 5th Miami Winter Symposia, University of Miami, Miami, Florida (January 15-17, 1973).

L. R. Gurley, R. A. Walters, and R. A. Tobey, Histone Phosphorylation in G_2 and Mitosis, presented at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey (April 15-20, 1973).

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F. N. Hayes, D. E. Hoard, and W. B. Goad, Radical Effects on Thymidine 5-Phosphate that Result in Loss of Chromophore, presented at the Gordon Research Conference on Radiation Chemistry, The New Hampton School, New Hampton, New Hampshire (July 26, 1973).

F. N. Hayes, D. E. Hoard, and W. B. Goad, Mechanisms of Chromophore Loss Due to the Action of Ionizing Radiation upon Aqueous Solutions of Thymine Derivatives, presented at the 29th Southwest Regional

Meeting of the American Chemical Society, El Paso, Texas (December 5-7, 1973).

C. E. Hildebrand, R. L. Ratliff, and R. A. Tobey, DNA-Membrane Complexes in Chinese Hamster Cells: Cell-Cycle Studies, presented at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey (April 15-20, 1973).

D. E. Hoard, F. N. Hayes, U. Hollstein, and W. B. Goad, Damage of DNA-Like Polymers and Their Constituents Produced by Ionizing Radiation, presented at the Laboratory of Nuclear Medicine and Radiation Biology, University of California, Los Angeles, California (August 14, 1973).

M. M. Kligerman and D. F. Petersen, Program Leading to Clinical Trials at LAMPF, presented at the Second Meeting on Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy, The Hague, The Netherlands (October 3-5, 1973).

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P. M. Kraemer, The Mammalian Cell Surface and the Role of Cell-Surface Heparan Sulfate, presented at the Noble Foundation, Inc., Ardmore, Oklahoma (January 17, 1973).

P. M. Kraemer, L. L. Deaven, H. A. Crissman, J. A. Steinkamp, and D. F. Petersen, On the Nature of Heteroploidy, presented at the Cold Spring Harbor Symposium on Quantitative Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (June 1-7, 1973).

P. M. Kraemer and D. A. Smith, High Molecular-Weight Heparan Sulfate from the Cell Surface, presented at the Thirteenth Annual Meeting of the American Society for Cell Biology, Miami Beach, Florida (November 14-17, 1973).

R. T. Okinaka, S. H. Cox, and B. J. Barnhart, The Development of Prophage HP1cl after UV Induction: An Electron Microscopic Analysis, presented at the Annual Meeting of the American Society for Microbiology, Miami Beach, Florida (May 6-11, 1973).

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Branch Meeting of the American Society for Microbiology, Las Cruces, New Mexico (October 19-20, 1973).

R. L. Ratliff, Enzymatic Synthesis of Polynucleotides, presented at the Division of Biology, University of Texas at Dallas, Dallas, Texas (January 25, 1973).

R. L. Ratliff, Studies with Biosynthetic Polynucleotides, presented at the Biochemistry Department, University of California, Riverside, California (April 10, 1973).

D. A. Smith, Some Studies on RNA Polymerase in *Escherichia coli*, presented at the Department of Biochemistry, University of Nebraska Medical School, Omaha, Nebraska (January 4, 1973).

G. F. Strniste, The DNA-Dependent RNA Polymerase: a. Effects of X-Irradiation on the Process of *In Vitro* RNA Polymerization; and b. Induction of Stable Complex Formation between Enzyme and Template by Ultraviolet Light, presented at the Division of Biomedical and Environmental Research, U. S. Atomic Energy Commission, Washington, D. C. (March 5, 1973).

G. F. Strniste, The DNA-Dependent RNA Polymerase: Effects of X-Irradiation on *In Vitro* RNA Polymerization, presented at the Molecular Biology Branch of Experimental Biology, Environmental Protection Agency, National Environmental Research Center, Research Triangle Park, North Carolina (March 6, 1973).

G. F. Strniste, X-Ray Inactivation of the DNA-Dependent RNA Polymerase, presented at the Biological Sciences Department, Lehman College, University of New York, West Bronx, New York (May 7, 1973).

G. F. Strniste and D. A. Smith, Formation of RNA Polymerase-DNA Stable Complexes with Ultraviolet Light, presented at the 166th Fall Meeting of the American Chemical Society, Chicago, Illinois (August 26-31, 1973).

R. A. Tobey, Mammalian Cell Synchronization: Techniques and Biochemical Studies, presented at the Department of Pathology, University of Colorado Medical Center, Denver, Colorado (January 18, 1973).

R. A. Tobey, Regulation of Proliferation in the Mammalian Cell, presented at a meeting of the Central Branch of the Tissue Culture Association, Ardmore, Oklahoma (May 3-5, 1973).

R. A. Tobey, L. R. Gurley, C. E. Hildebrand, R. L. Ratliff, and R. A. Walters, Sequential Biochemical Events in Preparation for DNA Replication and Mitosis, presented at a meeting on Control of Proliferation in Animal Cells, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (May 20-27, 1973).

R. A. Tobey, Control of Macromolecular Synthesis in Synchronized Cell Lines, presented at the Gordon Conference on Molecular Pathology, Regulation of Mammalian Cell Proliferation, Plymouth, New Hampshire (July 9-13, 1973).

