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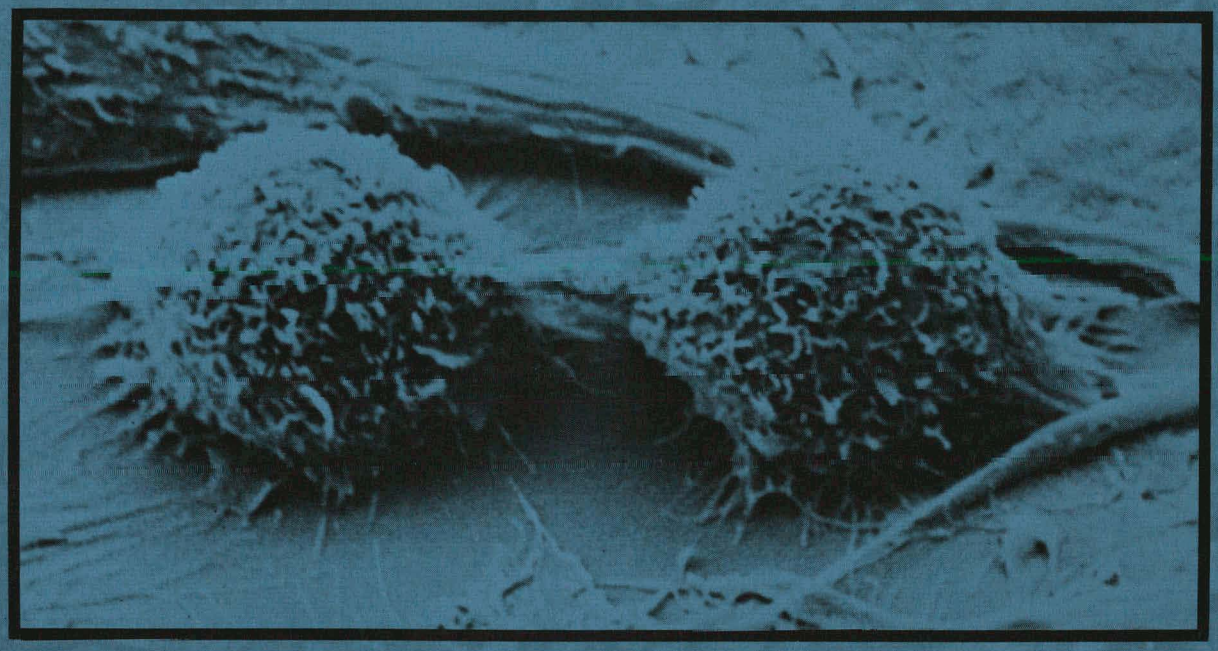
DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH

Annual Report

1976



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ARGONNE NATIONAL LABORATORY, ARGONNE, ILLINOIS

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DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH

ANNUAL REPORT
1976

Timothy E. O'Connor, Director
John F. Thomson, Associate Director
Steven S. Danyluk, Associate Director
Marcia W. Rosenthal, Editor

Preceding Report
ANL-76-99, December 1975

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COVER

LYMPHOSARCOMA FROM A DOG GIVEN COBALT-60 GAMMA IRRADIATION (See Section 3). PHOTOMICROGRAPH (BACKGROUND) AND SCANNING ELECTRON MICROGRAPH OF TUMOR CELLS GROWN IN CULTURE.

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ABSTRACTS

1. DIVISION DIRECTOR'S REPORT

Timothy E. O'Connor, Division Director

The primary mission of the Division of Biological and Medical Research, Argonne National Laboratory, is to explore the toxicological effects of effluents from various forms of energy production with a view to defining hazards and risk assessments for man. In order to meet this objective more effectively, the Division underwent extensive administrative organization in January 1976. The Organization Chart for the reorganized Division is shown in Figure 1.1.

One purpose of this reorganization was to provide for greater interaction and cohesiveness of the various scientific programs. Another objective was to provide a base for further evolutionary change that would match the scientific requirements of the Energy Research and Development Administration, the principal funding agency.

Two new scientific initiatives by the Division deserve special comment. During most of 1976, planning was underway for a new program to explore the toxicology of effluents from the fluidized bed coal combustion process. This new method of coal combustion is of particular importance for the utilization of coals of high sulfur content. Its successful development is especially relevant to eastern and midwestern portions of the United States, such as Illinois, where high-sulfur coal is abundant. These areas now must either rely on low-sulfur coal transported from western regions or employ stack-scrubbing technology to remove the sulfur dioxide from the effluents of combustion of regional coal. During the latter part of 1976, experimental work was undertaken with Laboratory support, and these initial efforts were subsequently expanded with ERDA funding in 1977. The initiation of these efforts required the close cooperation of the Chemistry, Chemical Engineering, and Engineering Divisions of Argonne National Laboratory.

A second project of note was the Molecular Anatomy Program (MAN), under the direction of Dr. Norman G. Anderson, which was initiated within the Division during 1976. In its early phases this program has emphasized the development of new methods of separation of proteins. The ISO-DALT procedure of two-dimensional gel electrophoresis already shows promise of providing a higher resolution of proteins than ever previously achieved. This method is being applied to the analysis of the protein constituents of plasma and urine with a view to detection of lesions arising from exposure to various energy pollutants. Successful development of this new technology could provide techniques for detection of pollutant-associated damage prior to development of

overt morbidity and thus prove invaluable in preventive medicine. The technology could provide a significant aid in routine medical diagnosis. The ISO-DALT procedure also permits delineation of the changing sets of proteins that are synthesized as cells undergo differentiation or malignant transformation and may thus permit new insights into the molecular aspects of differentiation, malignancy, and teratogenesis.

Research programs within the Division cover a range of topics in radiation biology, carcinogenesis, and biophysics. Perusal of this report indicates that significant progress was made in each of these categories during 1976, despite the inevitable trauma of reorientation of programs arising from national needs.

Two members of the Division achieved significant recognition during the present reporting period. Mr. George A. Sacher was awarded "The University of Chicago Award for Distinguished Performance at Argonne National Laboratory" for significant contributions to the field of radiation biology, specifically in developing a mathematical theory of chronic radiation lethality and by conducting an experimental program to test its main features. Argonne National Laboratory received an Industrial Research Magazine's 1976 IR-100 award for Dr. Y. E. Rahman's contribution in the field of liposome encapsulation of chelating agents and antitumor drugs.

The Division was co-host, together with the Division of Radiological and Environmental Research, Division of Environmental Impact Studies, Argonne Center for Educational Affairs, and Argonne Universities Association, of a bicentennial symposium on "Accomplishments and Challenges for American Life Sciences." Professor George W. Beadle, The University of Chicago, and Dr. Mortimer M. Elkind, Division of Biological and Medical Research, were co-chairmen of this memorable event which presented an overview of the interaction of the laboratory researcher, government agencies, and private philanthropy in the development of the present status of biology in America. The symposium was an occasion for joy over past achievements, and a time for evaluation of the tasks that lie ahead.

There is a growing appreciation that long-range solutions to the energy problem require the development of new technologies that are minimally toxic to man and nondeleterious to the environment. This Division has a major role to play in achieving these national goals. It is our intention to be responsive to immediate needs while at the same time to maintain our dedication to excellence in research, and our interest in basic research problems. Indeed, the solution to longer term problems may well require both a wider base of fundamental knowledge and the interest of first-rate minds that are attracted by the challenge of truly fundamental problems.

Timothy E. O'Connor
Director
Division of Biological
and Medical Research

DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH

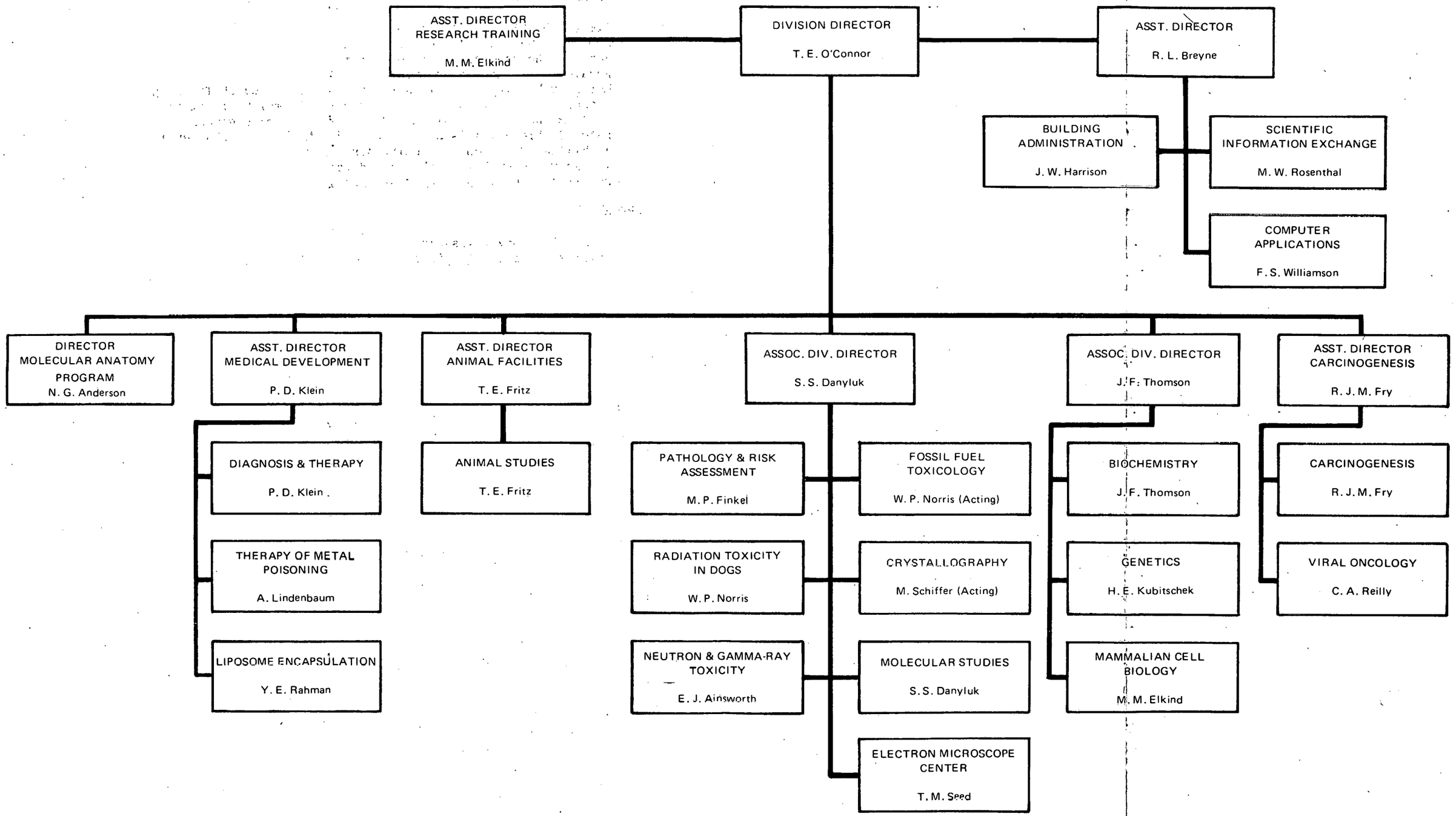

Fig. 1.1. Organizational Chart

ADMINISTRATIVE STAFF

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Steven S. Danyluk (Associate Director)
John F. Thomson (Associate Director)
Ronald L. Breyne (Assistant Director)
Mortimer M. Elkind (Assistant Director for Research Training)
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Peter D. Klein (Assistant Director for Medical Development)
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Marcia W. Rosenthal (Technical Editor)
Robert J. Robertson (Staff Assistant)

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Thomas J. Doody (Glassblower)
William J. Eisler (Engineering Specialist)

2. NEUTRON AND GAMMA-RAY TOXICITY STUDIES

ERDA RT-01-02
ANL 60300
NIH CA 18081-01

GROUP LEADER'S INTRODUCTION

John F. Thomson, Group Leader

Dr. E. John Ainsworth, who was the group leader for this program during the period covered by this report, transferred in early 1977 to the Environmental Impact Studies Division, along with Dr. Thomas B. Borak, who had been associated with the Dosimetry and Data Management Staff of the program.

The focus of the program is on the late effects of neutron and gamma radiation in experimental animals and the assessment of risk. The core of this program is a series of life-span experiments designed to define the responses to single, fractionated, or duration-of-life exposures to doses of neutron and gamma radiation, measured by analysis of life tables for cancer and noncancerous diseases. From the dose responses, models can be formulated for prediction of radiation hazards to man, particularly at low radiation doses.

There are a number of other end points deserving of study, as much for their inherent biological and radiobiological importance as for their contribution to risk assessment. Four of these end points--cardiovascular effects, host-defense mechanisms, hematology and hematopoiesis, and intestinal stem cell characteristics--are described in separate reports in this section.

On the basis of life shortening alone (histopathologic examinations are as yet incomplete), several conclusions can be drawn:

1) Whereas life shortening per rad is relatively constant for single doses of gamma irradiation, it varies inversely with dose for neutron irradiation (over a range of 20 to 240 rad); e.g., the number of days lost per rad after 20 rad is more than 3 times that observed after 240 rad.

2) Fractionation of the gamma dose (855 rad) produces a sparing effect, increasing with the number of fractions (6, 24, and 72), whereas fractionation of the neutron dose increases the life shortening, the increase seemingly independent of the number of fractions (this phenomenon is marked at 240 rad, not quite so convincing at 80 rad, and on the basis of preliminary analyses may be nonexistent at lower doses).

3) Female mice are somewhat more sensitive than males to fractionated neutron irradiation and considerably more sensitive to single dose exposures; there are no significant sex differences in life shortening after gamma radiation.

4) Life shortening per rad appears to be lower in older animals. These conclusions are based principally on data from relatively high doses; experiments are underway to validate these findings at lower doses.

Cardiovascular studies emphasize relationships between functional deterioration and histopathologic changes in the heart, major arteries, and capillaries. Functional changes, as measured by ^{133}Xe clearance time, were produced by relatively low doses of either neutrons or gamma rays, but there was no predictable dependence on radiation dose or quality. Similarly, there were only slight qualitative differences in the histopathologic effects of the two radiation qualities on the heart and aorta. However, studies of the heart, aorta, and microvasculature revealed that neutron radiation, especially when fractionated, was more damaging than gamma radiation.

Late radiation damage to the lung antibacterial mechanism occurred after doses of 240 neutron and 807 gamma rad. Animals receiving fractionated doses of gamma radiation were more severely impaired than those receiving single doses of either neutrons or gamma rays, or fractionated doses of neutrons. This finding is in marked contrast to the effect of these exposures on immune function, where a consistent enhancement of immunosuppression was observed after fractionation of the neutron dose. Late radiation damage has been observed in both the immune system and the microenvironmental stroma after doses as low as 40 rad of either neutrons or gamma rays.

The third set of pathophysiologic studies has been concerned with hematopoietic regulation and the functional capacity of stem cells from mice in which both the myeloid and lymphoid stem cell populations have been reduced. Injury to the hematopoietic system may be associated with deficiency in immune function, a propensity for leukemia and other neoplastic diseases, and nonspecific life shortening.

Finally, the differences in sensitivity of two strains of mice ($B6CF_1$ and BALB/c) have been shown to be explained, at least in part, by differences in the response of intestinal stem cells to colcemid block. In the BALB/c strain, the degree of stem cell killing during 18 hours of colcemid block was negligible, whereas in the $B6CF_1$ strain, there was nonlinear, parabolic, killing.

NEUTRON AND GAMMA-RAY TOXICITY STUDIES STAFF

REGULAR STAFF

*Ainsworth, E. John (Biologist)
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*Transferred to Environmental Impact Studies Division in 1977.

†Now in Pathology and Risk Assessment Group.

ERDA RT-01-02
ANL 60300

LIFE SHORTENING PRODUCED BY NEUTRONS AND GAMMA RAYS

John F. Thomson, E. John Ainsworth, Katherine H. Allen,† Jane L. Hulesch,† Donn L. Jordan, Louise S. Lombard,† V. Ann Ludeman,† Anthony R. Sallese,† Bobby R. Scott, Everett F. Staffeldt,† and Frank S. Williamson*

The purpose of the program is to obtain information, which ultimately will be of predictive value for assessing hazards to man, on the late effects in experimental animals of low doses of ionizing radiation. This information takes the form of dose-response curves, measured directly or indirectly, for two different qualities of radiation and for end points such as life shortening by various causes, particularly cancer; and the physical and biological factors affecting these responses, such as dose rate, fractionation, protraction, species of animal, age, sex, and hormonal and immunological status.

It has been recognized for many years that the precise shape of the dose-response curve for low levels of radiation cannot be determined directly without the use of exorbitant numbers of animals. Consequently, it is necessary to approach the problem by indirect means, so that extrapolation to lower dose ranges can be made with some certainty from the effects of doses that are amenable to study with manageable numbers of animals.

The use of two types of radiation, with quantitative differences in their biological effects, is an integral component of the program: gamma radiation from ^{60}Co sources, and fission neutron radiation from the JANUS reactor. One of the most important differences is the relative lack of dose-rate effects with the latter. It is our intention, therefore, to employ single exposures of neutrons at low doses, down to the lowest dose at which lethal effects or selected physiological changes can be reasonably expected to be observed with a "finite" number of animals. A limited number of experiments with fractionated doses will be carried out to assess the validity of the assumption of dose-rate independence.

The approach to the problem of gamma radiation will require considerable attention to dose protraction and fractionation. There is an extensive body of information available, much of it generated in this laboratory, concerning dose-rate effects on life shortening, based largely on duration-of-life exposure. Considerably less is known about protraction and fractionation effects on tumorigenesis.

Because some working hypothesis is essential to experimental design, we are using the "quadratic model," an empirical relation derived by G. A. Sacher that has in fact been validated by numerous studies, especially those of Grahn and Sacher with ^{60}Co gamma radiation.

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*Environmental Impact Studies Division.

†Pathology and Risk Assessment Group.

The obvious choice of experimental animals for economic reasons is the laboratory mouse (*Mus musculus*), specifically the $B6CF_1$/Anl hybrid. The extrapolation of *Mus* data to man, however, requires at least two intermediate stages. The first of these is the use of another rodent, the white-footed deer mouse (*Peromyscus leucopus*), about the size of *Mus* but with more than double the life-span and with a spectrum of tumors that is distinctly different from that of the $B6CF_1$ mouse. A limited number of single exposures to the same doses already employed in *Mus* should suffice to establish whether the life shortening per rad is an absolute value, or whether it is a percentage of total life-span.

The second stage involves the beagle, for which exposures to low-level continuous ^{60}Co gamma radiation have been underway for several years (see Section 3 of this report). The problems of dosimetry, numbers of animals needed, and the long life-span of the beagle militate against studying neutron effects. We expect that reasonable predictions can be made from the gamma-ray data by means of the comparisons established for the two radiation qualities between *Mus* and *Peromyscus*.

OPERATIONS

The JANUS program employs the combined skills of several groups of people involved with animal breeding and husbandry, radiation exposures, dose control and measurement, diagnostic observation, and collection of tissue and organ specimens for pathological evaluation. Of the nearly 10,000 mice now in the JANUS program, about 2000 are irradiated every week in a variety of exposure regimens. In the span of this reporting period, there have been 167 separate irradiations of 46 different neutron and gamma dose treatments, and nearly 5000 animals have passed through or are currently involved in this treatment phase. Each animal is observed at least once every day, 7 days a week, by personnel skilled in recognizing moribund symptoms.

The flow of animals into this system must be regulated to adjust to the limited manpower available for the task of tissue and organ collection and processing for pathological diagnosis. Gathering and verification of mortality data are fairly rapid, and the resulting mortality statistics are kept current. However, a considerable time lag occurs in the assessment of specific cause of death. Histopathologic examinations are carried out in collaboration with R. J. M. Fry (Carcinogenesis Group) and J. H. Rust (University of Chicago).