R. A. Tobey and H. A. Crissman, Differential Effects of Nitrosourea Analogues on Cell-Cycle Progression, presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, East Lansing, Michigan (August 19-23, 1973).

R. A. Tobey, (a) Isoleucine Deficiency Method; (b) Regulation of Proliferation in Cultured Mammalian Cells; and (c) Studies with Isoleucine-Deficient Cells, presented at the Principles of Cell Synchronization Course, W. Alton Jones Cell Science Center, Lake Placid, New York (September 8-11, 1973).

R. A. Tobey, L. R. Gurley, C. E. Hildebrand, P. M. Kraemer, R. L. Ratliff, and R. A. Walters, Sequential Biochemical Events in Preparation for Mitosis in Chinese Hamster Cells, presented at the Sixth European Study Group for Cell Proliferation, Moscow, U.S.S.R. (September 25-29, 1973).

R. A. Tobey, Sequential Preparations for DNA Replication and Division in Mammalian Cells, presented at the Imperial Cancer Research Fund Laboratories, London, England (October 4, 1973).

R. A. Tobey, Sequential Preparations for DNA Replication and Division in Mammalian Cells, presented at the Mergenthaler Laboratory for Biology, The Johns Hopkins University, Baltimore, Maryland (November 5, 1973); at the Central Research Department, E. I. DuPont de Nemours and Company, Wilmington, Delaware (November 7, 1973); at the Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania (November 9, 1973); at the Department of Biochemical Sciences, Princeton University, Princeton, New Jersey (November 12, 1973); at the Department of Physiology, School of Public Health, Harvard University, Boston, Massachusetts (November 13, 1973); and at

the Putnam Memorial Hospital Institute for Medical Research, Bennington, Vermont (November 15, 1973).

R. A. Tobey, Sequential Biochemical Events in the Mammalian Cell Cycle, presented at the Department of Cell Biology, University of Texas Medical Branch, Galveston, Texas (December 11, 1973); and at the Department of Developmental Therapeutics, M. D. Anderson Hospital and Tumor Institute, University of Texas, Houston, Texas (December 13, 1973).

R. A. Walters, L. R. Gurley, and R. A. Tobey, Effects of Caffeine on X-Ray-Induced Radiation Responses of Chinese Hamster Cells, presented at the 17th Annual Meeting of the Biophysical Society, Columbus, Ohio (February 27-March 2, 1973).

R. A. Walters and R. L. Ratliff, Effects of X-Irradiation on Deoxynucleoside Triphosphate Pools in Chinese Hamster Cells, presented at the 21st Annual Meeting of the Radiation Research Society, St. Louis, Missouri (April 29-May 3, 1973).

R. A. Walters, R. A. Tobey, and R. L. Ratliff, Cell-Cycle Dependent Variations of Deoxyribonucleoside Triphosphate Pools in Chinese Hamster Cells, presented at the 29th Southwest Regional Meeting of the American Chemical Society, El Paso, Texas (December 5-7, 1973).

D. L. Williams, The Use of Functional Blocking Groups in the Chemical Synthesis of Deoxyribonucleotide Sequences, presented at the University of New Mexico, Albuquerque, New Mexico (May 7, 1973).

BIOPHYSICS AND INSTRUMENTATION GROUP (H-10)

L. S. Cram, The USDA Program on Flow Microfluorometry at the Los Alamos Scientific Laboratory, presented at the Plum Island Animal Disease Laboratory, United States Department of Agriculture, Veterinary Sciences Research Division, Greenport, Long Island, New York (February 1, 1973).

L. S. Cram and C. E. Hildebrand, Effects of Temperature on the Mitotic Cycle of CHO Cells, presented at the 17th Annual Meeting of the Biophysical Society, Columbus, Ohio (February 27-March 2, 1973).

L. S. Cram, Techniques of Rapid, Single-Cell Analysis and Its Application to DNA Measurement, Quantitative Immunofluorescence, and Tumor Cell Identification, presented at the University of Oslo and Norsk Hydro Institute for Cancer Research, Oslo, Norway (June 1, 1973).

L. S. Cram, Application of Flow Microfluorometry (FMF) and Cell Sorting Techniques to the Control of Animal Diseases, presented at the Symposium on Rapid Methods and Automation in Microbiology, Stockholm, Sweden (June 3-8, 1973).

L. S. Cram, Techniques of Rapid, Single-Cell Analysis and Its Application to DNA Measurements, Quantitative Immunofluorescence, and Tumor Cell Identification, presented at the Karl-Friedrich-Bonhoeffer-Institut, Max-Planck-Institut für Biophysikalische Chemie, Goettingen, Germany (June 13, 1973).

L. S. Cram, Application of Flow Microfluorometry to the Differential Diagnosis of Newcastle Disease, presented at a meeting of the United States Animal Health Association, St. Louis, Missouri (October 15-16, 1973).

D. Freeman, J. C. Allred, E. S. Priori, L. Dmochowski, S. Cram, H. Crissman, P. Horan, P. Mullaney, J. Steinkamp, and M. Van Dilla, A Comparative Study of the Cellular DNA Content of Normal Cells, Tumor Cells, and Cells Infected with Oncogenic Viruses, presented at the Annual Meeting of the Southwest American Association for Cancer Research, Houston, Texas (November 9-10, 1973).

H. A. Crissman, Flow Microfluorometry -- Principles and Applications for Cell Biology, presented at The Salk Institute for Biological Studies, San Diego, California (August 14, 1973).

H. A. Crissman and J. A. Steinkamp, Rapid Measurement and Simultaneous Analysis of DNA, Protein, and Cell Volume in Single Cells from Large Mammalian Cell Populations, presented at the 13th Annual Meeting of the American Society for Cell Biology, Miami Beach, Florida (November 14-17, 1973).

H. A. Crissman, Multiparameter Analysis of Cells in Proliferative Cycle Traverse, presented at the Fourth Joint Working Conference, Cellular Kinetics and Pharmacokinetics Session, Hunt Valley Inn, Maryland (November 20, 1973).

H. A. Hopkins, C. J. Kovacs, M. N. Mitchell, P. K. Horan, and W. B. Looney, Determinations of Cellular DNA Contents of Hepatomas with Differing Chromosomal Complements by Flow Microfluorometry, presented at the National Cancer Institute Hepatoma Conference, National Institutes of Health, Bethesda, Maryland (May 10-12, 1973).

P. K. Horan, Techniques of Rapid, Single-Cell Analysis and Its Application to DNA Measurements, Quantitative Immunofluorescence, and Tumor Cell Identification, presented at Pennsylvania State University, University Park, Pennsylvania (August 7, 1973).

P. K. Horan, Some Parameters of SV-40 Infection as Measured by Flow Microfluorometry, presented at Pennsylvania State University, University Park, Pennsylvania (August 8, 1973).

P. K. Horan, Techniques of Rapid, Single-Cell Analysis and Its Application to DNA Measurements, Quantitative Immunofluorescence, and Tumor Cell Identification, presented at the Sloan-Kettering Cancer Hospital, New York City (August 16, 1973); at AEC Headquarters, Washington, D. C. (August 27, 1973); and at the University of Virginia, Charlottesville, Virginia (August 29, 1973).

P. K. Horan, J. Lehman, A. Romero, and J. H. Jett, Polyploid Formation Resulting from SV-40 Infection, presented at the Meeting on the Molecular Biology of SV-40, Polyoma and Adenoviruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (August 12-15, 1973).

P. F. Mullaney and J. A. Steinkamp, Multiparameter Cell Sorting and Analysis, presented at the 17th Annual Meeting of the Biophysical Society, Columbus, Ohio (February 27-March 2, 1973).

P. F. Mullaney, Tumor Cell Identification and Separation Using High-Speed Multiparameter Cell Sensing Techniques, presented at the Second International Symposium on Cancer Detection, Bologna, Italy (April 9-13, 1973).

P. F. Mullaney, A News Seminar on Atomic Medical Research, presented at the USAEC Atom Medic Meeting, Argonne National Laboratory, Argonne, Illinois (September 25-26, 1973).

P. F. Mullaney, FMF Research at the Los Alamos Scientific Laboratory, presented at the Physiology Department, University of New Mexico, Albuquerque, New Mexico (October 8, 1973).

P. F. Mullaney, Biological Applications of Cell Analysis and Sorting Instruments Developed at LASL, presented at the M. D. Anderson Hospital and Tumor Institute, Houston, Texas (November 20, 1973).

P. F. Mullaney, Cell Analysis and Sorting Research at LASL, presented at the Biophysics

Department, University of Houston, Houston, Texas (November 21, 1973).

M. R. Raju, Current Status of Pion Radiotherapy, presented at the San Francisco Medical Center, San Francisco, California (February 2, 1973).

M. R. Raju, Radiotherapy with High LET Radiations, presented at the National Bureau of Standards, Washington, D. C. (February 15, 1973).

M. R. Raju, Radiotherapy with Pions, presented at the Naval Research Laboratory, Washington, D. C. (February 16, 1973).

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M. R. Raju, Some Applications of Particle Accelerators and Particle Counting Techniques in Biology and Medicine, presented at the Symposium on Advanced Technology Arising from Particle Physics Research, Argonne National Laboratory, Argonne, Illinois (May 17, 1973).

M. R. Raju, Negative Pions in Radiotherapy: A Brief Review, presented at the Second Meeting on Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy, The Hague, The Netherlands (October 3-5, 1973).

M. R. Raju, High LET Radiations in Radiotherapy, presented at the Institute of Gustave Roussy, Val-de-Marne, France (October 9, 1973).

M. R. Raju, Biological Effects of Negative Pions, presented at the XIIIth International Congress of Radiology, Madrid, Spain (October 15-30, 1973).

M. R. Raju, Recent Developments in (a) Radiotherapy with Negative Pions and (b) Instrumentation for Therapeutic Radiobiology, presented at the Department of Therapeutic Radiology, University of Kentucky, Lexington, Kentucky (November 6, 1973).

M. R. Raju, Production and Application of Negative Pions in Radiotherapy, presented at a meeting of the American Nuclear Society, San Francisco, California (November 14, 1973).

M. R. Raju and J. H. Jett, RBE and OER of Plutonium Alpha Particles and X-Ray Mixtures for Damage to Human Kidney Cells (T_1), presented at the Joint Meeting of the Radiological Society of North America and American Association of Physicists in Medicine, Chicago, Illinois (November 25-30, 1973).