BIOLOGICAL STUDIES

The status of the irradiation experiments is as follows:

JM-2 involved a total of 7200 mice of both sexes (plus 2000 controls) exposed to single or fractionated doses of neutrons and ^{60}Co gamma rays. A number of animals were subsequently removed from the experiment, but 5869 irradiated and 1700 control mice were observed for their life-span. Preliminary analyses of life shortening data have been published (Ainsworth, E. J., et al., Proc. of the IRPA IVth International Congress, Paris, France, April 24-30, 1977, in press); histopathologic examination is nearing completion, and a

detailed analysis of causes of death should be available during 1977. In a supplementary experiment, JM-YZ, single doses (80 and 240 rad neutrons, 268 and 788 rad gamma rays) were given to older animals to compare the responses of 194- and 287-day-old mice with those of about 110 days, the age of the JM-2 mice at the time of their initial exposures; approximately 80% of this group of 2400 mice are now dead.

JM-3S is a single dose exposure series, 20 to 160 rad neutrons and 90-569 rad gamma rays, which is expected to produce 2% to 25% life shortening. Irradiations are 80% complete (2480 of 3100 animals).

JM-4K is a fractionated dose series given over 23 weeks, about 20% of the life-span. The total neutron doses are the same as those used in JM-3S, whereas the gamma-ray doses are higher (206 to 5110 rad total dose). The last replicate for irradiation treatment will be complete in April, 1977.

JM-4W and JM-5 are a series of single and fractionated doses for both serial evaluation and sacrifice for cardiovascular, hematopoietic, and immunologic studies. All irradiations have been completed for both series (3025 mice).

JM-6 involves irradiation of mice with both single and protracted exposures for the carcinogenesis study with pituitary hormones and isografts (in collaboration with R. J. M. Fry, Carcinogenesis Group). Irradiations have been completed for 1890 mice.

JM-7Q consists of fractionated doses given over 59 weeks (about 50% of the life-span) for comparison with JM-4K. As in most experiments, irradiation is begun when the mice are about 110 days old. Irradiations are 90% complete with 2520 mice done or in progress.

JM-7R is a companion experiment, in which single doses are given to mice at 523 days of age, i.e., at the end of the 59-week fractionation period used in JM-7Q. These experiments will extend the age-sensitivity studies that were represented in the JM-YZ series.

JM-8U employs one exposure per week for the duration of life of the animals, for comparison with similar total doses given for shorter intervals or as a single exposure. The original experimental design called for three dose rates of neutrons (0.667, 1.667, and 2.67 rad/exposure) and three of gamma rays (6.95, 17.4, and 31.4 rad/exposure); exposures have been initiated on 1200 mice.

JM-4L is an extremely low dose rate experiment employing the same constant weekly doses of gamma radiation as JM-4K, with the exposures protracted over about 110 hours rather than 45 minutes. Considerable dosimetry will be required before these exposures are begun.

MODELING

A kinetic model for radiation-induced life shortening in mice, due to late effects produced by low doses of ionizing radiation, has been developed. The model is based on the concept that an increase in the age-specific force

of mortality after exposure to radiation is a consequence of some form or forms of irreversible injury induced by the radiation. Life-shortening responses generated using the model have been shown to be consistent with available data for mice after single or fractionated doses of low- or high-LET radiation. Dose-RBE curves based on life-shortening responses have also been generated. It has been estimated that for gamma-ray doses of 100, 10, or 1 rad, the RBE's of fission or 1 MeV neutrons for life shortening, relative to ^{60}Co gamma rays, are about 10, 50, and 100, respectively, for RF or $B6CF_1$ mice irradiated at an early age. The model has also been shown to be convenient for extrapolating between sexes or strains; in order to do so, it is necessary to change only a single parameter.

Additional work has been done that concerns cell survival modification by wasted dose, after exposure to ultrahigh-LET particles (i.e., particles with LET of the order of 200 keV/μm or higher). Based on biophysical considerations, it is postulated that (1) when mammalian cells are irradiated with ultrahigh-LET radiation, they are likely to appear most sensitive to sterilization in the S phase, while appearing relatively resistant in other phases; (2) when cells are exposed to a conditioning dose of low-LET radiation immediately preceding a test dose of ultrahigh-LET radiation, the cells may appear to be protected against the high-LET radiation as a consequence of the conditioning dose.

The use of ultrahigh-LET radiation in the treatment of resistant tumors is likely to be beneficial, as a large proportion of the tumor cells are in the S phase, whereas normal cells, which are mostly responsible for late effects, are generally in the G_1 phase.

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DOSIMETRY AND DATA PROCESSING

Frank S. Williamson, Thomas B. Borak, Gordon L. Holmblad, Emil G. Johnson, Jr., and Joseph E. Trier*

DOSIMETRY

The purpose of this part of the JANUS program is to provide support by furnishing the best information obtainable on the radiation spectra used and on the absorbed-dose distribution within the biological specimens. For absorbed-dose distributions, we approach this goal in steps: (1) measure the spatial distribution of kerma in the radiation facility without biological specimens; (2) determine the perturbation of kerma caused by experimental specimens; (3) determine the absorbed dose distribution within the specimen, or its average if appropriate, as a function of the kerma determined in (1).

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*Environmental Impact Studies Division.

It is important to establish an audit trail of dosimetry data with efficient and accurate procedures for correction. The volume of data collected justifies the use of sophisticated computer data processing; for example, in scanning the JANUS high flux room, more than 5000 instrument readings were required.

The experimentally determined neutron spectra provide input to a Monte Carlo code, which then calculates the neutron spectra, absorbed dose, and LET distribution within mathematically modeled phantoms. It is most efficient to use this powerful computational tool as an adjunct to measurements of kerma in a field, verifying the calculations with selected measurements of absorbed dose in phantoms.

The following are some of the major areas of progress in neutron dosimetry during 1976:

The axial depth-dose distribution in a prolate spheroid tissue-equivalent phantom has been calculated using the Monte Carlo code and the dual-spectrum model (point source and isotropic). These data will establish a preliminary relationship between animal size and midline dose that is necessary for planning experiments with *Peromyscus leucopus*.

Monte Carlo calculation of the 5.5-MeV case (depth dose in 30-cm cube water phantom) of the 1973 International Neutron Dosimetry Intercomparison agrees very well with our measurements. These data have been transmitted to the evaluating committee, but we have not received any comments. Similar computations have been furnished to D. Bewley for the European Dosimetry Intercomparison and have been well received.

Calculations of kerma/fluence ratios, and of "W" for two standard tissue compositions, water, tissue-equivalent plastic, tissue-equivalent gas, acetylene, and ethylene have been made for two JANUS spectra. These data will permit the calculation of appropriate chamber constants and the recalculation of kerma distributions in the JANUS high flux room.

In collaboration with F. Kuchnir (Franklin McLean Memorial Research Institute) and T. Stinchcomb (DePaul University), LET and "Y" dose distributions have been measured in the JANUS high flux room "in air," in a dog phantom, in a 30-g phantom, and a 16-cm cube water phantom; and in the high level gamma room "in air" and in a dog phantom. From time to time, questions have been raised concerning our observations that the gamma-neutron dose ratio in the JANUS high flux room is less than 3%. These LET measurements confirm our assertion.

Measurements and Monte Carlo calculations have been made in an attempt to resolve the interface dosimetry problem experienced by M. M. Elkind when cells *in vitro* are attached to a polystyrene surface and irradiated with neutrons. Considerable progress was made, and modification of the Monte Carlo program to follow individual recoil particles could probably solve this problem.

Among accomplishments in gamma and X-ray dosimetry during 1976 were the following: (1) The dosimetry of the X- and Y-gamma rooms was documented and supplied to the Dog project. (2) Radiation effects in cables and connectors used with ionization chambers were studied in collaboration with J. Spokas,

Illinois Benedictine College. (3) A semiautomatic thermoluminescent dosimeter (TLD) readout system has been assembled and tested, and used to select a working set of TLD's. (4) In preparation for experiment JM-4L, the low-level gamma facility has been modified and the source mechanism replaced by a Gammabeam 150 (Atomic Energy of Canada Ltd.).

DATA PROCESSING

In any large-scale experiment involving the radiation treatment of thousands of animals or, indeed, in any epidemiological study, some form of computer-assisted data reduction and analysis is essential. In the JANUS program, we use computers for scheduling the animal irradiations, organizing and monitoring the random placement of animals in the irradiation facilities, and maintaining inventories of the animals in their living quarters. Wherever possible, operations are planned by computer and the records are automatically generated. Only errors in carrying out the planned operations are manually entered.

A computer system developed specifically for the JANUS program has proved invaluable for random allocation of animals to various experimental groups; directing the loading, irradiation, and unloading procedures; and the assignment of animals to specific locations within the geriatric ward or other components of the animal facilities. The system also prints cage cards, provides an audit trail of all manipulations and radiation doses to which the animals are subjected, and records survival and pathology data in files suitable for access by analysis and evaluation programs.

The gross autospy data for JM-2 (MACRO) are now stored in a file, and the few remaining animals from the aged animals addition, JM-YZ, are processed as they die.

The histopathology data for JM-2 (MICRO) are currently being processed. Extensive changes have been made to the programs in order to make many logical tests on the data. Since this file is, in effect, the final pathology diagnosis repository, a clear definition of logic in coding is critically important and a great deal of effort is being spent on thorough data checking and review. This experience confirms our belief that direct entry of such data from a terminal offering a selection list would be the method of choice. Death data from JM-3 through JM-8 are being kept up to date, but gross autopsy data are being held until JM-2 is analyzed.

The set of analysis programs now includes Hoel and Walburg cumulative mortality and age-specific death rates, with optional printer graphs and life tables. Causes of death and specific disease incidences can be extracted.

The JANUS record system has been interfaced to the BIM Animal Inventory System (see Section 17 of this report) so as to provide an audit trail for accounting.

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ANL 60300

PATHOPHYSIOLOGICAL EFFECTS OF EXTERNAL RADIATIONS ON THE CARDIOVASCULATURE

S. Phyllis Stearner, Emily J. B. Christian,* Rosemarie L. Devine,† and V. V. Yang**

Studies of the microvasculature, the heart, and the major blood vessels in the mouse evaluate long-term radiation effects of fission neutrons and ^{60}Co gamma rays, and compare these effects with normal aging. These studies complement those on life shortening and tumor incidence in the JANUS program, and provide improved definition of the role of systemic injuries in the late effects syndrome. Coronary artery damage and generalized vascular smooth muscle degeneration and fibrosis are end points of interest in connection with therapeutic use of low- and high-LET radiations, as well as with many phases of nuclear technologies.

Our studies of late damage to the cardiovascular system emphasize relations between (1) functional alterations, assessed by clearance of injected ^{133}Xe as an indicator of capillary blood flow, and (2) histopathologic changes in the subcutaneous microvasculature of the pinna and in the heart and aorta, as observed by light and electron microscopy.

The xenon-133 clearance technique (Stearner, S. P., and E. J. B. Christian, ANL 75-30, 1974, p. 59) showed a decrease in capillary blood flow between 3 and 30 months after irradiation with 80 neutron rad or 269 gamma rad, levels well below the acutely lethal range. A higher level of irradiation (but still below the acutely lethal range), either in fractionated or single exposures, resulted in little change in capillary blood flow compared with aged controls. The decrease in capillary flow rate after lower dose irradiation may be the result of degenerative changes in the medial layer of the blood vessel wall. Secondary increases in blood flow rates were observed at ages in excess of the mean life-span, and may represent compensatory aging changes reflecting increased need for O_2. Results clearly indicate that changes in capillary blood flow do not show a regular dependence on radiation dose and cannot be used as a basis for comparison of dose effectiveness for sources of different qualities.

Our capabilities for physiological observations have recently been extended by the development of a technique by G. Svihla (Molecular Studies Group) for the indirect measurement of blood pressure in mice. The method uses an inflatable cuff wrapped around the hind leg. The cuff pressure is rapidly raised above systolic pressure, then slowly reduced. An increase in tissue density, indicated by decrease in light transmission through the foot, reflects the systolic blood pressure. Comparison of blood pressure in irradiated mice with control values will allow correlation of any late postirradiation blood pressure changes with the changes in capillary flow rate and microvascular morphology already observed.

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†Electron Microscope Center Staff.

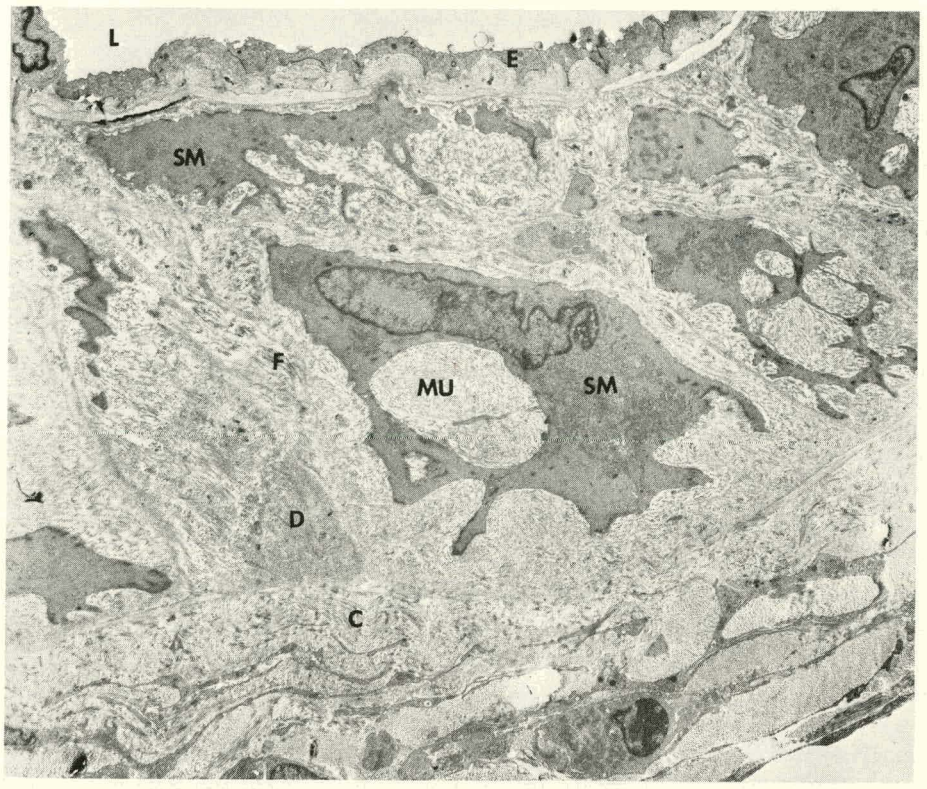

Fig. 2.1. Coronary artery at 18 months after a fractionated neutron dose of 80 rad, delivered in 24 fractions over 23 weeks. There is extensive degeneration of medial smooth muscle (SM), with accumulation of mucopolysaccharides (MU), debris (D), and fibrosis (F). L, lumen; E, endothelium; C, collagen. X2000.

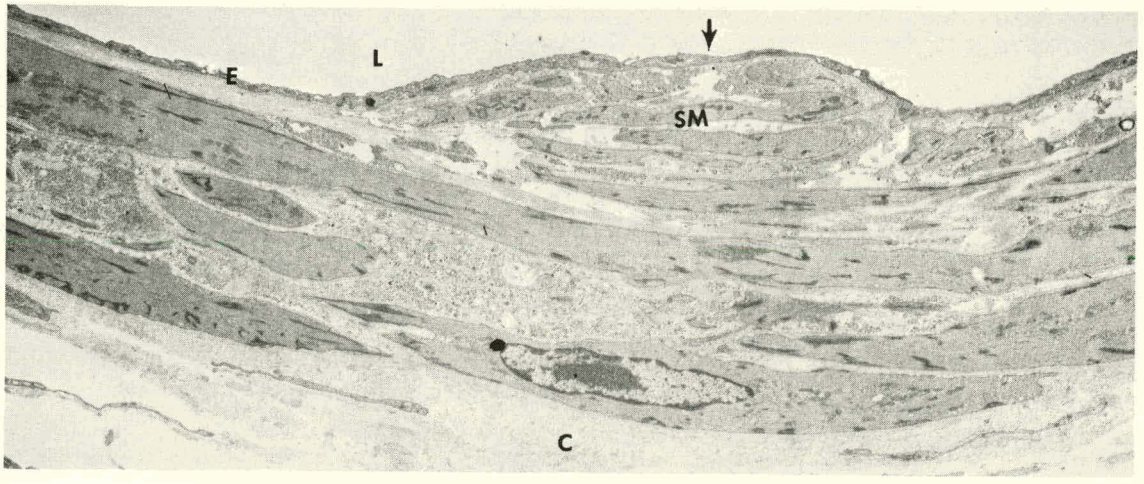

Fig. 2.2. Coronary artery at 12 months after a single gamma-ray dose of 788 rad. The intimal plaque (↓) contains smooth muscle cells (SM) and accumulated mucopolysaccharides. Note medial smooth muscle degeneration and debris. L, lumen; E, endothelium; C, collagen. X1600.

Late ultrastructural changes in the pinna microvasculature were compared with *in vivo* microscopic observations after neutron (240 rad) or gamma ray (788-2690 rad) treatment. Comparisons of single and fractionated fission neutron doses at 18-20 months after exposure suggested more severe medial smooth muscle degeneration with fractionated treatments. Less damage was seen in the pinna microvasculature after gamma than neutron irradiation. Recent work shows that even after a fractionated gamma dose of 2690 rad, changes in small arterioles were less prominent than after a neutron dose of 240 rad. Little endothelial damage was evident in any of the treatment groups.

Ultrastructural changes in the heart and aorta have been evaluated through 24 months after irradiation. Treatment groups include (1) single total-body doses of 80 or 240 neutron rad or 788 gamma rad, and (2) fractionated (24 fractions in 23 weeks) total-body doses of 20, 80, or 240 neutron rad, or 823 or 2690 gamma rad. Focal cardiac muscle degeneration and capillary damage, most severe at 1-3 months after irradiation, were less prominent at later times (12-24 months). In general, myocardial fibrosis increased with age. At 6-12 months, the endocardium was swollen, and there were increased accumulations of lysed materials and collagen fibers in the subepicardial region. Arterial degenerative changes, first noted in the larger coronary arteries at 3 months and in the aorta at 6 months, became progressively more severe with time. Major findings included smooth muscle degeneration, with fibrosis and extensive deposits of debris and extracellular matrix material. The matrix material was PAS positive, indicating accumulations of mucopolysaccharides. At 18 months after irradiation, degenerative changes in the coronary arteries were more extensive after fractionated neutron doses of 80 and 240 rad than after the same doses as single exposures. This is in sharp contrast to the sparing effect of fractionation for gamma irradiation. Our recent finding of marked coronary artery degeneration after a fractionated neutron dose of only 80 rad (Figure 2.1) indicates a special sensitivity to fission spectrum neutrons. For coronary artery damage, RBE estimates are in excess of 10 for fractionated exposures. Similar enhancement of neutron effects with fractionation has been noted for life shortening and tumor induction in the JANUS program (Ainsworth, E. J., et al., Proc. of an IAEA Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, IL, 1975, IAEA, Vienna, 1976, Vol. 1, p. 77). Among our more significant new findings are coronary arterial plaques, at 12 to 18 months after irradiation (Figure 2.2), that appear to involve smooth muscle proliferation. These plaques resemble lesions associated with human cardiovascular disease, except that they do not contain lipid accumulations.

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ANL 60300

EFFECTS OF NEUTRON AND GAMMA RADIATION ON HOST DEFENSE MECHANISMS

Patricia C. Brennan, David A. Crouse,* Wayne T. Kickels,* and Richard C. Simkins**

This study seeks to characterize host response to acute and chronic neutron and gamma irradiation with emphasis on late effects. Because of the generally accepted relationships between neoplasia and cellular immune function, our major effort is devoted to characterization of radiation effects on cell-mediated immunity. However, attention is also given to humoral immune function and to an assessment of the functional integrity of the pulmonary antibactericidal system.