M. R. Raju, Interaction of Heavy Particles with Tissue and Dosimetry, presented at the Bhabha Atomic Research Center, India (December 18, 1973).

M. R. Raju, Radiobiology Related to Radiotherapy, presented at the Bhabha Atomic Research Center, India (December 19, 1973).

M. R. Raju, Current Status of Heavy Particles in Radiotherapy, presented at the Bhabha Atomic Research Center, India (December 20, 1973).

C. Richman, Preparation for Pion Radiotherapy at LASL, presented at Stanford University, Stanford, California (June 7, 1973).

C. Richman, The Status of Pion Radiotherapy, presented at the Argonne National Laboratory, Argonne, Illinois (November 30, 1973).

G. C. Salzman, P. F. Mullaney, and J. R. Coulter, A Coulter Volume Spectrometer Employing a Potential Sensing Technique, presented at the 17th Annual Meeting of the Biophysical Society, Columbus, Ohio (February 27-March 2, 1973).

J. A. Steinkamp, The Los Alamos Cell Analysis and Sorting Program, presented at the Electrical Engineering Department, University of New Mexico, Albuquerque, New Mexico (April 12, 1973).

J. A. Steinkamp and P. M. Kraemer, Flow Microfluorometric Studies of Lectin Binding to Mammalian Cells: Measurement of Binding Sites per Unit Cell Surface Area, presented at the Thirteenth Annual Meeting of the American Society for Cell Biology, Miami Beach, Florida (November 14-17, 1973).

J. A. Steinkamp and H. A. Crissman, Multi-parameter Analysis and Sorting of Exfoliative Material and Tumor Cells, presented at the Engineering Foundation Conference, Asilomar Conference Grounds, Pacific Grove, California (December 2-7, 1973).

J. A. Steinkamp, Automated Analysis of Tumor and Other Mammalian Cells, presented at The Salk Institute, San Diego, California (December 10, 1973).

ORGANIC AND BIOCHEMICAL SYNTHESIS GROUP (H-11)

C. Gregg, D. Smith, D. Ott, J. Malanify, B. McInteer, N. Matwiyoff, and D. Neubert, Incorporation of Carbon-13 into Fetal and Maternal Protein and Nucleic Acid during Various Intervals of Pregnancy in Mice, presented as a poster session at the Ninth International Congress of Biochemistry, Stockholm, Sweden (July 1-7, 1973).

C. T. Gregg, Applications of Stable Isotopes in Clinical Pharmacology, presented at a Joint Meeting of the British and German Pharmacological Societies, Berlin, Germany (September 11-14, 1973).

V. H. Kollman, J. L. Hanners, and D. G. Ott, Large-Scale Photosynthetic Production of Carbon-13 Labeled Sugars, presented at the Conference on Stable Isotopes in Chemistry, Biology and Medicine, Argonne National Laboratory, Argonne, Illinois (May 9-11, 1973).

D. G. Ott, C. T. Gregg, V. N. Kerr, V. H. Kollman, and T. W. Whaley, Organic and Biosynthesis of Carbon-13 Labeled Compounds for Clinical Investigations, presented at the Symposium on New Developments in Radiopharmaceuticals and Labelled Compounds, Copenhagen, Denmark (March 26-30, 1973).

D. G. Ott, Synthesis of Compounds Labeled with Stable Isotopes, presented at the Department of Chemistry, Colorado State University, Fort Collins, Colorado (April 11, 1973).

D. G. Ott, Synthesis of Labeled Compounds, presented at the USAEC Atom Medic Meeting, Argonne National Laboratory, Argonne, Illinois (September 24-25, 1973).

D. G. Ott, Synthesis of Organic Compounds Labeled with Stable Isotopes, presented at the Eighth Annual Argonne National Laboratory-Argonne Universities Association Biology Symposium on "Applications of Stable Isotopes in Biological, Medical, and Environmental Research," Argonne National Laboratory, Argonne, Illinois (October 15-17, 1973).

W. W. Shreeve, J. E. Shoop, R. P. Eaton, C. H. Wu, M. S. Roginsky, D. G. Ott, C. T. Gregg, and R. Uphoff, Evaluation of Diabetes by Oxidation of ^{14}C - or ^{13}C -Labeled Glucose to CO_2 *In Vivo*, presented at the Symposium on New Developments in Radiopharmaceuticals and Labelled Compounds, Copenhagen, Denmark (March 26-30, 1973).

T. W. Whaley, The Isotope Program at Los Alamos, presented at Seaver Chemistry Laboratory, Pomona College, Claremont, California (January 4, 1973).

T. W. Whaley, Syntheses with Stable Isotopes, presented at the Department of Chemistry, University of New Mexico, Albuquerque, New Mexico (January 23, 1973).

T. W. Whaley, V. N. Kerr, and D. G. Ott, Organic Synthesis with Stable Isotopes, presented at the Conference on Stable Isotopes in Chemistry, Biology and Medicine, Argonne National Laboratory, Argonne, Illinois (May 9-11, 1973).