Radiation effects on humoral immunity, i.e., circulating antibodies, have been studied extensively but much less is known about cell-mediated immune competence. Virtually nothing is known about the late effects of chronic low-level exposure to neutron or gamma radiation.

We have reported the early and late changes in T-cell (thymus-derived) numbers following single doses of 240 neutron rad or 788 gamma rad, and have made preliminary observations on the proliferative response of T cells and B (bone-marrow derived) cells (Ainsworth, E. J., et al., Proc. of an IAEA Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, IL, 1975, IAEA, Vienna, 1976, Vol. 1, p. 77; see abstract, this report). We have also recently observed the enhanced suppression of immune competence by fractionated compared to a single administration of 240 rad of neutron irradiation, with the ability to induce a graft-versus-host (GVH) reaction and proliferative ability of T-cells and B-cells as end points (Ainsworth, E. J., et al., Proc. of the IRPA IVth International Congress, Paris, France, April 24-30, 1977, in press).

Enhanced susceptibility to experimental respiratory infection following chronic exposure to low-level gamma radiation has been reported, but no similar data have been available for neutron-irradiated animals. We recently reported that mice irradiated with neutrons repair the radiation-damaged clearance mechanism more slowly than those irradiated with gamma rays, and that mice irradiated with a dose of 240 neutron rad or 807 gamma rad in 24 weekly fractions had impaired ability to clear a challenge dose of *Pasteurella pneumotropica* 2 weeks after the last fraction; mice given the same doses in a single irradiation had normal clearance at this time (Brennan, P. C., and E. J. Ainsworth, Proc. of the 16th Annual Hanford Biology Symposium; see abstract, this report).

During the year, we have completed an experiment comparing single doses of 240 neutron and 807 gamma rad, and the same total doses administered in 24 weekly fractions. The systemic GVH reaction was assayed by spleen weight

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changes in allogeneic recipient mice after intravenous administration of lymph node cells from the irradiated mice. The resulting splenomegaly is proportional to the number of cells transferred. Less splenomegaly in the recipient test mouse reflects the presence of fewer functional cells and/or impaired function of the cells present. Mice that received fractionated neutron irradiation showed a reduced capacity to induce a GVH reaction when compared to those irradiated with a single dose or with age-matched controls. Gamma dose fractionation, on the other hand, produces a sparing effect, indicating repair during the fractionation sequence. These effects were observed from 1 day to 1 year after irradiation.

Spleen cells from the same donor mice in the GVH assays were stimulated *in vitro* with the T-cell mitogen phytohemagglutinin and the B-cell mitogen bacterial lipopolysaccharide. Quantitatively similar results to the GVH data were obtained, i.e., enhancement of immunosuppression following fractionated neutron exposure and a sparing effect following fractionated gamma exposure.

Taken together, these data support, on a cellular basis, the life-shortening results and tumor appearance rates in similarly exposed populations.

We are currently using the homing of radiolabeled lymphoid cells to evaluate late radiation injury to the microenvironmental stroma, by introducing normal lymphocytes from young mice into old, irradiated mice. In reciprocal experiments, young mice receive lymphocytes from old or old, irradiated mice. All experiments have the appropriate young controls as recipients or donors.

When lymph node cells from young donors were injected into aged, irradiated recipients, there was a decrease in homing to the lymph nodes and spleen independent of weight changes in these organs, but an increase in homing to the bone marrow. This increase may be related to a reduction in stem cell content in the old bone marrow. There was also an increase in lung retention of the labeled lymph node cells, which may reflect vascular damage and failure to clear clumps of cells from the capillary bed.

When bone marrow cells from young donors were put in old, irradiated recipients, similar results occurred, with more pronounced increase in bone marrow homing. When bone marrow cells prepared from young, old, and old, irradiated mice were injected into normal young recipients, both age- and radiation-related alterations were observed. Bone marrow homing was increased with the aged cells and unchanged with the aged, irradiated cells. These results reflect the total cellularity of the donor mice and may be a result of changing ratios of cell types.

These experiments were carried out on mice that received single doses of neutron or gamma radiation ranging from 40-240 neutron rad and 40-807 gamma rad. A clear dose response was evident with both radiations, but at any given neutron to gamma ratio the two radiations were qualitatively similar. The changes described above not only indicate stromal damage but also demonstrate changes in cell populations at late times after irradiation.

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EFFECTS OF NEUTRON AND GAMMA RADIATON ON HEMATOLOGY AND HEMATOPOIESIS IN THE MOUSE

David A. Crouse, Jane L. Hulesch,* Marietta Miller,* and E. John Ainsworth†*

The contributions of late injury from irradiation to the hematopoietic system, particularly to the stem cell compartment, may be related to life shortening, deficiencies in immune functions, and the propensity toward leukemia as well as other neoplastic diseases. Little is currently known about late radiation effects on any stem cell population. The hematopoietic stem cell, assayed by transplantation procedures, is used as a model system to evaluate relationships among stem cell killing, the extent of repopulation, and residual injury in the stem cells based on their capacity to differentiate and produce functional, mature cells.

During the past year, studies have focused on matters of hematopoietic regulation and various functional capabilities of stem cells from mice in which the total stem cell population in the spleen and the femur is reduced. The contribution of late damage to the hematopoietic stroma, as a mechanism for the observed reduction in stem cell content, has also been investigated.

The ability of stem cells from aged and aged, irradiated donor mice to be sequestered in the spleens of young irradiated animals at 2 and 24 hours after injection of cells has been evaluated by the f-factor technique, which measures the fraction of total stem cells that are found in the spleen. F-factor measurements were necessary because lower stem cell content observed in femur preparations from aged, irradiated animals could be spurious if f were different. Stem cells of aged and aged, irradiated animals demonstrated considerable variation in f-factor. Part of this variation may be a result of the presence of abnormal cell types, which frequently occur in old or irradiated populations; we have confirmed the presence of abnormal cells by histological preparations. F-factors for aged, irradiated mice showed some degree of reduction at 24 hours, but because of the variability we cannot reject the hypothesis that the stem cells from aged or aged, irradiated animals demonstrate significant differences in seeding ability at 2 or 24 hours postinjection.

The stem cell population in the femur can be divided into two populations with different microenvironmental distributions. Part of the stem cell pool is located in the central marrow and can be easily aspirated from the femur with a needle, syringe, and medium. Another population is intimately associated with the bone structure and can be released by enzyme treatment and/or grinding of the bony matrix. The latter population is often considered to be most

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representative of the pluripotential hematopoietic stem cell. We have examined the aspirated and residual stem cell content of the femurs of aged and aged, irradiated animals. It appears that the increase in cellularity seen in the whole femur is a function of the increase in cellularity of the central marrow without any significant contribution from those cells associated intimately with the bone. Although the stem cell reduction of aged and aged, irradiated femurs appears to be a result of reduction in stem cells of both femur compartments, the reduction in the central marrow is greater. Also, comparisons of the distribution of gamma- and neutron-exposed femur stem cells reveal differences that may be explained by LET effects in bone and soft tissue (central marrow). Further studies are now underway to verify these findings.

In collaborative studies with Dr. Clifford Gurney (The University of Chicago), we are evaluating the ability of aged and aged, irradiated animals to respond to hypoxic stress (high altitude chamber) by the differentiation of stem cell progeny into mature, functional erythrocytes. Aged and aged, irradiated mice typically have a reduced stem cell content as well as hematocrit; however, it appears that these mice still respond to hypoxic stress by increasing their red cell levels in a manner parallel to, yet somewhat below, the response of normal young mice. This test system seems suitable for the detection of alterations in the ability of stem cells to differentiate along the erythrocyte pathway. Studies are now in progress to determine whether sustained or intermittent hypoxic stress has a differential effect upon the stem cells of aged or aged, irradiated mice.

In another study related to the differentiation potential of hematopoietic stem cells, we have evaluated the ability of stem cells from aged or aged, irradiated mice to differentiate into megakaryocytes, which release platelets into the peripheral blood. Earlier we had shown that over a limited range of injected stem cells from young control animals, the number of platelets in the peripheral blood at 10, 12, 14, and 16 days was proportional to the number of stem cells injected. Platelet levels appeared to reach a plateau at about 18 days, and the proportionality was then lost. Stem cells from aged and aged, irradiated animals again show the same proportional pattern of platelets to stem cells. However, the number of platelets produced per injected stem cell is elevated in preparations from aged and aged, irradiated animals. The mechanism of this alteration is unknown at present.

Further studies on the stem cell content of the peripheral blood have shown that aged and aged, irradiated animals, which both have a reduced stem cell content in the spleen and femur, do not show a decrease in the circulating stem cell population.

Baseline studies on the hematology and radiation responses of *Peromyscus leucopus* have been extended to include fractionated exposure groups as well as split-dose $LD_{50/30}$ and $LD_{50/5,6,7}$ experiments. In general, the hematological responses of *P. leucopus* are very much like those observed in the $B6CF_1$/Anl mouse. Initial suppression and subsequent recovery of populations of lymphocytes, granulocytes, platelets, and red cells, as well as organ weights and other hematological variables, appear to be very similar to those previously reported for the $B6CF_1$ mouse. Lethality studies have proved difficult by virtue of the variability of response seen in such outbred mice. Nonetheless, no unusual characteristics of sensitivity or resistance are noted, and statistically reliable values for hematopoietic and gastrointestinal lethality will be completed with data and experiments currently in hand.

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INTESTINAL STEM CELL CHARACTERISTICS IN THE B6CF$_1$ AND BALB/c STRAINS OF MICE

Wayne R. Hanson, R. J. Michael Fry,* and Anthony R. Sallese†*

The intestinal microcolony assay has been used widely in the past as a test system for acute radiation effects, fractionation procedures, and drug effects. The use of this assay is being expanded greatly and will be used as a major assay for the effects of combined modalities on normal tissues. Certain assumptions were made during the development of this assay which have not been adequately tested. The two major assumptions were: (1) microcolonies come from single surviving clonogenic cells, and (2) the survival of these cells is independent of other surviving cells. Two other assumptions have been implicit in more recent investigations: namely, (3) crypt cells are fully oxygenated, and (4) the cells in amplification division with a cycle time of about 12 hours are capable of forming microcolonies.

It is likely that the first two assumptions are valid for the exponential portion of the survival curve, but neither one has been tested. The third assumption has not been tested, and the fourth appears to be invalid in that cytotoxic drugs such as high specific activity tritiated thymidine and colcemid, both of which reduce the number of cells per crypt by selectively killing cells in amplification division, have little effect on the clonogenic cell survival curve. Hydroxyurea, cytotoxic to cells in DNA synthesis and effective as a G_1-S interface block, produces stage-dependent cell killing. Cells released from the hydroxyurea block appear to be more sensitive in late G_1-early S and more resistant in mid to late S. The data from thymidine and colcemid suicide experiments suggest that the stem cells have a long cell cycle time with few cells in S. Further, cells in amplification division appear to be incapable of forming microcolonies. The data from hydroxyurea expriments suggest that stem cells in an extended G_1 may respond to hydroxyurea by going into a more rapid cycle.

Experiments were designed to block and kill cells with colcemid for an extended period of time. Since the stem cells appear to be slowly cycling and if it is assumed that the flux of stem cells through the cycle is uniform, a linear reduction of surviving stem cells during each time interval of colcemid block should be seen. Results of these experiments using the B6CF$_1$ strain of mouse showed a parabolic function of cell killing during colcemid block. However, in the BALB/c strain, the degree of stem cell killing during 18 hours of colcemid block was negligible.

The difference in response of the stem cells may in part explain the difference in radiosensitivity between the two strains. The constant increase in the degree of stem cell killing in the B6CF$_1$ strain during long-term colcemid

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block suggests that the stem cells are cycling faster. This may constitute a major compensatory capability of the intestine to injury. These findings have consequence in the interpretation of combined modality studies of drug and radiation effects. Further, caution in the evaluation of data from experiments dealing with fractionation regimens is suggested.

LIFE SHORTENING, NEOPLASIA AND SYSTEMATIC INJURIES IN MICE AFTER SINGLE OR FRACTIONATED DOSES OF NEUTRON OR GAMMA RADIATION*

E. J. Ainsworth, R. J. M. Fry, Patricia C. Brennan, S. Phyllis Stearner, J. H. Rust, and F. S. Williamson

The late somatic effects of fission neutrons from the Janus reactor or of ^{60}Co γ radiation are being evaluated in hybrid $B6CF_1$ mice (C57BL/6 x BALB/c) of both sexes. Single or fractionated doses were administered in an experiment designed to evaluate the effects of dose, dose rate, number of radiation fractions, age at exposure, and quality of radiation. Principal end points include life shortening, age-specific rates of neoplastic and non-neoplastic diseases, and late manifestations of damage to the vasculature, cell-mediated immune responsiveness, and haematopoiesis. Few survivors remain alive in the initial experiment, so provisional estimates of percent life shortening and RBE are derived from computations of mean after-survival. Over the range of single doses from which data are now available, the life shortening is inversely related to dose and the RBE ranges from $\sim$2 to 7. The effect of age at irradiation on life shortening was evaluated by administration of single doses at 115, 190 or 280 days of age. Increased age affords some sparing effect for both neutron and γ radiations, but an increased RBE was observed at 270 days. When neutron or γ doses are protracted over 6 months, similar mortality (rates) result from weekly doses of 3.3 neutron and 35 γ rad; under these conditions the RBE is estimated at 10-13. Data on lower weekly doses, which will be available in the future, will provide information on the extent to which RBE is dose-dependent under these conditions of long-term irradiation. Life shortening and age-specific death and tumour rates are compared when a total dose of 240 neutron rad is administered as a single dose or as fractionated doses distributed over 6 months; greater life shortening and higher age-specific death and tumour rates result from the dose fractionation. This enhancement phenomenon produced by neutron dose fractionation could result from a sparing effect on cell killing, with a concomitant increase in the expression of neoplastic transformation, or from increased cell killing and promotion of neoplastic growth due to repeated injury to susceptible cell populations. The structural and functional integrity of the vasculature, assessed by clearance of ^{133}Xe after a single dose or fractionated doses of neutron or γ radiation, indicates that the late effects of neutrons are at least total-dose-dependent, whereas a significant sparing effect is observed with fractionated γ irradiation. Late effects on cell-mediated immunity are evident in both γ and neutron-irradiated animals based on splenic repopulation by thymus-derived lymphocytes (T cells) and on the mitogen response of T cells that repopulate the spleen.

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*Abstract of a paper published in Biological and Environmental Effects of Low-Level Radiation, Vol. 1. IAEA International Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, IL, Nov. 3-7, 1975. International Atomic Energy Agency, Vienna, pp. 77-92, 1976.

DOSE-EFFECT RELATIONSHIPS FOR LIFE SHORTENING, TUMORIGENESIS, AND SYSTEMIC INJURIES IN MICE IRRADIATED WITH FISSION NEUTRON OR ^{60}Co GAMMA RADIATION*

E. J. Ainsworth, R. J. M. Fry, F. S. Williamson, P. C. Brennan, S. P. Stearner, V. V. Yang, D. A. Crouse, J. H. Rust, and T. B. Borak

Predictive models are essential for estimation of excess risk of cancers or other deleterious late effects of low- and high-LET radiation at dose levels where epidemiological data for man are incomplete and where dose levels are too low for direct confirmation by animal experiments. Models must benefit from human data, from results with experimental animals, at least in a qualitative mode, and from some understanding of critical biological events at the molecular, cellular, and tissue levels. Although reliable methods for quantitative extrapolation of risk estimates from experimental animals to man remain a challenge, animal experiments provide data needed to test existing models, formulate new models, and increase understanding of pathogenesis of late effects. Some results presented here are consistent with existing models, viz. RBE for life shortening is inversely related to dose and conforms to a slope of -0.5, and excess mortality after protracted gamma irradiation follows quadratic kinetics. However, other findings depart from expectations from existing models, viz. a slope of 0.6 rather than 1.0 describes the relationship between neutron dose and excess mortality between 20 and 240 rad, and enhanced effects of neutron dose fractionation must be considered in formulation of new predictive models. The observation that dose-response kinetics for excess mortality after neutron irradiation departs from expectations derived from short-term cellular responses emphasizes the myriad biology interposed between energy deposition, tumorigenesis and/or other events that culminate in death of an animal.

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*Conclusion of a paper to be published in the Proceedings of the IRPA IVth International Congress, Paris, France, April 24-30, 1977, in press.

DOSE RATE STUDIES WITH FISSION SPECTRUM NEUTRONS*

E. J. Ainsworth, D. L. Jordan, M. Miller, E. M. Cooke, and J. S. Hulesch

Fission spectrum neutrons produced by the JANUS reactor were used to test the hypothesis that no effect of dose rate or exposure time occurs when mouse lethality or hematopoietic injury is evaluated. Reduction of the dose rate from 13.1 to 1.2 rad/min resulted in a small increase in $LD_{50/30}$ and $LD_{50/7}$. Whereas no dose rate effect was found for cell killing (D_o) of hematopoietic stem cells in the femur or spleen, mice that received a sublethal dose at

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*Abstract of a paper published in Radiat. Res. 67, 30 (1976).

1.2 rad/min showed a small but reproducible advantage in repopulation of stem cells in the femur, and in reappearance of platelets in peripheral circulation. During a course of fractionated neutron irradiation, the femur stem cell content was higher in groups which were irradiated at 0.13 rad/min in comparison with others irradiated at 2.13 rad/min. Thus, the effects of fission spectrum neutrons are not totally independent of dose rate or exposure time based on the end points evaluated in these experiments. The effect of neutron dose rate on cell killing and on division delay or repopulation could vary independently.

EARLY AND LATE EFFECTS OF FISSION NEUTRON OR GAMMA IRRADIATION ON THE CLEARANCE OF *Pasteurella pneumotropica* FROM THE LUNGS OF B6CF$_1$ MICE*

Patricia C. Brennan and E. John Ainsworth

Enhanced susceptibility to experimental respiratory infection following chronic exposure to low-level gamma radiation has been reported, but no comparable information exists for neutron-irradiated animals. Such information is needed in view of the apparently greater additivity of repeated low fission neutron doses. Consequently, altered susceptibility to respiratory infection is being examined in the JANUS Neutron and Gamma-Ray Toxicity Program. B6CF$_1$ mice of various ages were challenged with *Pasteurella pneumotropica* either by intranasal instillation or by aerosol inhalation following single or fractionated doses of neutrons or ^{60}Co gamma radiation. Clearance of the bacteria from the lungs was assessed 4 days after challenge by a culture technique and by histological and immunofluorescence staining. From 5-21 days after a single dose of 288 neutron rad or 740 gamma rad, a ratio equal to the relative biological effectiveness (RBE) for cell killing, there was little repair of the radiation-damaged clearance mechanism evident in neutron-irradiated mice; 85% were unable to clear the organism as long as 21 days after irradiation. Over the same period only 25% of gamma-irradiated mice failed to eliminate *P. pneumotropica*. Immunofluorescent-stained lung sections at all time intervals between 5 and 21 days were strikingly similar among neutron- and gamma-irradiated mice and unirradiated mice. Alveolar macrophages were swollen with fluorescent *P. pneumotropica* cells, and macrophages surrounding the bronchi and in the bronchial exudate were also intensely fluorescent. These data, coupled with the culture data, indicate that pulmonary macrophages in the irradiated host are capable of engulfing *P. pneumotropica* cells, but that the ability to kill them is impaired.

The ability to clear a challenge of *P. pneumotropica* following single doses of 240 neutron rad or 807 gamma rad, or following the same total doses given in 24 weekly fractions, was assessed 2 weeks after the last fraction and 26 weeks after a single dose. Mice given single doses were able to clear the organism; mice that received the same total dose in 24 fractions were not. Interestingly, mice receiving fractionated gamma radiation were

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*Abstract of a paper to be published in the Proceedings of the 16th Annual Hanford Biology Symposium, Richland, WA, Sept. 27-29, 1976, in press.

more severely impaired than those receiving fractionated neutron radiation. Consequently, under conditions of protracted irradiation the RBE of fission neutrons is less than 3.6 based on susceptibility at ∿ 300 days of age.

MIXED-POPULATION TYPE SURVIVAL CURVE IN THE ABSENCE OF A MIXED POPULATION*

B. R. Scott

Based on biophysical considerations, it is shown that survival curves (dose-log surviving fraction curves) that appear to be due to the presence of a mixed population can occur in the absence of a mixed population when 1-hit biological targets are exposed to ionizing radiation. This phenomenon is predicted to occur when some of the targets are inactivated by electrons in the slowing-down spectrum that are tightly clustered in space while others are inactivated by electrons in the slowing-down spectrum that are dispersed in space. Targets inactivated by electrons that are tightly clustered will appear to be more resistant than those inactivated by electrons that are dispersed owing to a greater waste of dose in the former case. Spatial regions where the tightly clustered electrons are found will define a separate compartment from spatial regions when they are not found. Based on these compartments and the targets therein, the dose-response survival fraction is shown to be given by the sum of exponentials rather than their product.

*Abstract of a paper submitted for publication.

ON THE THEORETICAL RELATIONSHIPS BETWEEN CELL SURVIVAL MODIFICATION AND WASTED DOSE*

B. R. Scott

Based on biophysical considerations, it is postulated that when cells are irradiated with ultrahigh linear energy transfer (LET) radiation (LET of the order of 200 KeV per micrometer or higher):

1. The radiosensitivity as a function of phase of the cell cycle may vary in a nonconventional manner. The phase in which cells are most sensitive to low-LET radiation may be the phase in which they are most resistant to ultrahigh-LET radiation. Conversely, the phase in which the cells are most resistant to low-LET radiation, may be the phase in which the cells are most sensitive to ultrahigh-LET radiation. In order for this phenomenon to

*Summary of a paper to be submitted for publication.

be observed experimentally, the choice of the radiation and cells should be as follows. The low-LET survival curve should have a large shoulder when the cells are irradiated in the most resistant phase of the cell cycle. The high-LET survival curve should have no shoulder whatsoever, for any phase of the cell cycle. Furthermore, the cells must be highly synchronized.

2. If cells are exposed to a conditioning dose of low-LET radiation immediately preceding a test dose of ultrahigh-LET radiation, the cells may appear to be protected against the high-LET radiation, as a consequence of the conditioning dose. The magnitude of this protection should decrease as the interval between the conditioning and test doses increases, and should increase as the conditioning dose increases.

ON THE POSSIBLE EXISTENCE OF A NEGATIVE CURVATURE ON DOSE-RESPONSE CELL SURVIVAL CURVES FOR CELLS IRRADIATED WITH HIGH-LET IONIZING RADIATION*

B. R. Scott and E. J. Ainsworth

A kinetic model is presented that is equivalent to the ion-kill gamma-kill model of Katz *et al.* By use of a second kinetic model it is shown that if one abandons the assumption of the independence of the ion- and gamma-kill modes of inactivation, then dose-log surviving fraction curves with negative curvature (i.e., the magnitude of the slope decreases as the dose increases) are predicted to occur when $P/[(1-P)L]$ is greater than λ_0 and less than ∞, where P is the fraction of the dose in the ion-kill mode, L is the LET_∞, and λ_0 is a positive constant that depends on the type of cell.

It is pointed out that cell survival curves with negative curvature for cells of the same type irradiated with high-LET radiation have the following biological implications:

(1) The cells may accumulate and repair nonlethal damage.

(2) Fractionating the dose may cause enhanced cell killing when compared to that observed after a single exposure. This enhancement would only occur if the cells repaired nonlethal intracellular damage.

(3) Cell killing at low dose rates may be greater than that observed at high dose rates when the total dose is fixed. This enhancement would also occur only if the cells repaired nonlethal intracellular damage.

Some dose-response curves showing negative curvature are presented. It is also suggested that an exponential survival curve does not necessarily imply the absence of nonlethal cellular damage in the surviving cells. This may be of some importance in regard to the induction of tumors by high-LET radiations.

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*Abstract of a paper submitted for publication.

STATE VECTOR MODEL FOR LIFE SHORTENING IN MICE AFTER BRIEF EXPOSURES TO LOW DOSES OF IONIZING RADIATION. I. THEORY*

B. R. Scott and E. J. Ainsworth

A model for radiation-induced life shortening caused by late radiation effects is presented. The model is based on the concept that life shortening is caused by some form or forms of irreversible injury induced by the radiation. The model is kinetic in nature. In the development of the model, the different age-specific forces of mortality to which injured or uninjured animals are subjected are considered. Equations are derived for the mean survival time after irradiation, mean age at death, percent life shortening, and percent life shortening per rad, as a function of the midline tissue dose, dose rate, and age at exposure.

*Summary of a paper to be submitted for publication.

STATE VECTOR MODEL FOR LIFE SHORTENING IN MICE AFTER BRIEF EXPOSURES TO LOW DOSES OF IONIZING RADIATION. II. APPLICATION TO AVAILABLE DATA*

B. R. Scott and E. J. Ainsworth

The state vector model for life shortening in mice is shown to be useful in simulating dose-response curves for the mean survival time, mean age at death, percent life shortening, percent life shortening per rad, and relative biological effectiveness (RBE) when mice are exposed to low or high linear energy transfer (LET) radiation.

The following conclusions are drawn concerning life shortening after brief exposures to low radiation doses administered at high dose rates:

a. Like the RBE for cell killing, the RBE for life shortening depends on radiation quality, number of dose fractions, dose rate, and generally decreases as the dose increases. Unlike the RBE for cell killing, the RBE for life shortening also depends on sex, age at irradiation, and strain.

b. In some cases, the percent life shortening per rad may decrease as the dose increases over a range of doses. It is likely that this phenomenon is due to delays in the time of appearance of specific causes of death (e.g., tumor latent period), rather than to physical saturation of the dose.

c. It is unlikely that the percent life shortening (% LS) after exposure to fission neutron doses to tens of rad or higher can be characterized by the linear-quadratic relationship, $\% LS = k_1 D + k_2 D^2$; where k_1 and k_2 are

*Summary of a paper to be submitted for publication.

positive constants, and D is the midline dose. However, after exposure to 250 or 300 kVp X-rays, 60 MeV protons, or ^{60}Co gamma rays, the linear-quadratic relationship may be adequate to doses of the order of 100 rad.

d. Neutron RBE values for life shortening, relative to ^{60}Co gamma rays, may be underestimated at low doses, when one assumes the neutron dose-response curve to be linear for doses to tens of rad.

LATE CHANGES IN THE IRRADIATED MICROVASCULATURE: AN ELECTRON MICROSCOPE STUDY OF THE EFFECTS OF FISSION NEUTRONS*

S. P. Stearner, R. L. Devine, and E. J. B. Christian

Microvascular changes in the pinna were studied *in vivo* and recorded photographically over a period of 12-18 months after irradiation of 4-month-old B6CF$_1$ mice. Radiation treatment consisted of total-body exposure to 240 rad fission neutrons either in a single dose or in 72 fractions of 3.3 rad each over 24 weeks. Neutrons with a mean energy of 0.8 MeV were supplied from the JANUS reactor. A fission neutron dose of 240 rad is below the acutely lethal range. At 20 months after treatment, after a series of *in vivo* observations of the microvasculature, animals were sacrificed for study of changes in vascular fine structure in the pinna. Blood vessels were selected from regions that had been identified on photomicrographs. After single or fractionated neutron exposures, the surviving functional blood vessels had relatively minor late ultrastructural changes in the endothelium. Many arterioles, however, showed extensive degenerative changes in the subendothelial intima (including the elastica) and marked necrosis of smooth muscle. Accumulations of fibrillar material and debris frequently occupied much of the media and replaced regions of smooth muscle lost by focal necrosis. Arteriolar degeneration and sclerosis appeared to be more extensive after fractionated treatments. Corresponding small veins or venules also showed smooth muscle degeneration and increased fibrosis, but changes were somewhat less severe than in arterioles. Capillary changes included a thickened basal lamina and increased fibrosis. Endothelial swelling and increased vacuolization were sometimes observed.

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RADIATION-INDUCED CHANGES IN THE FINE STRUCTURE OF THE HEART: COMPARISON OF FISSION NEUTRONS AND ^{60}Co γ RAYS IN THE MOUSE*

V. V. Yang, S. P. Stearner, and S. A. Tyler

The ultrastructural changes in mouse cardiac muscle and cardiac vasculature from 4 days to 1 yr after irradiation are described. Radiation treatment consisted of single-dose, total-body exposure to 240 rad fission neutrons or 788 rad ^{60}Co γ rays.

Cardiac muscle showed areas of focal myofibrillolysis, myofibrillar degeneration with loss of entire myofibrils, presence of lipid bodies and of lysosomal-like bodies, and partially vacuolated mitochondria in some myocytes. There was apparently no Z-band thickening or disruption. Intercalated disks were sometimes dissociated and interstitial fibrosis was occasionally seen surrounding myocytes.

The cardiac microvasculature showed progressive degenerative lesions, including swollen endothelial cells, cytoplasmic blebs and extensions, myelin-like figures, vacuolated mitochondria, and thrombi that adhered to irregularities of the endothelial surface. After both forms of irradiation, smooth muscle degeneration and fibrosis in coronary arteries first appeared at 3 months and became progressively more severe at 6 and 12 months.

Quantitative estimations of myofibrillar and capillary degeneration revealed that damage was most severe at 1-3 months in both neutron- and gamma-irradiated groups. At 12 months, significant capillary degeneration was still present, but the condition of the myofibrils was comparable to that in controls. Quantitative differences between neutron- and gamma-irradiated groups were not statistically significant.

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*Abstract of a paper published in Radiat. Res. 67, 344 (1976).

3. RADIATION TOXICITY IN DOGS

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GROUP LEADER'S INTRODUCTION

Thomas E. Fritz, Group Leader

Dr. William P. Norris, who was the group leader for this program during most of 1976, became group leader of the new Fossil Fuel Toxicology Group in December.

The objective of these studies is to determine the detailed responses of beagle dogs subjected to continuous (22 hr/day) irradiation from a ^{60}Co gamma-ray source. The effects of three major factors associated with the irradiation procedures are being explored: (1) the daily irradiation dose rate, (2) the accumulated total exposure to radiation, and (3) the influence of age at the time irradiation is initiated on the effects observed. The work compares the responses of young adult (about 400 days of age) beagles of both sexes, kept in the gamma-ray field until they die, with those of similar dogs that have accumulated predetermined total exposures ranging from 600-4000 R. In addition, the developing beagle fetus is being irradiated continuously throughout its gestation period, and this work is being extended to include the postnatal period.

Considerable work has already been completed on defining the responses of young-adult and fetal beagles irradiated at rates ranging from 5-300 R/day. In these studies, major interest became centered on the effects observed at 5-35 R/day where, with decreasing daily dose rate, there were systematic, consistent, dose-rate-dependent responses limited primarily to the hematopoietic and reproductive organs. These results have been discussed in previous reports (Norris, W. P., et al., ANL-76-99, 1975, p. 10; Norris, W. P., et al., ANL-75-30, 1974, p. 9).

The aggregate of the data from dogs irradiated at the above rates argued strongly in favor of pursuing this work in dogs at daily dose rates substantially below 5 R/day, with the three purposes of (1) acquiring data on the mortality rates in young-adult beagles that will extend our present comparisons of responses between species, (2) determining the nature of the responses and pathologic changes that may occur, and (3) defining the exposure rates that allow continued reproduction.

Construction of a new gamma-ray facility that will house 200 beagles exposed at rates of either 2.5, 1.0, or 0.4 R/day was completed during the past year and, following dosimetric measurements, it was put into operation. The anticipated response of the animals to these low exposure rates will be slow enough to allow us to redirect a portion of our time to investigate, in more detail, the cellular mechanisms associated with the previously defined radiation-induced responses. Work in this area was initiated during the past year. An especially important aspect of this new research is to gain an insight into the changes in hematopoietic control mechanisms and functions that lead to leukemia and its pathologic opposite, aplastic anemia. Comparison of the hematopoietic effects of chronic irradiation with effects of other agents, particularly chemicals, that also have a marrow-suppressive and leukemogenic effect, also is an important and relevant goal.

Four related, but separate, studies are now in progress. In the first, young-adult beagles of both sexes are placed in the gamma-ray field for duration of life at one of a number of daily exposure rates. In the second, young-adult beagles are exposed in a similar fashion, at the same exposure rates, until they have accumulated predetermined amounts of total exposure ranging up to 4000 R. They are then removed from the radiation field and kept for the rest of their lives to allow development and study of late radiation effects. In the third study, pregnant beagles are irradiated at one of the several exposure rates, for all or part of the gestation periods, to evaluate the effects of continuous irradiation of the developing fetus. The fourth study, initiated during the past year, consists of irradiating dogs at 10 R/day, the rate shown by the first study to be the most efficient (in terms of time to induction and percent developing the disease) for the induction of leukemia. These dogs are being followed through sequential blood and bone marrow biopsies that are evaluated by cytologic, light and electron microscopic, and cultural procedures to determine the changes leading to the onset of leukemia.

Two important aspects of the above studies of protracted whole-body external irradiation are the use of the data for interspecies comparisons, and for comparisons with the effects of irradiation from internally deposited radionuclides. In the case of interspecies comparisons, the ultimate intent is extrapolations to man. In the case of comparisons with effects produced by internal radionuclides, accurate relationships between effects, exposure rate, and total dose can be established for ^{60}Co irradiation that are difficult to determine with radionuclides because most are nonuniformly distributed in the body.

Our studies have now reached the point where we can develop dose-rate specific end points in the beagle for use in the comparative studies just described. The following list summarizes the most relevant conclusions and observations from the four studies that will be discussed in the reports to follow.

1) Myelogenous leukemia occurs in about half of dogs exposed to 5-10 R/day.

2) Implantation of fertilized ova is not impeded in bred bitches exposed to 5 R/day, but is substantially reduced at 17 R/day.

3) Organogenesis in the beagle fetus proceeds normally at 5-35 R/day.

4) Testicular development in the male beagle fetus proceeds normally at 5 R/day, but not at 10 R/day.

5) Ovarian development in the female beagle fetus proceeds normally at 2.5 R/day, but not at 5 R/day.

6) Normal body size is not attained by beagles exposed to greater than 5 R/day during fetal life.

7) Normal dental development and eruption is inhibited in beagles exposed during fetal life at about 10 R/day.

During the past year the results of our studies with dogs, including those points listed above, were compared with data taken from mice previously published by Sacher and Grahn (Norris, W. P., et al., Proc. of an IAEA Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, IL, 1975, IAEA, Vienna, 1976, Vol. 1, p. 147). These comparisons will be strengthened and extended as additional data become available from the newly initiated studies of dogs irradiated at exposures as low as 0.4 R/day.

RADIATION TOXICITY IN DOGS STAFF

REGULAR STAFF

Doyle, Donald E. (Scientific Assistant)
Fritz, Thomas E. (Veterinary Pathologist)
*Kaspar, Lillian A. (Scientific Assistant)
Kretz, Norbert D. (Scientific Assistant)
*Lombard, Louise S. (Veterinary Pathologist)
†Norris, William P. (Biochemist)
*Polk, Patrick H. (Scientific Assistant)
Seed, Thomas M. (Assistant Biologist)
*Tolle, David V. (Scientific Associate)

TEMPORARY STAFF DURING 1976

Gutzeit, Diane L. (Postdoctoral Appointee)

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*Now in Pathology and Risk Assessment Group.

†Now in Fossil Fuel Toxicology Group.

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RESPONSE OF YOUNG-ADULT BEAGLES TO PROTRACTED EXPOSURE TO ^{60}Co GAMMA RAYS

Thomas E. Fritz, William P. Norris, Thomas M. Seed, Calvin M. Poole,† David V. Tolle,‡ Louise S. Lombard,‡ Susan M. Cullen,‡ Donald E. Doyle, Lillian A. Kaspar,‡ William G. Keenan,† Norbert D. Kretz, and Patrick H. Polk‡*

Two separate studies utilizing protracted whole-body gamma irradiation from ^{60}Co are being conducted. The first consists of irradiating groups of young-adult beagles at one of several exposure rates continuously until death, while the other consists of similarly irradiating beagles but removing them from the radiation field at predetermined levels of total exposure.

The objective of the first study is to characterize the responses of the young-adult beagle dog subjected for duration of life to continuous, whole-body ^{60}Co gamma radiation delivered at exposure rates ranging as low as those allowing for survival approaching a normal life-span. The objective of the second study is to define the relative importance of total dose and dose rate to the delayed effects of whole-body gamma irradition (Norris, W. P., et al., ANL-76-99, 1975, p. 13). In addition to determining the decrease of life-span associated with each exposure protocol, the progression of radiation-induced damage will be followed in each experiment in detail, by clinical, hematological, and pathological evaluations (Norris, W. P., et al., ANL-76-99, 1975, p. 10).

During the past year, the last survivor irradiated continuously until death at 5 R/day died of nephritis and multiple tumors after 2,878 days of irradiation. The study of the four groups of dogs irradiated at 35, 17, 10, and 5 R/day is, therefore, completed except for examination of tissue specimens and final analyses. Norris et al. (ibid.) summarize the dose-rate dependent survival and causes of death, up to the death of the last dog.

Our present work with continuous irradiation will largely utilize the newly completed facility that will house 200 dogs exposed to either 2.5, 1.0, or 0.4 R/day. Following pre-exposure clinical and hematological evaluation, litters of dogs, randomized among groups, enter the radiation field for duration of life as they reach 400 days of age. About 45 dogs have already been entered into the experiment. As in previous studies, all dogs will be evaluated regularly and at necropsy (ibid.).

By using the technique of rotating cages, and systematically migrating dogs daily, we have gone further in establishing uniform exposure conditions than any chronic gamma study made elsewhere. There remain some considerations

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of dose distribution within dogs, as the consequences of extremes of individual behavior patterns. We plan to address these questions on a limited scale by using implanted thermoluminescent dosimeters, and by observation of dog behavior using time-lapse TV recording, in collaboration with the Dosimetry Section of the Neutron and Gamma-Ray Toxicity Group.

Previous experience with young-adult beagles has already shown that, except for the anticipated reduction in the sperm counts of the males, little or no clinically discernible change will be produced by continuous ^{60}Co gamma irradiation delivered at 0.4-2.5 R/day during the next 2 years.

We anticipate, however, that the hematological evaluations during the next year or two will define the exposure rates at which all bone marrow elements, and particularly the platelet-producing segment, become essentially nonresponsive to irradiation.

A total of 342 dogs have been given protracted whole body exposures terminated at fixed values of total exposure between 600-4000 R, as described previously (Norris, W. P., et al., ANL-76-99, 1975, p. 13). The rate at which these exposures are being completed was increased during the past year because dogs originally scheduled for continuous irradiation in the new irradiation facility were diverted to these groups. Of all the groups scheduled to be irradiated, only six dogs remain to be entered into the gamma field to receive terminated exposures; three each at 17 and 35 R/day for total exposures of 600 R.

Ninety-six of the dogs given terminated irradiation have died, either of septicemia or anemia, during the irradiation period or within 100 days after exposure was terminated. A total of 28 have died at times longer than 100 days after exposure was terminated. These include 11 of 20 irradiated for a total of 4000 R at 10 R/day and 8 of 20 irradiated for a total of 1400 R at 35 R/day. Only three dogs have died at these later times in any of the other groups. There were six malignancies, including four leukemias (three myelogenous and one lymphosarcoma) among the 11 dogs dead at 4000 R (10 R/day) and two malignancies including one leukemia (lymphosarcoma) among the 8 dead at 1400 R (35 R/day). The mean time to death in the 4000 R group was 979 days for dogs dying with malignancies, and 1424 days for all dead dogs. In the 1400 R group, the mean time to death for the two malignancies was 2395 days, but for all causes it was 1894 days.