RESEARCH INTERESTS OF DIRECTIVE STAFF MEMBERS

BIOMEDICAL AND ENVIRONMENTAL RESEARCH PROGRAM

- E. C. Anderson, Ph.D., University of Chicago (1949)
Electronic spectrometry of cell population distributions
Analysis of cell growth and division dynamics by mathematical and computer models
Biochemistry of mammalian cell life cycle
Biological effects of hot particles
- B. J. Barnhart, Sc.D., Johns Hopkins University (1962)
Biochemistry of bacterial genetic transformation
Photobiology: Bacterial responses to ultraviolet light and DNA repair processes
- E. E. Campbell, M.S., University of Denver (1953)
Analysis of trace metal contaminants, excretion rates of metabolites, and calculation of body content based on excretion data
- L. S. Cram, Ph.D., Pennsylvania State University (1969)
Macromolecular structure and ultraviolet radiation action
High-speed cellular analysis and biomedical instrumentation
- H. A. Crissman, Ph.D., Pennsylvania State University (1971)
Application of cytology and histochemistry techniques to high-speed, flow-system cell analysis instrumentation
- P. N. Dean, M.A., Rice University (1958)
Low-level radioactivity counting
Radiological physics
Computer applications
- L. L. Deaven, Ph.D., University of Texas (1969)
Structure and function of mammalian chromosomes
- M. D. Enger, Ph.D., University of Wisconsin (1964)
Mammalian RNA synthesis and modification
- H. J. Ettinger, M.C.E., New York University (1958)
Aerosol generation, characterization, and sampling
Air cleaning for fine particulates
Occupational health and safety
- J. E. Furchner, Ph.D., University of New Mexico (1955)
Metabolism of radionuclides
Internal emitters
Radiation protection guides
- C. T. Gregg, Ph.D., Oregon State University (1959)
Energy metabolism of cultured cells
Biochemical applications of stable isotopes
- L. R. Gurley, Ph.D., University of North Carolina (1964)
Histone chemistry and metabolism
- T. E. Hakanson, Ph.D., Colorado State University (1972)
Ecological investigations of radionuclides in the natural environment, including pathways, transport mechanisms, and ecosystem modeling
- W. C. Hanson, Ph.D., Colorado State University (1973)
Radiation ecology of radionuclides in natural systems
Ecological studies of arctic ecosystems
- F. N. Hayes, Ph.D., Northwestern University (1949)
Radiation chemistry and biology of nucleic acids
- C. E. Hildebrand, Ph.D., Pennsylvania State University (1970)
Synthesis of cell-free protein
- D. E. Hoard, Ph.D., University of California (1957)
Organic synthesis of oligodeoxynucleotides
Effects of radiation on nucleic acids

- L. M. Holland, D.V.M., Colorado State University (1958)
Laboratory animal medicine
Hematology and surgery
Mammalian radiobiology
- P. K. Horan, Ph.D., Pennsylvania State University (1970)
Tumor cell diagnosis using LASL cell sorter techniques
- J. H. Jett, Ph.D., University of Colorado (1969)
Nuclear physics applications and instrumentation for radioactivity counting
Computer applications for data analysis and system control
- P. M. Kraemer, Ph.D., University of Pennsylvania (1964)
Structure and function of mammalian cell-surface complex carbohydrates
- J. F. McInroy, Ph.D., Colorado State University
Metabolism, distribution and concentration of metallic environmental contaminants in humans
Physical and chemical characterization of particulates in the lung
- J. C. Martin, B.A., Drury College (1965)
Cell sorting and biomedical engineering
- P. F. Mullaney, Ph.D., University of Delaware (1965)
Optical cell sensing
Light scattering by cells
Simultaneous measurement of multicellular parameters
- J. W. Nyhan, Ph.D., Colorado State University (1972)
Soils and systems ecology
Radioecology of transuranics
- R. T. Okinaka, Ph.D., University of California, Los Angeles (1971)
Ultrastructural problems related to virus maturation
Mammalian chromosomal organization
- D. G. Ott, Ph.D., Washington State University (1953)
Organic syntheses with isotopes
Nucleotide chemistry
Biochemical applications of stable isotopes
- D. F. Petersen, Ph.D., University of Chicago (1954)
Biochemistry of mammalian cell life cycle
Synchronization of mammalian cell cultures
- J. R. Prine, D.V.M., Texas A and M University (1951)
Board certified veterinary pathologist
Pathology of small laboratory animals and primates
Radiation pathology
- R. L. Ratliff, Ph.D., St. Louis University (1960)
Isolation of DNA polymerase and deoxy-nucleases
Enzymatic synthesis of polynucleotides
- M. R. Raju, Sc.D., Andhra University (1961)
Dosimetry and radiobiology of high LET radiations in general, with reference to radiotherapy
Dosimetry and radiobiology of negative pions
Application of FMF instrumentation to normal and tumor cell kinetics
- C. Richman, University of California, Berkeley (1943)
Dosimetry and radiobiology of negative pions
- C. R. Richmond, Ph.D., University of New Mexico (1958)
Metabolic kinetics and biological effects of radionuclides
Water and electrolyte metabolism
Mammalian radiobiology
- G. C. Salzman, Ph.D., University of Oregon (1972)
Light scattering of mammalian cells
Computer analysis
- A. G. Saponara, Ph.D., University of Wisconsin (1964)
Isolation and characterization of minor nucleotides from mammalian cell nucleic acids
- G. R. Shepherd, Ph.D., Stanford University (1961)
Cellular differentiation
Structure and function of histones
Structure of chromatin

D. A. Smith, Ph.D., University of Southern California
(1964)

Nucleic acid enzymology

Ultracentrifugation

Molecular radiobiology

J. F. Spalding, Ph.D., Texas A and M University
(1953)

Somatic and genetic effects of ionizing
radiation from external sources

J. A. Steinkamp, Ph.D., Iowa State University
(1970)

Electronic instrumentation design and testing

Electromagnetic radiation monitoring

Identification of cells by fluorescent methods

Automated cell analysis and sorting and
biomedical instrumentation

R. A. Tobey, Ph.D., University of Illinois (1963)

Biochemical regulation of mammalian cell-
cycle events

M. I. Tillery, M.S., University of Rochester (1972)

Aerosol generation, characterization, and
sampling

Air cleaning for fine particles

R. A. Walters, Ph.D., Colorado State University
(1967)

Biochemistry of radiation effects on mamma-
lian cells

Analytical biochemistry of macromolecules

T. W. Whaley, Ph.D., University of New Mexico
(1971)

Organic scintillators

Organic synthesis with stable isotopes

D. L. Williams, M.S., University of Colorado (1944)

Organic synthesis of oligodeoxynucleotides

SYMPOSIA AND SPECIAL ACTIVITIES

THE THIRD ANNUAL WORKSHOP ON CELLULAR AGING, ADVANCED TECHNIQUES IN CELL CULTURE (July 22- August 3, 1973)

The Third Annual Workshop on Cellular Aging, Advanced Techniques in Cell Culture, was held at Los Alamos on July 22-August 3, 1973, under the sponsorship of the National Institute of Child Health and Human Development of the National Institutes of Health in collaboration with the U. S. Atomic Energy Commission, Los Alamos Scientific Laboratory, and the Tissue Culture Association. The theme was to promote excellence in research on the biology of aging by providing a learning environment addressing recent advances in the research and technology of cell culture. Invited speakers addressed current topics in cell biology during the morning sessions, and laboratory course centers designed to provide experience in selected areas of cell culture research and technique were conducted in the afternoon sessions. The workshop was limited to 21 doctorate or equivalent participants, with a total of 30 supporting staff, including speakers and local personnel. The following are brief descriptions of the course centers, representing five major areas of laboratory activities.

I. Basic Tissue Culture Methodology

The principal theme of this laboratory workshop was to provide instruction in tissue culture laboratory quality control. Procedures were included for glassware washing, water purification and nutrient media preparation, screening for mycoplasma and other contamination, preparation and continuance of primary and established cell lines, microscopical and cinematographic surveillance of cultures, cell identification, and preservation of cell lines.

II. Perfusion and Mass Culture Techniques

Experiments conducted in this workshop included the setup and operation of automated systems for culture of cells on microscope stages, in replicate

stationary flasks, in rolling bottles, and in suspension culture. A specific and recently perfected method for producing relatively massive quantities of cells, using plastic roller bottles fitted with plastic spiral inserts, was also conducted by each student, particularly those who wished to use the cell packs obtained in cell fractionation and plasma membrane projects conducted during the second week of the course. The theory and applications of open versus closed culture systems were discussed and illustrated.

III. Somatic Cell Genetics

Modern techniques for karyologic analysis, G banding, C banding, fluorescence banding, and pattern recognition were discussed in detail and each of the techniques included as a laboratory exercise. Also included were techniques including hybridization for the localization of genetic information on individual chromosomes.

IV. Cell Synchronization and Cell Preparative Protocol for High-Speed Cell Analysis

This center was designed, in part, to provide a working knowledge of several methods currently employed for providing large populations of cells in a specific phase of the life cycle (i.e., G_1 or mitosis). Another part of the center addressed the participant to cell preparative techniques including cell dispersal, fixation, and staining of tissue culture cells for flow microfluorometric (FMP) analysis. The operational features of FMP and its application to cell analysis were explored.

V. Cell Surface Phenomena

Cell membranes are crucially involved in such diverse processes as cell and molecule recognition and transport. Specific experiments designed to demonstrate these phenomena quantitatively were

conducted using appropriate cellular models (for example, plant lectin binding, aggregation, agglutination reactions with specific antigens, and membrane isolation techniques). Students acquired practical experience with each of these systems and a working knowledge of the use of required laboratory equipment.

LECTURE TOPICS

Tissue Culture in Perspective
Theory and Application of Open and Closed Culture Systems
Properties of the Human Fibroblast, WI-38
Cell Synchrony
Biological Applications of Rapid Cell Sensing and Sorting Techniques
Mammalian Cytogenetics
The Cell Surface
Cellular Immunology
The Biochemistry of Cells in Culture
Functional Properties of Organelles
Topography of the Cell Surface
Membrane Structure and Assembly
Somatic Cell Genetics
Cell-Cycle Regulation
Nuclear Cytoplasmic Interactions
The Biology of Aging
Cell Fractionation
The Organized Cell versus the Cell in Culture

PARTICIPANTS

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Burlington, Vermont

Dr. Matthews O. Bradley
Stanford University School of Medicine
Stanford, California

Dr. Francoise Farron
Cancer Research Institute
New England Deaconess Hospital and
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Boston, Massachusetts

Dr. Roy C. Foster
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Mr. Gary L. Grove
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University Park, Pennsylvania

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Boston, Massachusetts

Mr. George S. Roth
Gerontology Research Center
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland

Ms. Marie Shatos
The Catholic University of America
Washington, D. C.