The malignancies now include a total of three cases of myelogenous leukemia, two lymphosarcomas, and one monocytic leukemia. The three myelogenous leukemias all occurred within 1-1/2 years after irradiation, as did one of the lymphoid tumors. The monocytic leukemia, closely related to the myelogenous, occurred at 1241 days while the other lymphoid tumor occurred 2341 days after termination of irradiation.

Other than the malignancies, which seem excessive for the ages of the dogs in these groups, there is as yet no other pattern emerging as regards significant pathological end points or lesions specifically related to the irradiation.

Because of the schedule on which dogs were staged into the gamma field, survival time, to date, varies considerably. Some of these dogs have been observed for more than 3000 days after exposure. Subsequent to the early wave

of myelogenous leukemias described above, there were no further deaths that appear to be related to the irradiation. Apparently, the incidence of leukemia and related diseases decreases rapidly in dogs that survive beyond this time.

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EFFECT OF RADIATION DOSE RATE ON THE DEVELOPMENT OF THE REPRODUCTIVE AND ENDOCRINE SYSTEMS OF FETAL AND YOUNG GROWING BEAGLES

Thomas E. Fritz, William P. Norris, Thomas M. Seed, Diane L. Gutzeit, Calvin M. Poole,† David V. Tolle,‡ William G. Keenan,† Patrick H. Polk,‡ and Margaret M. Sanderson***

The developing mammalian fetus is thought to be highly sensitive to the effects of ionizing irradiation, and it is well known that mammals develop temporary and permanent dose-rate dependent sterility. However, the relationships between effects and dose and dose rate remain to be defined, especially those following low dose exposures given during fetal life.

Previous work in this laboratory has shown that female beagles, irradiated continuously at rates from 5-17 R/day from conception to parturition, produce apparently normal litters (Norris, W. P., and C. M. Poole, ANL-8070, 1973, p. 44). Similarly, bred females exposed at rates of 17 and 35 R/day for the last two thirds and middle one third of gestation, respectively, also produce normal litters. (The dogs in these litters, however, are significantly smaller than the normal beagle in adult life.) Much more striking, however, is the effect of fetal irradiation on the reproductive systems. At the lowest daily exposure previously available for study (5 R/day), all females irradiated *in utero* had hypoplastic anovular ovaries (about one third normal weight) and were sterile. Male littermates, similarly irradiated at 5 R/day, had normal sperm and were regarded as fertile. At higher exposure rates (10 R/day and above) both sexes are sterile (males are aspermic) (Norris, W. P., et al., ANL-76-99, 1975, p. 21).

We regard it important to determine, therefore, the exposure rate that, delivered to the developing fetus throughout gestation, allows for apparently normal gonad development and effective reproduction in adulthood. Once the exposure that allows normal ovarian development to proceed has been determined, newborn puppies so irradiated *in utero* will be maintained in the gamma field at the same exposure until they reach reproductive age. Ultimately, this

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approach will produce an estimate of the daily rate of continuous irradiation (conception to reproductive age) that allows for survival of the species.

Clinical observation of adult female beagles irradiated at 5 R/day and above had revealed atypical and irregular estrous periods. Because they copulate in spite of anovular ovaries (biopsy observation), we believe there are important aberrations in the control mechanisms regulating ovarian function, with obvious implications regarding related secondary changes in the pituitary gland. Preliminary assays of ovarian and pituitary hormones have supported these impressions (Fritz, T. E., et al., ANL-75-30, 1974, p. 14). Expanded studies are needed to explain the dysgenesis of the ovary and the paradoxical aberrant sexual behavior of the anovular females, and to investigate basic mechanisms of ovarian function and endocrine interactions. A total of 40 bred bitches has now been irradiated at rates between 0.4 and 34 R/day. One hundred and fifty-two pups were delivered, of which 67 still survive for lifetime evaluation. They appear normal except, as noted above, both sexes are smaller than unirradiated dogs.

Because single, brief, sublethal doses of irradiation at exposure rates significantly higher than those used in our studies impair normal tooth development and eruption, we evaluated the teeth of the 67 adult dogs that were irradiated *in utero*. The existing teeth were apparently normal, but there was a significant decrease in the number of permanent teeth, especially the premolars, as compared to unirradiated control dogs. There appears to be a dose-rate-dependent response with a greater effect at 17 R/day than at lower exposure rates.

Five bred bitches were irradiated during the past year at exposure rates of either 5.0, 2.5, 1.0, or 0.4 R/day. All produced apparently normal litters, and the gonads from each pup were removed at selected intervals between 4 and 180 days of age for light and electron microscopy to evaluate their differentiation, growth, and maturation. Preliminary evaluation by light microscopy indicates that ovaries from puppies irradiated *in utero* at 2.5 R/day may be functional. As in previous examinations, ovaries from puppies similarly irradiated at 5 R/day were hypoplastic and anovular.

Electron microscopic examination of ovaries from puppies exposed at 5 R/day shows severe progressive damage with absence of oogonial development and with associated changes in the interstitial tissue and microvasculature. Exposure rates of 2.5 R/day and less do not appear to affect normal maturation of oogonia and pregranulosal cells to primordial follicles. Although there were areas of damage to the microvasculature, it was not as severe as in ovaries from dogs exposed to 5 R/day.

Also during the past year, we began a collaborative study (with Dr. G. Niswender, Colorado State University) to assay hormonal levels in estrous females that were irradiated *in utero*. We have previously shown that most come into estrus, but none have conceived. Daily serum samples collected during the estrous period (proestrus, estrus, and metestrus) are being assayed for levels of luteinizing hormone (LH), follicle-stimulating hormone, estradiol, progesterone, cortisol, and thyroxin. Incomplete assays on 14 females representing dogs irradiated *in utero*, unirradiated breeders, and unirradiated nonbreeder controls have shown severe imbalances in LH, estradiol, and progesterone levels

in the bitches irradiated *in utero*. The data indicate that there is little or no feedback regulation from the ovary to the pituitary, and that ovulation is not occurring.

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CELLULAR MECHANISMS OF RESPONSES TO CONTINUOUS EXPOSURE TO ^{60}Co GAMMA IRRADIATION AND OTHER MYELOSUPPRESSIVE AND LEUKEMOGENIC AGENTS

Thomas M. Seed, Thomas E. Fritz, David V. Tolle, Calvin M. Poole,† Patricia C. Brennan,* Lillian A. Kaspar,* and Susan M. Cullen**

Myeloproliferative disorders (MPD), occurring mostly as myelogenous leukemia, have been previously shown in this laboratory to be a prominent pathological consequence of protracted ^{60}Co irradiation of the beagle (Fritz, T. E., et al., ANL-8070, 1973, p. 37; Fritz, T. E., et al., Bibl. Haemat. 39, 170, 1973). The objectives of this study are: (1) to evaluate the causal relationship between protracted exposure to myelosuppressive agents (i.e., protracted ionizing irradiation and, in the near future, benzene) and the genesis of life-shortening leukemias in the beagle; and (2) to identify early prognostic indicators of impending disease.

The design of the experiment entails a comprehensive, multiphasic examination of morphological, hematological, immunological, and possible virological characteristics altered by the continuous insult of low daily doses of myelosuppressive agents that influence the dynamic balance of the hematopoietic system and result in the onset of leukemias. Biological features are being analyzed in a sequential fashion into the period of patent disease. In this fashion, the course of events leading up to and eventually culminating in a terminal myeloproliferative disorder will be described longitudinally in individual experimental animals.

A morphological assessment of the architectural changes within sequentially sampled bone marrow tissues is being made by standard light and electron microscopic techniques. Quantitation of hematopoietic stem cell numbers and the titers of regulatory humoral factors are being assayed by *in vitro* soft agar culture techniques (Marsh, J. C., et al., J. Lab. Clin. Med. 79, 1041, 1972). The immunological characteristics being measured include quantitation of thymus-derived and bone marrow-derived lymphocytes (T- and B-cells, respectively), and their responses to specific mitogens. An exposure rate of 10 R/day allows the hematopoietic system of half of the dogs to accomodate to this stress, and the dogs to survive for extended periods before their final death from leukemia. Leukemia usually develops after the dog has received an accumulated dose of approximately 2000 R (i.e., after approximately 200 days

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in the field). The half that do not accomodate develop aplasia of the bone marrow and die of anemia. Those that accomodate and eventually develop leukemia have thrombocytopenia and anemia, but only moderate leukopenia. Eventually, this hypoactive condition of the marrow becomes hyperplastic and, in turn, malignant.

During the past year, 12 dogs designated for intensive longitudinal studies of the development of leukemia were put into the gamma field at 10 R/day of continuous exposure. The first group of six has been irradiated 360 days for an an approximate total dose of 3600 R, while the second group has accumulated about 2000 R after 200 days of continuous exposure. All irradiated animals exhibited the characteristic radiotoxic response. The nadir of their cellular response occurred at about 200 days in the gamma field. As expected from previous studies, at least half of the dogs showed partial hematopoietic recovery. Four out of the 12 irradiated dogs that did not accomodate hematologically have died of marrow aplasia. Absolute granulocytopenia, reflecting severe depression of bone marrow function, and a prognostic indicator of developing aplastic anemia, is a common terminal finding. To date, the blood chemistry assays of serially collected sera from these dogs have not shown significant trends, except for a drop in serum iron reflecting developing anemia.

In related but separate experiments, we have evaluated for 500 days after irradiation the cell-mediated immune function of four dogs exposed to 1400 R at a dose rate of 10 R/day. The dogs showed elevated responses to the T-cell mitogens concanavalin A and phytohemagglutinin (PHA) while in the gamma field. These elevated responses were still evident 60 days after termination of irradiation, but at 100 days and at later times, the response was significantly depressed from pre-exposure levels, although total lymphocyte numbers and T- and B-cell numbers were normal. These results thus demonstrate a functional defect in T-lymphocytes long after the radiation sequence is completed. Using the same technique, we are evaluating the immunological response of the 12 dogs irradiated at 10 R/day in the longitudinal studies of the development of aplastic anemia or myelogenous leukemia. The response of these dogs to the mitogens had equilibrated at a level substantially below pre-exposure levels. In one dog that died, the response dropped dramatically shortly before death.

Electron microscopy of serially biopsied bone marrow of the dogs being irradiated at 10 R/day for study of leukemia development has revealed significant cellular and structural changes in the early phases that long precede the onset of leukemia. These changes may be important prognostic features. By scanning electron microscopy, a fibrous network becomes prominent in the marrow following 100-200 days of irradiation. Viewed by transmission electron microscopy, the marrow has increased proportions of fixed reticular cells with Golgi-rich cytoplasm and free hematopoietic cells with prominent monocytoid features. Budding C-type viral particles have not been observed in any of the autopsied tissues to date.

The hypoplastic condition of the marrow during these early phases is reflected quantitatively by a depletion of granulocyte reserves and also by an impaired leukocyte release mechanism, as measured by the endotoxic stress assay (Marsh, J. C., and S. Perry, Blood 23, 581, 1964). The release rate of leukocytes from the marrow into the peripheral circulation drops by more than

60% from average pre-exposure values following total radiation doses as low as 1000 R. Recovery times (time required for peripheral leukocyte counts to come back to baseline following standardized endotoxin stress) lengthen with increasing exposure. Dogs exhibiting the weakest response developed marrow aplasia and died.

A dog that developed monocytic leukemia, resembling Schilling's type in man, following a terminated exposure of 2000 R at 17 R/day was studied in some detail. Juvenile monocytes, characterized ultrastructurally, were the predominant cell type in the bone marrow. Leukemic monocytes maintained in tissue culture retained their basic morphology, except for the presence of voluminous cytoplasmic vacuoles. In both the leukemic cells taken from the dog and those grown in culture, there was no morphological evidence of C-type virus within these leukemic monocytes. The soft agar culture technique was employed to assess, *in vitro*, cloning and growth characteristics of cells from this leukemic marrow. As in the acute leukemias in man, we observed few colonies developing after 8-10 days in culture. However, there were great numbers of viable cell clusters. Suspending cells for short periods with low concentrations of the mitogen PHA prior to plating greatly stimulated colony formation. This suggests, as others have proposed, that leukemic cells with appropriate stimuli can exhibit normal growth characteristics, despite prominent maturation defects.

We plan in the near future to initiate a pilot study in which benzene will be utilized (instead of ionizing irradiation) as the myelosuppressive-leukemogenic agent. Benzene is a ubiquitous chemical with known leukemogenic activity. It is associated with a wide variety of industries (including fossil fuels) and has caused serious medical problems to exposed populations (Federal Register 42, 22516, May 3, 1977).

It is likely that leukemia(s) in both dog and man, in particular those myelogenous forms induced by irradiation and chemicals (e.g., benzene), share common early developmental sequences, despite etiological differences. Therefore, a comparative longitudinal study of the beagle hematopoietic system, stressed continuously with either radiation or benzene, should provide novel insights into early events that eventually culminate in leukemia, and should aid in our prognostication of impending leukemia in dogs and, perhaps, in man. It is also possible that application of antileukemia drugs may reverse or retard the developmental sequence that ends in leukemia.

IMPROVED TEMPERATURE CONTROL OF THE TECHNICON TISSUE PROCESSOR PARAFFIN BATH*

William J. Eisler and Patrick H. Polk

Modifications of the AutoTechnicon Tissue Processor Paraffin Bath are described. The modifications are a combination of electrical and mechanical changes that will ensure long-term reliability of the unit and dependable temperature maintenance of the paraffin. Extensive testing has verified that modified units are able to stabilize temperature within a range of 0.25C over many months of continuous operation.

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*Abstract of a paper published in Stain Technol. 51, 301 (1976).

PATHOLOGY AND FAMILIAL INCIDENCE OF ORCHITIS AND ITS RELATION TO THYROIDITIS IN A CLOSED BEAGLE COLONY*

T. E. Fritz, L. S. Lombard, S. A. Tyler, and W. P. Norris

Lymphocytic orchitis occurs spontaneously in a closed colony of beagle dogs with an incidence of 32% among 69 untreated males over 1 year of age. This disease is genetically influenced as determined by an analysis of the ancestral composition of each animal. Orchitis is related in its occurrence to lymphocytic thyroiditis which has been previously described in this colony. The incidence of both diseases increases with increasing degrees of relatedness to three sibling progenitors of a partially inbred line which comprises a significant portion of the colony. Among the dogs that derived all of their genes from the three progenitors, 85% had lymphocytic thyroiditis and 65% had lymphocytic orchitis. The pathologic changes in the testes result in a significant reduction in testis size and weight as well as sterility or reduced fertility.

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*Abstract of a paper published in Exp. Mol. Pathol. 24, 142 (1976).

AN INTERSPECIES COMPARISON OF RESPONSES OF MICE AND DOGS TO CONTINUOUS ^{60}Co γ IRRADIATION*

W. P. Norris, S. A. Tyler, and G. A. Sacher

Young-adult purebred beagle dogs were exposed continuously, 22 h/d, to ^{60}Co γ rays until they died. The daily dose rates ranged from 3.5 to 210 rad. At 3.5 rad/d the mean survival time was in excess of 1860 days (the study was still in progress in Nov. 1975), while at 210 rad/d mean survival time was 13 days. The data are compared with those from previously published information from similarly irradiated mice. The comparison is made in terms of radiation-specific death rate, defined as $1/MAS_i - 1/MAS_c$, where MAS_i and MAS_c are the mean after-survival times of the irradiated and control populations, respectively. In both species, when log radiation-specific death rate is plotted against log dose rate, the response has a slope = 2, i.e. the death rate increases with the square of dose rate. This occurs over the entire dose rate range where damage to haematopoietic injury is no longer a primary cause of death. In the mouse, at daily dose rates below 20 rad/d, haematopoietic injury is no longer a primary cause of death and the response curve shifts to slope = 1, where injury is dependent only on total accumulated dose and is independent of dose rate. The data available so far suggest that a similar inflection may occur with the dog at dose rates below 3.5 rad/d. An experiment is being initiated to determine whether this will be the case. Statistical considerations, essential to the design of the study, are presented.

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*Abstract of a paper published in Biological and Environmental Effects of Low-Level Radiation, Vol. 1. IAEA International Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, IL, Nov. 3-7, 1975. International Atomic Energy Agency, Vienna, pp. 147-155, 1976.

RADIATION-INDUCED ERYTHROLEUKEMIA IN THE BEAGLE DOG*

David V. Tolle, Thomas E. Fritz, and William P. Norris

Eleven cases of myeloproliferative disease occurred among 24 beagle dogs placed in a ^{60}Co γ-ray field at about 13 months of age and irradiated at an exposure rate of 5 R/22-hour day for duration of life. Of these eleven cases, five, described in this paper, were diagnosed as erythroleukemia. The bone marrow showed marked erythroblastic hyperplasia with maturation arrest of the erythroid elements, and increased numbers of myeloblasts and promyelocytes. The terminal peripheral blood was characterized by marked anemia and

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*Summary of a paper to be published in Amer. J. Pathol.

thrombocytopenia, with circulating erythrocytic precursors and abnormal erythrocyte morphology. Splenomegaly and hepatomegaly occurred in four of the five animals. In the spleens and livers of all five there was extensive leukemic infiltration and proliferation. The extent of leukemic involvement in other tissues and organs varied in individual dogs.

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4. FOSSIL FUEL TOXICOLOGY

GROUP LEADER'S INTRODUCTION

William P. Norris, Group Leader

During 1976 plans were initiated for a program for the investigation of the toxicology of coal-derived effluents. W. P. Norris was appointed to coordinate the development of the program, under the direction of T. E. O'Connor and S. S. Danyluk.

As currently planned, this program will utilize a battery of cellular and mammalian test systems and end points to evaluate the toxicological effects of acute, sub-acute, and long-term, low-level exposure to gaseous and particulate effluents from combustion of coal with special emphasis on fluidized bed combustion (FBC). The program will bring together investigators from the Divisions of Biological and Medical Research, Chemistry, and Chemical Engineering, and the Environmental Control Technology Program at ANL into a fully integrated multidisciplinary effort. Among the principal elements proposed for the program are:

1) Construction of an "in-house" FBC facility and toxicant delivery system dedicated expressly to studies of the biological, environmental, and health effects of its effluents.

2) Physical and chemical characterization of gaseous and particulate effluents from FBC.

3) Assays of effluent toxicity by a battery of cellular systems.

4) Toxicological studies in selected animal model systems.

5) Evaluation of molecular toxicological properties of organic compounds entrained in particulate effluents.

A key feature of the overall plan is to develop a unique system for delivery of aged effluents from FBC that will provide the stable, well-defined source term that is essential for determination of reliable toxicological data. In the initial phase, the principal focus will be on *in vitro* assays for cellular damage, and on selected preliminary studies of mammalian responses to exposures to the effluent streams from coal combustion facilities. The latter include the FBC source at ANL, as well as a conventional coal combustion unit expected to become operational soon at ANL. These studies are designed to provide an early estimate of the toxicity of the effluents from coal combustion, and are the starting point for planning of subsequent experiments and improvement of test systems.

As the program evolves and the FBC in the Division becomes operational, the core of the program will center around the Divisional facility, with supporting studies using effluents from other sources continuing, as needed, to define correlations generic to coal combustion. Correspondingly, it is expected that the major focus of the program will shift progressively to investigation of toxicological effects in selected animal systems, with particular emphasis on dose-response effects for long-term, low-dose exposure conditions.