Dr. Inderjit Singh
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New York University Dental Center
New York City, New York

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Dr. John J. Yemma
Youngstown State University
Youngstown, Ohio

LECTURERS

Dr. Marlene Absher
University of Vermont College of Medicine
Burlington, Vermont

Dr. Milton Adesnik
New York University School of Medicine
New York City, New York

Dr. Paul Atkinson
Albert Einstein College of Medicine
Bronx, New York

Miss Sandra Barnard
Baltimore City Hospital
Baltimore, Maryland

Dr. Olga Blumenfeld
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Dr. William R. Brinkley
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Galveston, Texas

Dr. Vincent Cristofalo
The Wistar Institute
Philadelphia, Pennsylvania

Dr. Robert Dell'Orco
The Noble Foundation, Inc.
Ardmore, Oklahoma

Professor Harry Eagle
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Bronx, New York

Dr. Leonard Hayflick
Stanford University School of Medicine
Stanford, California

Mr. W. House
Imperial Cancer Research Fund Laboratories
London, England

Dr. Raju Kuchelapati
Yale University
New Haven, Connecticut

Dr. Donald G. Murphy
Adult Development and Aging Branch
National Institutes of Health
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Dr. Leslie Orgel
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San Diego, California

Dr. Keith Porter
University of Colorado
Boulder, Colorado

Dr. Stephen Roth
Mergenthaler Laboratory for Biology
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Baltimore, Maryland

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Ms. Evelyn C. Campbell

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Ms. J. Grilly

Dr. P. M. Kraemer

Dr. P. F. Mullaney

Dr. D. F. etersen

Dr. C. R. Richmond

Ms. Phyllis C. Sanders

Dr. J. A. Steinkamp

Dr. R. A. Tokey

FIRST ANNUAL LIFE SCIENCES SYMPOSIUM, MAMMALIAN CELLS: PROBES AND PROBLEMS (October 17-19, 1973)

The Los Alamos Scientific Laboratory's First Annual Life Sciences Symposium on "Mammalian Cells: Probes and Problems," sponsored by the Los Alamos Scientific Laboratory, the U. S. Atomic Energy Commission, and the National Cancer Institute, was held on October 17-19, 1973. The general scope of the symposium was as follows:

Macromolecular alterations, both within and on the surface of eukaryotic cells, can be measured conveniently and rapidly with a high degree of precision by applying new methods for measuring parameters such as DNA content and distribution, surface receptor sites, cell and nuclear volume, and light scattering. Because these measurements can be performed very rapidly (flow systems) or with markedly reduced ambiguity (microscopy involving fluorescence or Giemsa banding measurements), we can now contemplate experiments which previously were impractical. This symposium explored the validation, correlation, and application of these new analytical techniques to both applied and basic biological problems. Hopefully, we can review the current state-of-the-art and summarize observations on biological behavior of growing systems: cell-cycle analysis, DNA synthesis, cell-surface receptors, karyotypic stability, and changes associated with malignant transformation

Each of the five general sessions was opened

by the appointed chairman, and there were two full-length presentations covering the individual session topics, followed by shorter presentations as backup developments in specialized fields of instrumentation and cell analysis. Each session was concluded with summary remarks by the chairman or appointed participant. A short question and answer period followed the formal presentations in which all attendees were invited to participate. An outline of the program follows:

October 17, 1973

SESSION I: IMAGE ANALYSIS TECHNIQUES

Chairman's Remarks

G. Wied, M.D., Department of Obstetrics and Gynecology, University of Chicago

"Scanning Cytophotometry: Principles and Practices"

B. Mayall, Biomedical Division, Lawrence Livermore Laboratory

"Extraction and Evaluation of Information from Cell Images"

P. Bartels, Department of Microbiology and Optical Sciences, University of Arizona, Tucson

"Slit-Scanning Cell Analysis Methods"

L. L. Wheelless, Department of Pathology, University of Rochester Medical Center

"Analysis of DNA Synthesis Changes in Cells Infected with SV₄₀ Virus"

J. Lehman, Department of Pathology, University of Colorado Medical Center, Denver

"Image Analysis of Defined Populations of Irradiated Lymphocytes"

R. E. Anderson, Department of Pathology, University of New Mexico School of Medicine, Albuquerque

Session Summary

M. Menzelsohn, Biomedical Division, Lawrence Livermore Laboratory

SESSION II: FLOW-SYSTEMS CELL ANALYSIS AND SORTING

Chairman's Remarks

M. A. Van Dilla, Biomedical Division, Lawrence Livermore Laboratory

"Multiparameter Cell Sorting and Analysis"

J. A. Steinkamp, Biophysics and Instrumentation Group, Los Alamos Scientific Laboratory

"FMF Applications to Studies of Transformed Cells"

A. Vogel, Cold Spring Harbor Laboratory

"Applications of Cytophotographic Analysis"

M. Melamed, Sloan-Kettering Institute for Cancer Research, New York City

"Cell Preparation and Staining for Flow Systems"

H. A. Crissman, Biophysics and Instrumentation Group, Los Alamos Scientific Laboratory

"The Stanford Cell Sorter and Its Applications"