FOSSIL FUEL TOXICOLOGY STAFF

REGULAR STAFF

Norris, William P. (Biochemist)

5. CARCINOGENESIS

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ANL 60400
ANL 60401
ANL 60402
NASA-T-3568E
EPA-IAG-D5-E681

GROUP LEADER'S INTRODUCTION

R. J. Michael Fry, Group Leader

The Carcinogenesis Program, instituted in January 1976, is composed of two groups, the Viral, Radiation, and Environmental Oncology Group (Section 6 of this report) and the Carcinogenesis Group. The research of the Carcinogenesis Group, reported here, is broad in scope, with the main emphasis on mechanisms of tumor development. Recent studies of liver, thyroid, and Harderian gland carcinogenesis have also concentrated on the problem of cocarcinogenesis. The identification and assessment of cocarcinogens and an understanding of the underlying mechanisms of their action are important and difficult problems that involve the interaction of radiation, viruses, and chemical agents.

Studies of the mechanism of liver tumorigenesis resolve into two basic approaches. The first involves the study of the tumorigenic process itself, i.e., the response of the liver to tumorigenic stimuli and to agents that modulate the effects of these stimuli. The second involves the study in normal liver of the putative targets for tumorigenic agents; i.e., those intracellular molecular processes which, through derangement by interaction with a carcinogen, might lead to tumorigenesis. The observations that phenobarbital enhances hepatic tumorigenesis, irrespective of the nature of the initiator, and that drugs that have features in common with phenobarbital, either in structure or action, do not enhance, led to the development of a test system for the study of chemical agents and other factors that enhance, promote, or prevent chemically induced liver tumors. In the past year, the common food additive butylated hydroxytoluene (BHT) has been shown to be an enhancer of liver tumorigenesis, but a considerably less potent enhancer than either phenobarbital or DDT. Studies to investigate the persistence of initiated or transformed cells have shown that, although some of the events initiated by chemical carcinogens are lost with time, there is a prolonged persistence of potential tumor cells. The characteristics that have been found in liver tumorigenesis suggest that a two- or multistage process is involved.

A collaborative study with the Neutron and Gamma-Ray Toxicity Group is being carried out on the factors that promote tumor expression after irradiation or exposure to chemical carcinogens. The Harderian gland has proved suitable as the test system. We have shown that increased levels of anterior pituitary hormones increase the incidence and the malignancy of Harderian gland tumors induced by radiation.

The fundamental question of the role of catalase and peroxidation in carcinogenesis has been studied in mice with a genetic defect in catalase, and treated with aminotriazole. This study has led to work on the carcinogenic and cocarcinogenic effects of aminotriazole on the thyroid and the liver. Techniques have been developed for the study of peroxidases, and marked tissue differences in active peroxidase have been found. Adjunct studies are concerned with isozymes and particularly with differences in enzyme expression between "induced" tumors and "naturally occurring" tumors.

A new area of enzyme studies concerned with cytochrome P-450 has been introduced this year. The experiments on this enzyme fall into two categories. In the first, the characterization of structure and properties of cytochrome P-450, Dr. Haugen was invited to join the team led by Dr. M. J. Coon (University of Michigan) to determine the primary structure of liver cytochrome P-450. This collaborative study will involve 3 months of work in 1977 at the University of Hawaii with Dr. K. T. Yasunobu. The second category includes an investigation of the age- and strain-dependence of induction of aryl hydrocarbon hydroxylase in the mouse lung. Parallel studies are being carried out on cell proliferation and the induction of lung tumors by benzo(a)pyrene.

Skin tumors induced by UV radiation provide a model for studying a number of factors involved in carcinogenesis. Certain of the furocoumarins react with nucleic acids when exposed to near-UV light; they also sensitize the skin to the UV light so that squamous cell carcinomas subsequently appear. The objective of this project is to determine whether the initial step in carcinogenesis in this system is the photoaddition of furocoumarins. To do this, the molecular interaction of 8-methoxypsoralen with DNA is being studied in mouse skin exposed to UV light of various spectra. The current results suggest that the psoralen bifunctional adducts are not, at least by themselves, the molecular lesion responsible for the skin tumors, but the results underline the importance of synergistic effects of lesions induced by different wavelengths. Mutation studies, which were introduced last year, have also revealed interactions of lesions induced by different spectra.

In related studies with skin, we have found a marked difference in susceptibility to carcinoma induction between two strains of hairless mice. Much of this difference is related to the expression or promotion of tumors rather than in the initiation events. In contrast to accepted views, there is a lack of additivity for tumor induction and a dependence on fractionation pattern. In the case of the photosensitized skin, there was no clear-cut correlation between erythema induction and tumor induction after UV irradiation. This finding suggests the use of other criteria, such as DNA damage, for indication of subsequent tumor expression.

As a continuation of studies on the induction of DNA damage in mouse skin, experiments have been initiated with short-term cultures of intact skin *in vitro* to enhance radionuclide labeling of epithelial DNA and to facilitate the measurement of various repair characteristics.

We have also succeeded in transplanting human skin onto nude athymic mice, and are about to start studies of the acute effects of UV light.

CARCINOGENESIS STAFF

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Jacobson, Gunnard K. (Postdoctoral Appointee)
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*Terminated during 1976.

†Now in the Electron Microscope Center.

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**Transferred to Environmental Impact Studies Division in 1977.

††Now in the Pathology and Risk Assessment Group.

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MODULATION AND MECHANISMS OF TUMOR DEVELOPMENT IN LIVER, THYROID, AND HARDERIAN GLAND

Robert N. Feinstein, R. J. Michael Fry, David A. Haugen,* V. Ann Ludeman,† Carl Peraino, Aldona M. Prapuolenis, Anthony R. Sallese,† Surendra T. Shenoy, and Everett F. Staffeldt†*

MECHANISMS OF PHENOBARBITAL ENHANCEMENT OF TUMORIGENESIS IN THE LIVER

In previous experiments (Peraino, C., et al., Cancer Res. 35, 2884, 1975), we observed that the short-term feeding of 2-acetylaminofluorene (AAF) at a low dietary concentration resulted in the late appearance of well differentiated hepatic tumors at low incidence levels. When the AAF treatment was followed by the feeding of a phenobarbital-supplemented diet, the appearance of these tumors was accelerated and their overall incidence levels were increased. The present study examined the characteristics of this interaction between the effects of AAF and phenobarbital by measuring tumor production after changing either the duration of the interval between the AAF and phenobarbital treatments, or the duration of the post-AAF exposure to phenobarbital.

A sufficiently long delay in the onset of phenobarbital feeding, following the termination of AAF treatment, reduced the level of enhancement of AAF-induced liver tumors. Increasing the duration of the phenobarbital treatment (begun immediately after the AAF treatment) shortened the time to tumor development and increased overall tumor incidence levels. These enhancement effects occurred and were sustained after the cessation of the phenobarbital treatments. The results of this study suggest that:

1) Some of the tumorigenic changes induced by brief AAF treatment are subsequently lost, either through intracellular repair or through the loss of initiated cells. Alternatively, though less likely, the changes may persist but the enhancement of their expression may be impaired as a result of age-dependent changes in the responsiveness of the rats to phenobarbital.

2) The effects of phenobarbital on AAF-modified cells are irreversible. Therefore, the increases in enhancement produced by lengthening the phenobarbital treatments may reflect phenobarbital-mediated responses in cells with progressively lower intrinsic capacities for tumorigenic expression, i.e., cells bearing successively fewer AAF-induced modifications.

It remains unknown how phenobarbital enhances the incidence of chemically induced liver tumors. As part of the studies to investigate the mechanism of

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*Biochemistry Group.

†Pathology and Risk Assessment Group.

the phenobarbital effect, rats were fed diets containing 0.002, 0.01, 0.05, or 0.25% phenobarbital for 11 days. At daily intervals rats were killed and livers were analyzed for thymidine uptake, thymidine kinase, ornithine decarboxylase, ornithine aminotransferase, serine dehydratase, cytochrome P-450, cytochrome b_5, cytochrome oxidase, and microsomal protein. It was found that the microsomal enzymes showed a dose-dependent and sustained positive response to dietary phenobarbital.

As another part of the study of the mechanism by which phenobarbital enhances hepatic tumorigenesis induced by AAF, we have measured the levels of template-active RNA that contains poly A, total RNA, and protein in the liver during the stimulation of liver growth by phenobarbital, in order to examine the molecular changes associated with the stimulation. Poly(A)-RNA was isolated by phenol extraction and purified by affinity chromatography on oligo d(T) cellulose. The template activity was assayed in a cell-free protein synthetic system derived from wheat germ. The cell-free system was sufficiently sensitive to detect differences of less than a microgram of mRNA when optimal concentrations of Mg^{++} and K^{+} were used.

Rats injected with phenobarbital exhibited a 26% increase in liver weight over untreated controls without undergoing a significant change in body weight. There were no significant differences in the hepatic content of poly(A)-RNA between the control and the treated groups. The DNA level was also unchanged, but total RNA and protein levels increased significantly in the treated group. These results suggest that phenobarbital-induced hepatomegaly (1) is at least in part characterized by hypertrophy (cell enlargement), (2) is mediated by a generalized increase in the synthesis of RNA other than functional message, and (3) involves an increased translation of mRNA *in vivo*.

STUDIES OF THE EFFECTS OF AMINOTRIAZOLE

The herbicide 3-amino-1,2,4-triazole (AT) is an antithyroid agent (Alexander, N. M., J. Biol. Chem. 234, 148, 1955) which results in thyroid tumors (Innes, R. J. M., et al., J. Nat. Cancer Inst. 42, 1101, 1969). This compound is also an inhibitor of catalase. It has therefore been used in the current experiments to reduce the tissue catalase in acatalasemic mice in order to study the role of hydrogen peroxide in both the naturally occurring and radiation-induced tumors. Although the effects of aminotriazole are complex, we have obtained some interesting findings about the effects of the compound on tumorigenesis in both acatalasemic mice and mice with normal catalase levels. AT is in some cases a carcinogen, in others an anticarcinogenic agent. The C3H strain of mice shows a high incidence of naturally occurring liver tumors in males, but a low incidence in females. If C3H mice are maintained on a diet containing AT, the incidence of liver tumors increases considerably in the female; in both sexes on an AT diet, liver tumors appear earlier and in greater incidence in acatalasemic mice than in their normal catalase counterparts. On the other hand, a period of AT treatment delays the appearance of mammary tumors in either normal or acatalasemic C3H females carrying the murine mammary tumor virus. In C57BL/6 mice, which are very susceptible to the induction of thymic lymphomas by irradiation, treatment with AT reduces the incidence of postirradiation lymphoma but appears to increase the incidence of other tumors.

One aim of these experiments was to investigate aminotriazole as a co-carcinogen, especially in the case of thyroid tumors. It is not yet clear whether or not AT enhances radiation-induced thyroid tumors, but an interesting AT-radiation relationship has been observed: a preliminary irradiation with 80 R of fission neutrons considerably speeds the induction of liver tumors by AT.

Further work will be necessary to unravel the mechansims involved in the tumorigenic effects of 3-amino-1,2,4-triazole, but clearly the compound provides a way to investigate some of the cellular metabolic events that may be involved in tumor initiation and expression.

HORMONAL MODULATION OF HARDERIAN GLAND TUMORS

Pituitary hormones from pituitary isografts increase the incidence and malignancy of radiation-induced Harderian gland tumors (Fry, R. J. M., et al., Proc. of an IAEA Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, IL, 1975, IAEA, Vienna, 1976, Vol. 1, pp. 213-226). The present experiment was designed to determine whether the pituitary hormone, presumed to be prolactin, also enhances chemically induced tumors. A subsidiary question was asked, namely, whether this tumor system shows age-dependent susceptibility. Urethane was the chemical carcinogen used, and assay was at 1 year of age. When six injections of 0.5 mg/g of this carcinogen were given to $B6CF_1$/Anl mice, starting when the animals were 7 days old, 48% of the mice had Harderian gland tumors within 1 year. Twenty-seven percent of the total number of glands had tumors.

When this carcinogen treatment was followed by pituitary isografts at 71 days of age, 73% of the mice and 57% of the glands had tumors by 1 year. When the urethane was given at 50 days of age, without pituitary isografts, only 9% of the mice and 5% of the glands had tumors. When pituitary isografts were made at 44 days of age and the urethane was given at 88 days, 76% of the mice and 57% of the glands had tumors.

It is clear that: (1) the Harderian gland of the $B6CF_1$ mouse is susceptible to tumor induction by chemical carcinogens; (2) the expression of this tumor is enhanced by the presence of pituitary isografts; and (3) the incidence of tumors is greater when the carcinogen was given from 7-24 days of age (48%) than from 50-67 days of age (9.3%). Because no age-dependent difference in incidence was found in the mice with pituitary isografts, it appears that age-dependent susceptibility may be due to differences in tumor expression rather than initiation. We will investigate whether this test system responds similarly to a polycyclic hydrocarbon carcinogen.

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SKIN AND LUNG CARCINOGENESIS

Evelyn M. Buess, R. J. Michael Fry, Donald D. Grube, David A. Haugen, Gunnard K. Jacobson, Walter E. Kisieleski,† Ronald D. Ley, Kathy A. Rettman, Beverly A. Sedita, and Ruth L. Willey‡*

Two objectives of the project on skin are to investigate the role of photoadducts, induced by combined treatment with certain psoralen-type furocoumarins and ultraviolet radiation (UV), in skin carcinogenesis and the reasons for the marked differences in susceptibility to skin cancer in two strains of hairless mice.

Exposures to topically applied 8-methoxypsoralen (8-MOP) and UV induce squamous cell carcinomas in the two strains of hairless mice that we use (SKH-hr-1 and HRS/J/Anl). The incidence and the time of appearance of tumors was strain dependent, particularly for the exposures to broader spectra, namely 300-400 and 320-400 nm light. We have previously established that the induction of psoralen bifunctional photoadducts was wavelength dependent, but strain independent. Therefore we examined the possibility that the strain differences were due to another form of photomediated damage or its repair. Graded exposures to UV resulted in the induction of the same number of endonuclease-sensitive sites in both strains. Furthermore, there was no significant repair of this damage in either strain within 24 hours. It was also shown that the endonuclease technique measured pyrimidine dimers induced by the UV.

In further experiments, a small number of fractionated UV exposures was given, followed by treatment with the potent tumor promoter phorbol ester. From these it is evident that a large part of the strain difference in skin tumor response is due to differences in events that determine tumor expression, rather than those responsible for tumor initiation. Examination and understanding of host factors that influence tumor expression are therefore important in the interpretation of cancer experiments and in making estimates of tumor risk.

In parallel studies of the mutagenic effects of psoralen and UV, we determined yeast cell survival, petite mutation, and gene conversion in response to combined treatments with the psoralen (8-MOP) and 365-nm light and 254-nm light. Cells were irradiated with 365-nm light in the presence of 8-MOP prior to exposure to 254-nm light in the absence of 8-MOP. A positive interaction, possibly synergistic, was found to exist between the two treatments with respect to cell survival and petite induction. Although the rates

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*Biochemistry Group.

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of cell killing and petite formation were the same for 254-nm exposure following 8-MOP plus 365-nm exposure as with 254-nm light alone, the shoulders of the survival curve and petite induction curve as seen with 254-nm light alone were eliminated. The increase in gene convertants per unit dose of 254-nm light is higher when 254-nm irradiation follows 365-nm light plus 8-MOP treatment; but on the basis of gene convertants versus survival, the rate of convertant increase in combined treatment is the same as in 254-nm irradiation. alone.

The data indicate that cell killing and petite mutation are enhanced by the interaction of DNA lesions produced by 8-MOP plus 365-nm light (mono- and bifunctional adducts) and 254-nm light (pyrimidine dimers), while recombinational events such as gene conversion are not.

The risk for cancer in a number of tissues varies with the age at exposure to carcinogens. The factors that determine the age dependency in different tissues are still poorly understood. Factors such as cell proliferation can influence the number of cells at risk, while hormonal levels can influence the expression of certain tumors. In the case of chemical carcinogens that undergo metabolic activation and deactivation, age-dependent susceptibility is influenced by the age-dependent changes in the levels of the enzymes involved.

The metabolism of benzo(a)pyrene (BaP) was examined *in vitro* in pulmonary tissue from mice of different ages and strains. In the lungs from mice not previously exposed to BaP it was found that the capability of metabolizing BaP increased during the 7-21 day postnatal period. On the other hand, the relative degree of induction of metabolic activity after exposure to either BaP or 3-methylcholanthrene was greatest in the 7-day-old mice.

We have also studied the morphological development of the mouse lung in the first 14 days of life, with particular emphasis on the pattern of proliferative activity in the various pulmonary structures. Details about both the proliferative activity and the metabolism of the carcinogens are essential for an understanding of the age dependency for lung cancer induction. The results show that cell proliferation, and thus growth, in the trachea and bronchi occurs early, reaching a maximum rate 2 days after birth, whereas the alveolar portion of the lung shows the greatest cell proliferation at about 8 days of age. Up to about 4 days of age, the lung volume increases with the general somatic growth but this increase appears to be accomplished by stretching of the alveolar portion rather than growth by cell proliferation. Between 4 and 8 days of age the increase in lung size involves active cell proliferation.

INCREASING THE FLEXIBILITY OF POLYACRYLAMIDE GEL-SLAB ISOELECTRIC FOCUSING*

Robert N. Feinstein

The flexibility and usefulness of polyacrylamide gel-slab isoelectric focusing is greatly increased by performing separations with glass slides embedded in the gel. After separations, each gel-coated slide can be treated separately; as many as eight individual reactions can be observed after a single electrofocusing or electrophoresis run. Other modifications described include the simultaneous electrofocusing of two different gel compositions and a simple technique for permanent recording which does not require photography.

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*Abstract of a paper published in Anal. Biochem. 72, 533 (1976).

NEW ALDEHYDE DEHYDROGENASE ISOZYMES IN CHEMICALLY INDUCED LIVER TUMORS IN THE RAT*

Robert N. Feinstein, R. J. Michael Fry, Erma C. Cameron, Carl Peraino, and Harold P. Morris

All liver tumors induced in rats by AAF or DAB, but only one of 29 tumors induced by ethionine, exhibited new isozymes of aldehyde dehydrogenase. These new isozymes, which differed from those of normal liver with regard to activity, pI, stability, cellular distribution, and coenzyme specificity, were not found in fetal or regenerating rat liver, nor in spontaneous liver tumors of the mouse or mastomys, nor in several other tumors appearing in rats bearing AAF-induced liver tumors, nor in any of several transplanted Morris hepatomas. The activity of the new isozymes did not correlate with histological criteria of differentiation, and the new forms were sometimes detectable in the liver of AAF-fed rats in areas where no tumor was macroscopically or histologically visible.

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*Summary of a paper published in Proc. Soc. Exp. Biol. Med. 152, 463 (1976).

RADIATION ONCOGENESIS *IN VIVO**

R. J. Michael Fry

This contribution is concerned with the basis for estimates of risk of radiation-induced tumors, and the factors that influence that risk. Data and generalizations that relate to man are needed, but (fortunately) data for radiation-induced tumors in man, especially by particle radiation, are limited. Therefore, I include what animal experimentation has revealed, or can help reveal, specifically studies on external particle radiation.

As the risk of iatrogenic tumors is still quite minor in comparison to the benefits of cure, all energies will be concentrated on improving cure rates. The estimates of risk in relation to LET will have an impact only if it is shown that the increase in cure rates with particle radiation is countered by an increased risk of tumorigenesis with relatively short latent periods. The most difficult problem may be evaluating the comparative risks and benefits of the different treatment modalities, especially for the treatment of children.

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*Abstract of a paper for Particle Radiation Therapy, Proceedings of an International Workshop, Oct. 1-3, 1975, Key Biscayne, FL.

RADIATION INJURY: SOME ASPECTS OF THE ONCOGENIC EFFECTS*

R. J. M. Fry and E. J. Ainsworth

The late effects of irradiation stem from cell killing, mutation, and malignant transformation. Cancer is the major somatic late effect of exposure to low dose levels of radiation, and estimates of risk of cancer in man after irradiation are based entirely on human experience. The data for dose-response relationships for the induction of tumors by external irradiation in man have been obtained from a single exposure or a small number of exposures delivered at high dose rates. In contrast, exposure to environmental irradiation is mainly protracted over a long period of time and is delivered at a low dose rate. As yet no allowance has been made for the effect of protraction of the exposure time in estimating the risk of cancer, although an adjustment has been made in the case of estimates of genetic risk. Incidence of tumors has been the only end point used for risk estimates, but latent period and degree of malignancy, which are probably both dose and dose-rate dependent, influence the nature of the risk from radiation. As the knowledge about the effects of low-level radiation has been accumulated and assimilated over the last seventy years, so has the concern for reasonable standards of safety. There are still problems in estimation of radiation risks, but at least many of the relevant

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*Abstract of a paper published in Fed. Proc. 36, 1703 (1977).

questions can now be framed. The problems of estimating risks for chemical carcinogens are clearly greater, but the experience gained from radiation studies should help in the design of the necessary experiments.

EFFECT OF PITUITARY ISOGRAFTS ON RADIATION CARCINOGENESIS IN MAMMARY AND HARDERIAN GLANDS OF MICE*

R. J. M. Fry, A. G. Garcia, Katherine H. Allen, A. Sallese, E. Staffeldt, T. N. Tahmisian, Rosemarie L. Devine, Louise S. Lombard, and E. J. Ainsworth

The effects of single-dose, fractionated and protracted exposures to ^{60}Co γ radiation and of single-dose and fractionated exposures to Janus reactor neutrons (fn) on mammary and Harderian gland tumorigenesis were studied in B6CF$_1$ mice. The incidence of mammary tumors is about 1% in the hybrid B6CF$_1$ mouse (C57BL/6J x BALB/cJ)F$_1$An1 and shows little increase with low-dose protracted ^{60}Co γ irradiation. After exposure to fission neutrons from the Janus reactor with single doses of 20, 80 and 240 rads, or with 80 and 240 rads given in 24 fractions, or to single and fractionated doses of ^{60}Co γ irradiation, the incidence of mammary tumors somewhat increased. However, the maximum incidence (in the 80-rad fn group) was only 3.8%. To determine the influence of certain hormone levels on mammary and Harderian gland tumorigenesis, two types of experiment were carried out. First, the authors determined by autoradiographic analysis the proliferative activity in the mammary and Harderian gland after isografting two pituitaries under the spleen capsule or after administration of diethylstilbestrol diphosphate or prolactin. In the mice with pituitary isografts, the labelling index (percent of cells in DNA synthesis) in the mammary gland epithelium was increased about tenfold. This increased synthetic activity was maintained at least up to 525 days after grafting. After seven days of administration of 1 µg/litre diethylstilbestrol diphosphate in the drinking water, the DNA synthetic activity was increased over tenfold. The presence of pituitary isografts did not have any effect on proliferative activity in the Harderian gland at 22 or 525 days after grafting but prolactin administered for three days caused about a twofold increase in DNA synthetic activity. In the second type of experiment, mice with pituitary isografts were exposed to a single dose of 20 or 80 rad Janus fn, or to 90 or 269 rad ^{60}Co γ radiation. The preliminary results showed that in comparison with unirradiated control mice, with or without grafts, two major effects of the combined treatment of pituitary isografts and neutron irradiation on tumorigenesis occurred: (1) There was a reduced latent period and increased incidence of mammary tumors. (2) For Harderian tumors there was: (a) a reduced latent period, (b) a marked increase in incidence (e.g. from 2.5% in controls to about 37% after a single dose of 80 rad fn), and (c) an increase in the fraction of Harderian gland tumors with

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*Abstract of a paper published in Biological and Environmental Effects of Low-Level Radiation, Vol. 1. IAEA International Symposium on the Biological Effects of Low Level Radiation Pertinent to Protection of Man and His Environment, Chicago, IL, Nov. 3-7, 1975. International Atomic Energy Agency, Vienna, pp. 213-226, 1976.

metastases. Since results were not yet available from mice irradiated prior to pituitary transplantation, it was not yet possible to distinguish whether the pituitary isograft had altered the susceptibility to the induction of tumors by radiation or acted as a promoter.

A SOLUBLE PEROXIDASE IN HEART AND SKELETAL MUSCLE OF RAT AND MOUSE*

Zoilo Gonzalez-Lama and Robert N. Feinstein

Several peroxidase isozymes have been demonstrated in the 0.25 *M* sucrose-soluble portion of mouse and rat heart and skeletal muscle. Isoelectric focusing on polyacrylamide gel was used to show that the peroxidatic activity is not due to hemoglobin, catalase, or cytochrome *c*. No true peroxidase activity was detected in either the soluble or insoluble fraction of mouse liver, kidney, brain, spleen, or lung.

*Summary of a paper published in Proc. Soc. Exp. Biol. Med. 154, 322 (1977).

THYROID PEROXIDASE OF THE PIG, DOG, RAT, AND MOUSE: SOLUBILIZATION AND IDENTIFICATION OF ISOZYMES BY ISOELECTRIC FOCUSING*

Zoilo Gonzalez-Lama and Robert N. Feinstein

Dog and pig thyroid peroxidase, which exist naturally in a largely insoluble form, can be solubilized by the use of 4 M urea, or of chlorhexidine, with small losses of total activity. In the mouse and the rat, the thyroid peroxidase occurs in a soluble form. The demonstration of these rodent thyroid peroxidases is therefore complicated by unavoidable contamination with peroxidatically acting hemoglobin and catalase; the demonstration of the presence of true peroxidase was achieved by isoelectric focusing on polyacrylamide gel slabs, which separates the various factors, and by the use of the catalase and peroxidase inhibitor 3-amino-1,2,4-triazole.

*Abstract of a paper to be published in Biochem. Med.

PHOTOSENSITIZING EFFECTS OF 8-METHOXYPSORALEN ON THE SKIN OF HAIRLESS MICE--I. FORMATION OF INTERSTRAND CROSS-LINKS IN EPIDERMAL DNA*

R. D. Ley, D. D. Grube, and R. J. M. Fry

The photomediated induction of interstrand cross-links by 8-methoxypsoralen has been measured in the epidermal DNA of hairless mice. Equivalent efficiencies for cross-link induction were determined for HRS/J/Anl and SKH:hairless-1 mice. A wavelength dependence on the relative efficiency of cross-link induction was observed; a broad spectrum light source, 300-400 nm, was approximately 5 times more effective in cross-link formation than a 365 nm light source. Repeated exposure to 8-methoxypsoralen followed by ultraviolet light, 5 times a week for 6 weeks, altered epidermal thickness and resulted in a decreased efficiency for DNA cross-link formation.

*Abstract of a paper published in Photochem. Photobiol. 25, 265 (1977).

PHOTOSENSITIZING EFFECTS OF 8-METHOXYPSORALEN ON THE SKIN OF HAIRLESS MICE--II. STRAIN AND SPECTRAL DIFFERENCES FOR TUMORIGENESIS*

D. D. Grube, R. D. Ley, and R. J. Michael Fry

The photosensitizing effects of 8-methoxypsoralen on the skin of two strains of hairless mice was studied using fractionated exposure to UV. Spectral dependent differences for tumorigenesis were studied by comparing the tumor responses to three different spectra; the exposure levels for each spectrum were adjusted in proportion to their relative efficiencies for tissue damage and cytokinetic responses. There were no strain differences in the spectral dependent induction of cutaneous damage or estimates of the photomediated interstrand cross-linking of epidermal DNA by 8-methoxypsoralen.

Squamous cell carcinomas were induced in the photosensitized skin of both strains of mice after fractionated exposures to emissions at principally 365 nm. Exposures to a broader spectrum of light resulted in the earlier appearance of tumors in the photosensitized skin of the SKH:hairless-1 mice, but produced few or no tumors in the HRS/J/Anl strain. In a second series of experiments, mice were exposed to a fluorescent sun lamp prior to each combined treatment of psoralen and exposure at 365 nm to determine the influence of shorter wavelengths of UV on the tumor response. These treatments resulted in an enhanced expression of tumors in the SKH:hairless-1 mice as compared to the HRS/J/Anl strain.

Under the conditions of the experiments, the marked strain and spectral dependent differences for tumorigenesis demonstrated that although treatments that induce psoralen photoadducts also induce tumors, there was no apparent

*Abstract of a paper published in Photochem. Photobiol. 25, 269 (1977).

quantitative correlation between the occurrence of DNA cross-links and the incidence of tumors. The results also suggested, first, an interaction between UV (280-400 nm) induced photoproducts and psoralen photoadducts and secondly, a strain difference in the oncogenic effects of this interaction.

SAMPLE OXIDATION FOR LIQUID SCINTILLATION COUNTING: A REVIEW*

Walter E. Kisieleski and Evelyn M. Buess

The liquid scintillation spectrometer is a versatile instrument for the measurement and analysis of low-energy beta emitters, especially hydrogen-3 (tritium) and carbon-14. On the other hand, biological materials as well as environmental samples are most difficult to prepare as true solutions for liquid scintillation counting and present unique problems in sample preparation. To overcome problems of sample solubility, quenching, and chemiluminescence, a more universal preparation technique can be achieved if the sample is burned at red heat in an atmosphere of oxygen and the carbon and hydrogen converted into carbon dioxide and water and quantitatively dissolved in a scintillator to produce an unquenched sample. A number of methods of oxidizing samples for liquid scintillation counting are discussed. Experimental studies using carbon-14 and tritium are presented and potential applications to biological and environmental problems are considered.

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* Abstract of a paper published in Liquid Scintillation Science and Technology, Proceedings of the International Conference on Liquid Scintillation Science and Technology, Banff, Canada, June 14-17, 1976. Academic Press, New York, pp. 299-308, 1976.

DNA CROSSLINKS, SINGLE-STRAND BREAKS AND EFFECTS ON BACTERIOPHAGE T4 SURVIVAL FROM TRITIUM DECAY OF [2-^{3}H]ADENINE, [8-^{3}H]ADENINE AND [8-^{3}H]GUANINE*

Frank Krasin, Stanley Person,† Ronald D. Ley, and Franklin Hutchinson‡

Escherichia coli and bacteriophage T4 DNA containing [2-^{3}H]adenine accumulated crosslinks between the complementary strands. For T4 DNA stored in frozen solution there were 0.41 to 0.54 crosslinks formed per tritium decay. The crosslinks were demonstrated both by an increased DNA sedimentation rate in alkaline sucrose gradients and by an increasing amount of DNA

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* Abstract of a paper published in J. Mol. Biol. 101, 197 (1976).

† The Pennsylvania State University.

‡ Yale University.

that renatured quickly after denaturation by heat or alkali. Single-strand breaks were also formed with an efficiency of 0.08 to 0.50 breaks per tritium decay. DNA containing both [8-^{3}H]adenine and [8-^{3}H]guanine showed no cross-linking but did undergo single-strand breaks at a rate of 0.08 per tritium decay. T4 bacteriophage containing [2-^{3}H]adenine lost plaque-forming ability when stored at 4°C, with 0.34 lethal hits per tritium decay, whereas the same phage labeled with a mixture of [8-^{3}H]adenine and [8-^{3}H]guanine sustained only 0.12 lethal hits per tritium decay. The loss of plaque-forming ability in the latter case is probably due to a radiation effect from the emitted beta particle; the high lethal efficiency for tritium decay at 2-adenine is probably caused either by crosslinks between complementary strands or from some undetected lesion produced in the DNA.

PURIFICATION AND IMMUNOCHEMICAL CHARACTERIZATION OF ALDEHYDE DEHYDROGENASE FROM 2-ACETYLAMINOFLUORENE-INDUCED RAT HEPATOMAS*

Ronald Lindahl and Robert N. Feinstein

1. A series of aldehyde dehydrogenase isozymes (aldehyde:NAD(P)$^+$ oxidoreductase, EC 1.2.1.5), has been purified from heptatomas induced in Sprague-Dawley rats by 2-acetylaminofluorene.

2. The functional hepatoma-specific aldehyde dehydrogenase isozymes exist as 105 000-dalton dimers composed of two subunits of 53 000 daltons. Isoelectric points of the purified isozymes are 6.9-7.2.

3. Antiserum to these purified hepatoma-specific aldehyde dehydrogenases has been produced and the immunological relationships of these isozymes to their normal liver counterpart have been studied. Results of Ouchterlony double diffusions, agar-gel immunoelectrophoresis and polyacrylamide gel and agar immunoelectrophoresis indicate that anti-hepatoma aldehyde dehydrogenase antiserum cross-reacts with normal liver aldehyde dehydrogenase.

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*Summary of a paper published in Biochim. Biophys. Acta 452, 345 (1976).

IMMUNOCHEMICAL STUDIES OF SERINE DEHYDRATASE AND ORNITHINE AMINOTRANSFERASE REGULATION IN RAT LIVER *IN VIVO*[*]

J. Emory Morris and Carl Peraino

Previous studies of serine dehydratase (EC 4.2.1.13) and ornithine aminotransferase (EC 2.6.1.13) adaptation in rat liver showed that in rats on a high protein diet, glucocorticoid administration increased serine dehydratase activity while simultaneously reducing the activity of ornithine aminotransferase. The present study examines the role of enzyme synthesis in the expression of these and other dissimilar adaptive characteristics of the two enzymes.

Both enzymes were purified to crystallinity and used to prepare specific antibodies. Changes in the rate of synthesis of each enzyme during adaptation were then measured immunochemically.

In rats fed *ad libitum*, the synthetic rates for both enzymes exhibited circadian rhythm, although enzyme levels remained relatively constant. The circadian cycle for ornithine aminotransferase synthesis was in phase with the cycles for body weight and relative liver weight (maxima at 9 a.m., minima at 9 p.m.) but was approximately 12 hours out of phase with the cycle for serine dehydratase synthesis. 9α-Fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxypregna-1,4-diene-3,20-dione (triamcinolone), injected at 9 a.m., increased serine dehydratase synthesis and simultaneously decreased the synthesis of ornithine aminotransferase. When triamcinolone was injected at 9 p.m., however, serine dehydratase synthesis was not stimulated, although the reduction of ornithine aminotransferase synthesis was still produced.

These results suggest that: (a) circadian cycling of synthesis may be a general phenomenon in enzyme regulation even though for enzymes with relatively long half-lives, such cycling may not be reflected as fluctuations in enzyme levels; (b) such circadian rhythmicity may also involve cyclic changes in the responsiveness of the enzyme-forming system to regulatory stimuli; (c) whereas the adaptive behavior of serine dehydratase typifies that of amino acid-catabolizing enzymes in general, the responses of ornithine aminotransferase denote a functional association of this enzyme with anabolic processes. On this basis, the possibility that ornithine aminotransferase plays a pivotal role in the regulation of urea cycle activity and nitrogen balance is discussed.

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[*]Abstract of a paper published in J. Biol. Chem. 251, 2571 (1976).

ENHANCING EFFECTS OF PHENOBARBITONE AND BUTYLATED HYDROXYTOLUENE ON 2-ACETYLAMINOFLUORENE-INDUCED HEPATIC TUMORIGENESIS IN THE RAT*

C. Peraino, R. J. M. Fry, E. Staffeldt, and J. P. Christopher

A comparison has been made between the enhancing effects of 0.05% dietary phenobarbitone and of 0.5% dietary butylated hydroxytoluene (BHT) on hepatic tumorigenesis in rats previously fed 2-acetylaminofluorene for a brief period. Prolonged feeding of the BHT diet produced a significant degree of enhancement which, however, was both lower in magnitude and delayed, in comparison with the enhancement produced by the phenobarbitone diet. Daily phenobarbitone injections stimulated persistent liver enlargement and a transient fourfold increase in DNA synthesis over a 5-day period. Similar treatment with BHT produced less pronounced liver enlargement after a delay of 1 day and did not stimulate DNA synthesis as did phenobarbitone. The results suggest that the dissimilar tumorigenic-enhancing abilities of BHT and phenobarbitone may result from differences in the effects of these agents on biochemical processes related to liver growth.

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*Abstract of a paper published in Fd. Cosmet. Toxicol. 15, 93 (1977).

EVIDENCE FOR DIFFERENT MECHANISMS IN THE CIRCADIAN AND GLUCOCORTICOID CONTROL OF RAT LIVER ORNITHINE AMINOTRANSFERASE SYNTHESIS*

Carl Peraino, J. Emory Morris, and Surendra T. Shenoy

In untreated rats fed a 60% casein diet *ad libitum* the rate of ornithine aminotransferase synthesis, measured immunochemically, decreased 50% between 9 AM and 9 PM. (Previous studies showed that this decrease represents the descending phase of a circadian cycle for ornithine aminotransferase synthesis.) This decrease was not affected by actinomycin D administration. When triamcinolone was given at 9 AM the rate of synthesis was reduced to 25% of the 9 AM value but this additional reduction was completely blocked by actinomycin D or α-amanitin treatment. These results suggest that the repression of ornithine aminotransferase synthesis by triamcinolone requires mRNA synthesis, and that the repression may occur at a post-transcriptional stage in the synthesis of the enzyme. However, the circadian decline in ornithine aminotransferase synthesis in untreated rats is apparently not dependent on the synthesis of a post-transcriptional repressor.

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*Summary of a paper published in Life Sci. 19, 1435 (1976).

CHANGES IN LIVER COMPOSITION IN PHENOBARBITAL-INDUCED HEPATOMEGALY*

Surendra T. Shenoy and Carl Peraino

In a study of molecular events occurring during phenobarbital-induced hepatomegaly, the levels of poly(A)-RNA (heterogeneous nuclear RNA + cytoplasmic mRNA), total RNA, DNA, and protein were compared in untreated rats and those injected with phenobarbital (80 mg/kg/day) for 3 days. Total poly(A)-RNA isolated by phenol extraction and purified by affinity chromatography on oligo d(T) cellulose, was assayed for its template (messenger RNA) activity in a cell-free protein synthetic system derived from wheat germ. The cell-free system was sufficiently sensitive to detect differences of less than a microgram of mRNA when optimal concentrations of Mg^{+2} and K^{+} were used. Using this system, no significant differences in the hepatic content of poly(A)-RNA were observed between the control and treated groups. The DNA level was also unchanged, but the liver weight was increased by 26% in the treated group. Total RNA and protein levels also increased significantly (25 and 38%, respectively, per 100 g body weight, and 21 and 30%, respectively per unit of DNA) in the treated group. Protein levels, when expressed in terms of poly(A)-RNA, showed a significant (22%) increase in the treated group. These results suggest that phenobarbital-induced hepatomegaly: (i) is characterized by hypertrophy (cell enlargement), (ii) is not mediated by a generalized increase in the level of poly(A)-RNA, but involves a generalized increase in the synthesis of RNA other than functional message, and (iii) involves an increased translation of mRNA *in vivo*.

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*Abstract of a paper to be published in Exp. Mol. Pathol.

6. VIRAL, RADIATION, AND ENVIRONMENTAL ONCOLOGY

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ANL 60500
ANL 60501
NCI Y01-CP-70504

GROUP LEADER'S INTRODUCTION

Christopher A. Reilly, Jr., Group Leader

The Viral, Radiation, and Environmental Oncology Group was established in January of 1976 as an outgrowth and extension of the Experimental Radiation Pathology and Oncology (ERP) program. Investigations in that program suggested that bone-seeking radionuclides induce bone cancer in mice through activation of a quiescent, endogenous oncornavirus. The long-term goal of the present program is to determine the mechanism of carcinogenesis with specific emphasis on the role of oncornavirus activation by radiation and other energy-related environmental pollutants. Current studies involve the characterization of the physical, biological, and biochemical properties of the murine oncornaviruses isolated at Argonne by the ERP group (ERP/ANL oncornaviruses), and a search for similar viruses in canine and human osteosarcoma. Capabilities in molecular virology, viral biochemistry, and immunology, which are essential to a more definitive examination at the molecular level of virus-cell interactions and virus activation, are now an integral part of the program. A spectrum of animal and human tumors will be studied. Animal tumors will be derived from the diverse radiotoxicity programs at ANL. Human tumors will be obtained through existing collaboration with Mayo Clinic, Rochester, Minnesota; Department of Radiology, University of Missouri, Columbia; Billings Hospital, Chicago; and numerous orthopedists throughout the United States.