M. H. Julius, Department of Genetics, Stanford University School of Medicine

Session Summary

M. A. Van Dilla, Biomedical Division, Lawrence Livermore Laboratory

October 18, 1973

SESSION III: CELL-CYCLE ANALYSIS

Chairman's Remarks

D. F. Petersen, Cellular and Molecular Radiobiology Group, Los Alamos Scientific Laboratory

"A Biochemical Dissection of S-Phase"

G. C. Mueller, McArdle Laboratory, University of Wisconsin

"Cycle-Dependent Enzyme Induction"

G. M. Tomkins, Department of Biochemistry and Biophysics, University of California, San Francisco

"Biochemical Events in the Cell Cycle"

R. A. Tobey, Cellular and Molecular Radiobiology Group, Los Alamos Scientific Laboratory

"Cells in Metabolic G₁ Arrest"

R. W. Holley, The Salk Institute, San Diego

"Cycle-Dependent Therapeutic Agents"

V. H. Bono, Division of Cancer Treatment, National Cancer Institute

Session Summary

D. F. Petersen, Cellular and Molecular Radiobiology Group, Los Alamos Scientific Laboratory

SESSION IV: THE CELL NUCLEUS

Chairman's Remarks

J. H. Taylor, Institute of Molecular Biophysics, Florida State University, Tallahassee

"Discontinuous DNA Synthesis in Mammalian Cells"

J. A. Huberman, Department of Biology, Massachusetts Institute of Technology

"Mechanisms of Cell Banding and Practical Applications"

F. Arrighi, M. D. Anderson Hospital and
Tumor Institute, University of Texas Medical
Center, Houston

"Chromosome G-Banding and DNA Constancy"

L. L. Deaven, Cellular and Molecular Radio-
biology Group, Los Alamos Scientific
Laboratory

"Genetic Markers Associated with Hamster
Chromosomes"

E. H. Y. Chu, Department of Human Genetics,
University of Michigan Medical School,
Ann Arbor

Session Summary

J. H. Taylor, Institute of Molecular Bio-
physics, Florida State University,
Tallahassee

October 19, 1973

SESSION V: THE CELL SURFACE

Chairman's Remarks

P. M. Kraemer, Cellular and Molecular Radio-
biology Group, Los Alamos Scientific Lab-
oratory

"Chemical Nature of Binding Sites"

S. Kornfeld, Washington University Medical
School, St. Louis

"Molecular Organization of Membranes"

G. Nicolson, The Salk Institute, San Diego

"Speculations on the Binding of Multivalent Antigens
to Cell Surfaces"

G. I. Bell, Theoretical Division, Los Alamos
Scientific Laboratory

"Heterogeneity of Antibody Binding Affinity at the
Cellular and Serum Antibody Levels"

G. Siskind, Department of Pathology, Cornell
University Medical Center, New York City

"Cell Adhesion"

C. G. Hellerqvist, Department of Biology,
The Johns Hopkins University

Session Summary

P. M. Kraemer, Cellular and Molecular Radio-
biology Group, Los Alamos Scientific Lab-
oratory

SYMPOSIUM ATTENDEES

Dr. C. W. Abell
University of Texas Medical Branch
Galveston, Texas

Dr. H. M. Agnew, Director
Los Alamos Scientific Laboratory
University of California
Los Alamos, New Mexico

Dr. E. C. Anderson
Los Alamos Scientific Laboratory
University of California
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R. E. Anderson, M.D.
University of New Mexico School of Medicine
Albuquerque, New Mexico

Dr. Frances E. Arrighi
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Houston, Texas

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Tucson, Arizona

Dr. Mary L. Bartlett
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Los Alamos, New Mexico

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Nashville, Tennessee

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Dr. B. K. Bhuyan
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Dr. D. Billen
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Gainesville, Florida

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National Institutes of Health
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National Institutes of Health
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ICONS NEWSLETTER

The Health Division's first ICONS Newsletter (Isotopes of Carbon, Oxygen, Nitrogen, and Sulfur) was distributed late in 1973. T. W. Whaley (Group H-11) is the editor, and associate editors are D. G. Ott (Group H-11) and N. A. Matwiyoff (Group CNC-4). The Newsletter is not intended to be a periodical but will be issued routinely to disseminate information to those individuals with an interest in the uses and properties of the stable isotopes. The format will be flexible and will incorporate several feature articles, notes on conferences, letters to the editor, references to current journal articles and books, and anything else pertaining to the general subject. Current plans for future articles include detection methods, instrumentation, clinical investigations, synthesis of compounds, biological effects, production, and uses of stable isotopes as molecular probes.

Environmental Tissue Contaminants (etc.) Newsletter

The Health Division's first etc. Newsletter (Environmental Tissue Contaminants) was distributed in June 1973 to inform interested individuals of current environmental topics related to the tissue analysis program at the Los Alamos Scientific Laboratory. The tissue analysis program at present is directed toward the measurement of $^{239,240}\text{Pu}$ in selected tissues of both occupationally exposed

workers and members of the general public. It is very possible that the program may be expanded significantly to include the determination of non-radioactive metals. Some tissues have been analyzed for mercury, cadmium, beryllium, and lead. In February 1974 a second issue was mailed to pathologists participating in the LASL program. Future issues of the etc. Newsletter will present items of interest from the LASL tissue analysis studies.