Increased effort in the area of virus activation has been made possible through interagency funding in the form of a contract entitled "In Vivo Radiation-Activation of Endogenous Sarcoma Virus Genome" with the National Cancer Institute. Emphasis at this time is on the question: Does ^{90}Sr induce murine bone cancer through activation of the quiescent state of one of the ANL-isolated sarcoma viruses? Tissue culture studies are now aimed at large scale production of the ERP/ANL oncornaviruses from which ^{3}H-labeled DNA probes and viral antigens will be prepared. These reagents will permit the use of molecular hybridization and radioimmunoassay techniques to search for evidence, at the subcellular level, of viral activation by radiation and energy-related environmental pollutants.

Perhaps the two most important observations of the year were made in *in vitro* systems. First was the preliminary nucleic acid hybridizations that indicated there are substantial genetic differences between both FBJ and FBR and the well characterized Rauscher mouse leukemia virus. Second, and perhaps most central to radiation-induced viral activation studies, was the successful isolation of viruses from tumor cell cultures established from ^{90}Sr-induced osteosarcomas in CF#1 mice. Of the ten tumors now in culture, seven are producing infectious viruses detectable by reverse transcriptase assays. Future characterization as to the oncogenicity of these isolates is, however, necessary to evaluate their significance fully.

VIRAL, RADIATION, AND ENVIRONMENTAL ONCOLOGY STAFF

REGULAR STAFF

Chan, Emerson W. (Assistant Biochemist)
Dale, Phylis J. (Scientific Assistant)
Finkel, Miriam P. (Senior Biologist)
*Flynn, Robert J. (Senior Veterinarian)
Greco, Isabel L. (Scientific Assistant)
Lee, Chung K. (Assistant Biologist)
O'Connor, Timothy E. (Senior Biologist)
Pahnke, Vernon A. (Scientific Assistant)
Reilly, Christopher A., Jr. (Microbiologist)
Rockus, Gabriele (Scientific Assistant)

TEMPORARY STAFF DURING 1976

†Gutzeit, Diane L. (Postdoctoral Appointee)

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*Transferred to Environmental Impact Studies Division in 1977.

†Now in Radiation Toxicity in Dogs Group.

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ANL 60500
ANL 60501
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ACTIVATION OF ENDOGENOUS ONCORNAVIRUS BY RADIATION AND ENERGY-RELATED ENVIRONMENTAL POLLUTANTS

Emerson W. Chan, Miriam P. Finkel, Chung K. Lee, and Christopher A. Reilly, Jr.; and Phylis J. Dale, Robert J. Flynn, Isabel L. Greco, Diane L. Gutzeit,† Vernon A. Pahnke, Timothy E. O'Connor, and Gabriele Rockus*

Physical and chemical agents are known to cause cancer in man. Viral agents have also been implicated. In our program, viruses have been isolated from malignant bone tumors in two strains of mice, CF#1 and X/Gf. In the CF#1 strain, the tumor was spontaneous, whereas in X/Gf it was induced by ^{90}Sr. In their respective hosts, these two viruses are potent, specific inducers of osteosarcomas (malignant bone tumors).

^{90}Sr, a bone-seeking radionuclide, is also a very effective inducer of osteosarcomas (Figure 6.1). Although electron microscopy shows viral particles in most of these ^{90}Sr tumors (Figure 6.1), virus isolation has only occasionally been successful.

^{90}Sr and our murine bone tumor viruses provide a unique system to determine whether radiation causes cancer by activating latent cancer tumor viruses. With molecular probing techniques, we are searching for such evidence, which would support the idea that viruses are fundamentally involved in cancer induction and would show how radiation causes cancer. Such evidence also would encourage testing chemotherapeutic and immunotherapeutic antiviral measures for the prevention and cure of radiation-induced cancers.

During 1976, new information basic to the assessment of the role of the ERP/ANL oncornaviruses in ^{90}Sr-induced osteosarcoma was obtained. The two sarcoma viruses most relevant to sarcoma virus activation studies, FBJ and FBR, were shown to possess 60-70S RNA, a reverse transcriptase, and a density in sucrose of 1.16 g/cc, all of which are characteristic of type C RNA tumor viruses. Tests were run to select a tissue cell culture system for large-scale production of these viruses, and the following facts were established: (1) Host Range--The infectivity of both viruses is limited to murine cells. Whereas they infect and replicate efficiently in cell lines derived from NIH Swiss, BALB/c, and SC-1 mice, they are noninfectious for cell lines of bat, hamster, dog, and human origin. (2) Transforming Activity--Both viruses transform normal mouse fibroblasts with transforming titers of up to 10^4 focus forming units/ml. (3) Nontransforming Viruses--In association with the transforming agents, both virus preparations have nontransforming components 2-3 logs in excess of the transforming titer.

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*Environmental Impact Studies Division.

†Radiation Toxicity in Dogs Group.

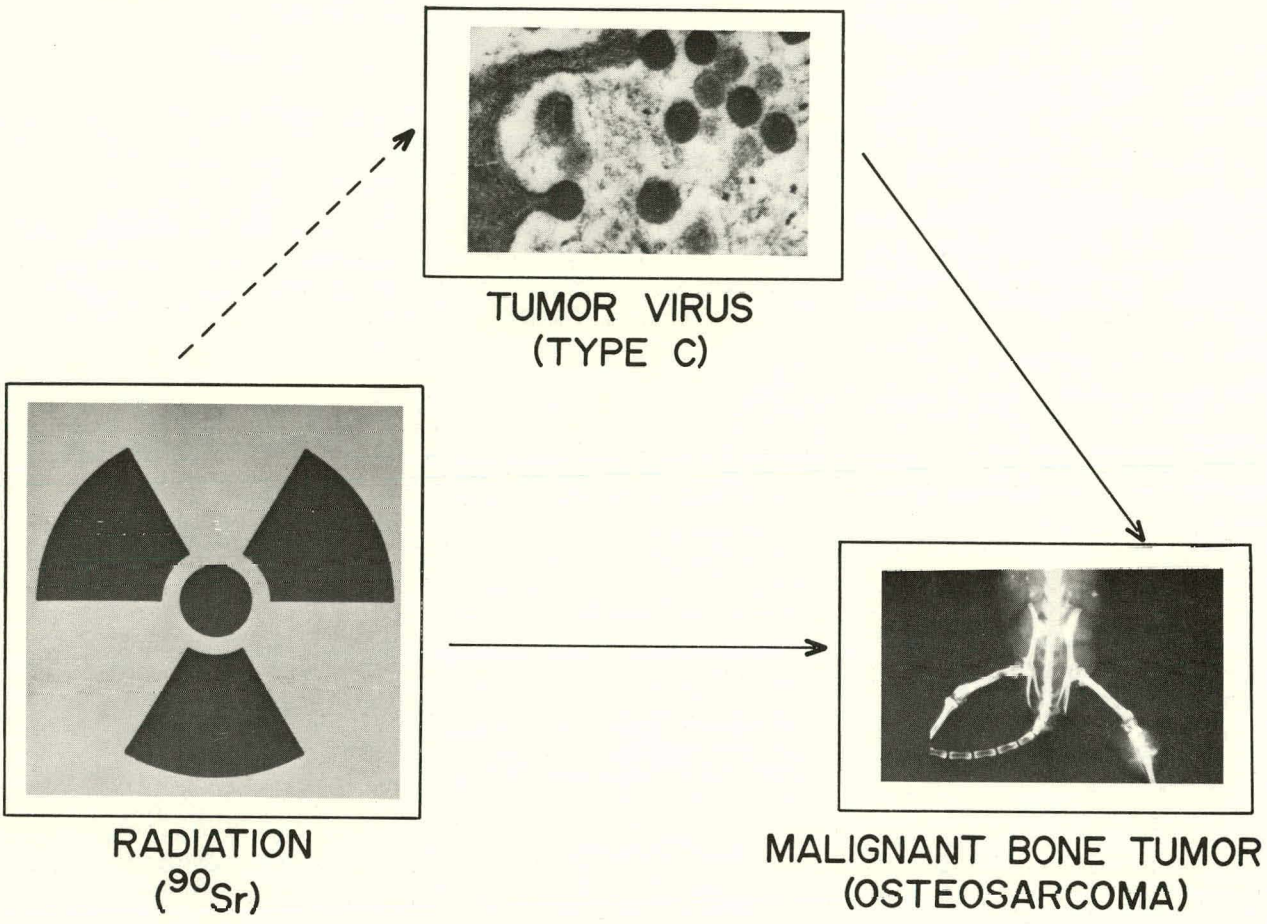

Fig. 6.1. Are oncornaviruses involved in the basic mechanism of induction of murine osteosarcomas by ^{90}Sr?

Of special interest is the extent of relatedness of FBJ and FBR to other murine oncornaviruses. We have found substantial differences in sequence homology between RLV (Rauscher mouse leukemia virus) and both FBJ and FBR. The differences are found with a ^{3}H-cDNA probe made from RLV which hybridized 86% to RLV RNA but only 69% to FBJ, and 68% to FBR RNA's. Conversely, they are also found with a ^{3}H-cDNA probe, made from FBJ, which hybridized 63% to FBJ RNA, but only 29.5% to RLV RNA.

Virus has been detected by reverse transcriptase assay in seven of ten tumor cell cultures established from ^{90}Sr-induced osteosarcomas in CF#1 mice. These new virus isolates are fully infectious and are currently being characterized.

In vitro study of the third ERP/ANL oncornavirus, the benign osteoma virus, RFB, revealed that it does not induce morphologic transformation of murine embryonic cell cultures derived from CF#1, CBA, NIH Swiss, and X/Gf strains. However, RFB was capable of infecting and replicating to high titers

in cells of all strains except X/Gf, as determined by recovery of ^{3}H-uridine-labeled virus and electron microscopic detection of C-type particles.

Additional biological experiments with the five ERP/ANL oncornaviruses provided the following information:

1) Injection of FBJ virus into the bone marrow cavity of weanling Syrian hamsters fails to improve FBJ's weak oncogenicity in that species.

2) Antiserum prepared in rabbits against FBJ virus completely neutralizes both bone tumor viruses of the CF#1 mouse, FBJ osteosarcoma virus, and RFB osteoma virus. Similarly, rabbit antiserum to RFB neutralizes both RFB and FBJ.

3) FBJ and RFB viruses, harvested from infected mouse embryo cell cultures, are fully tumorigenic in mice.

4) In addition to the previous findings that both FBJ and FBR osteosarcoma viruses are highly oncogenic in newborn mice, new tests show that they are also highly oncogenic when injected intramuscularly in young-adult mice of their natural host strains, CF#1 and X/Gf, respectively.

5) Unlike the above osteosarcoma viruses, the benign RFB osteoma virus is oncogenic only when injected into susceptible newborn mice.

6) The CF#1 reticular tissue tumor virus (RTTV) has a host range similar to the CF#1 bone tumor viruses, FBJ and RFB. The RTTV induces tumors of the reticular tissues in 25 to 50 days when injected into CF#1, CBA, and NIH Swiss mice but rarely produces tumors in the X/Gf strain.

7) Similarly, RTTV from X/Gf mice shows the same restrictive host range as the X/Gf osteosarcoma virus, FBR. X/Gf RTTV induces reticular tissue tumors in X/Gf mice but not in CF#1, NIH Swiss, and CBA mice.

The formerly demonstrated interference with FBJ's ability to induce osteosarcomas by prior infection with RFB osteoma virus was examined further by treating the osteoma virus with β-propiolactone (B-PL), which leaves viruses immunologically intact but unable to replicate and induce tumors. Newborn CF#1 mice, given B-PL-inactivated RFB followed in 24 hours with normal FBJ virus, developed half as many osteosarcomas as a comparable group of mice given FBJ only, and the time to death with bone cancer was doubled. This interference, while significant, was no better than that seen with live osteoma virus.

Interference studies were also carried out *in vitro* with both CF#1 and NIH Swiss mouse secondary embryo-cell cultures. The transforming efficiency of FBJ virus is greatly reduced in terms of the number of foci of transformed cells if cultures are preinfected or simultaneously infected with RFB virus.

The interference potential of *Herpes simplex* viruses against FBJ osteosarcoma virus was also tested in mice. These viruses were chosen because they are ubiquitous in man, primarily in a quiescent stage, and because they are DNA viruses. Results of several experiments indicated that UV-inactivated virus, both Type 1 and Type 2, reduces the effectiveness of FBJ virus, as evidenced by a decrease in the incidence of bone cancer and an increase in the time to death with cancer.

Since FBJ and FBR viruses have such contrasting host ranges, we initiated studies designed to examine host genetic control of susceptibility to these viruses. FBJ is highly oncogenic in CF#1 mice and almost non-oncogenic in the X/Gf strain, whereas FBR is highly oncogenic in X/Gf mice and almost non-oncogenic in the CF#1 strain. We tested the cancer-inducing properties of each virus in the hybrid offspring of a CF#1-X/Gf cross. Preliminary data indicate that the hybrids develop osteosarcomas with either virus. However, there is a significantly longer latent period (time to death with tumor) than in the susceptible parental strain.

In search of a human osteosarcoma virus, tissues from 168 patients have been either extracted for injection into neonatal Syrian hamsters or put into tissue culture. Thirty-eight malignant mesenchymal tumors, 21 of which were osteosarcomas, have been diagnosed to date in the experimental animals; more tumors may be added to this list because some autopsy specimens are awaiting histological examination, and a few hamsters are still alive. Spontaneous sarcomas of all types except those of the reticuloendothelial tissues are very rare in Syrian hamsters. In our control animals, there has been only one nonreticuloendothelial sarcoma, a fibrosarcoma, and we are aware of only eight osteosarcomas described by the published literature in Syrian hamsters. Depending upon the selection of base population, the incidence of mesenchymal tumors is 1.5 to 6% in experimental hamsters, and 0.25% in controls. Extracts of tissues from 36% of the bone tumor patients from whose tissues extracts were prepared have resulted in one or more of these hamster tumors.

PLASMA ALKALINE PHOSPHATASE IN MICE WITH EXPERIMENTALLY-INDUCED OSTEOSARCOMAS*

Janice M. Bailey, William D. Hill,† Alvin G. Fiscus,†
Christopher A. Reilly, and Miriam P. Finkel

No statistically significant difference in alkaline phosphatase levels was demonstrated in animals injected with the FBJ virus. However, there was a significant increase associated with the development of osteosarcomas in response to the iv injection of 1.0 μCi ^{90}Sr/g body weight in 11-18-mo-old An1:CF1 females. It was proposed that alkaline phosphatase determinations can be used as well as roentgenographic analysis to detect ^{90}Sr-induced tumors in mice.

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*Summary of a paper published in Lab. Anim. Sci. 26, 66 (1976).

†Montana State University.

APPROACHES TO ANTIVIRAL CHEMOTHERAPY: A STATUS REPORT*

Timothy E. O'Connor

Over the past half century, immense strides have been made in elucidation of the molecular biology of viruses. Yet control of viral disease, where it has been achieved, has usually resulted from effective employment of vaccines rather than from specific chemotherapy. Although the usefulness of antiviral chemotherapy might therefore be in doubt, there are indications for a brighter future. The present paper outlines the current state of the art, and presents some possibilities for future directions.

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*Introduction of a paper published in Advances in Pathobiology, Vol. 5, Eds. C. Borek and D. W. King. Stratton Publishers, New York, pp. 151-158, 1976.

INTERRUPTION OF ONCORNAVIRUS REPLICATION BY RIFAMYCIN ANTIBIOTICS*

Timothy E. O'Connor, Charles D. Aldrich,† and V. Sagar Sethi†

Thirteen rifamycin SV derivatives containing 3'-alkylaminomethyl substituents failed to inhibit the activities of the simian sarcoma virus Type I DNA polymerase, and of cellular DNA, RNA, and poly(A)polymerases prepared from NIH Swiss mouse embryos. These compounds show a range in their toxicities for NIH Swiss mouse 3T3 cells and in their capacities to inhibit production of foci of morphologically altered cells by murine sarcoma virus (MSV). Three compounds--the *N*-methyl-*N*-hydroxyethylaminomethyl, the *N*,*N*-dimethylaminomethyl, and the N^4-methylpiperazinomethyl rifamycin derivatives--are comparable to adenine arabinoside and ribavirin in their toxicity for 3T3 cells, but these compounds show superior focus inhibition. These compounds inhibit oncornaviruse production apparently by exacerbation of a delay in growth that results from infection of 3T3 cells with MSV.

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*Summary of a paper published in Third Conference on Antiviral Substances, Ed. C. Herrmann, Jr. New York Academy of Science, NY. Ann. N. Y. Acad. Sci. 284, 544 (1977).

†Litton Bionetics, Kensington, MD.

IN VIVO INTERFERENCE OF VIRUS-INDUCED OSTEOSARCOMAS BY A BENIGN BONE TUMOR VIRUS*

Christopher A. Reilly, Jr. and Miriam P. Finkel

Two bone tumor viruses isolated at Argonne National Laboratory provide a unique system for examining viral oncogenesis. These viruses, which induce malignant (FBJ) and benign (RFB) bone tumors, were isolated from a spontaneous osteosarcoma and osteoma, respectively, in CF1 mice. This strain normally has a high incidence of bone tumors: 1-2% for osteosarcomas and 10-20% for osteomas. Unraveling the natural role of viruses in tumor induction should be more reliable when native viruses rather than laboratory isolates are used.

When both viruses were given to the same animal, osteosarcoma development was retarded. Animals that received RFB-osteoma virus 24 hours before FBJ-osteosarcoma virus developed fewer malignant bone tumors, and these appeared later than usual. This interference was specific: osteosarcoma virus given 24 hours before osteoma virus had no effect on osteoma development. Interference was then tested in NIH Swiss mice, a strain not normally harboring these two viruses but capable of responding to them with tumor production. In these mice, also, the osteoma virus decreased the number of osteosarcomas and increased the time to death with tumor. In another experiment, RFB-FBJ virus-treated newborn CF1 and Swiss mice were foster-nursed: the CF1 pups

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*Abstract of a paper published in Bibl. Haematol., No. 43, Eds. J. Clemmesen and D. S. Yohn. S. Karger, Basel, pp. 441-444, 1976.

were given to Swiss dams and the Swiss pups to CF1 dams. In Swiss mice, interference was the same whether or not the mice were foster-nursed. In CF1 mice, however, interference was enhanced when they were nursed by Swiss dams so that osteosarcomas were almost completely inhibited.

This specific *in vivo* interference of virus-induced malignant tumors by a virus inducing benign tumors may have importance in normal tumor expression. For example, such interaction might explain why osteomas are ten times more frequent than osteosarcomas in untreated CF1 mice, or why, in general, carcinomas occur more frequently than sarcomas.

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7. PATHOLOGY AND RISK ASSESSMENT

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ERDA RT-05-01
ANL 68100
ERDA RT-05-03
ANL 65902

GROUP LEADER'S INTRODUCTION

Miriam P. Finkel, Group Leader

The programs of the Pathology and Risk Assessment Group are directed toward providing basic data for use in evaluating the hazard to man from exposure to radiation and other energy-related environmental pollutants. The eight present programs fall into three main categories: (1) characterization of normal human and laboratory animal populations in order to establish baselines for recognizing changes that might be brought about by environmental pollutants, (2) examination of human and animal populations exposed to known or potentially hazardous agents, and (3) animal experiments designed to answer specific questions concerning dosages responsible for deleterious changes and mechanisms by which these changes are brought about.

The first category contains two programs. One deals with human populations, and is directed toward establishing a standardized approach to health surveillance by use of only the normal resources of vital, census, and economic statistics and also toward developing statistical and actuarial models for risk analysis. The other involves animal studies in which comparisons are made of the anatomy, physiology, and diseases of two rodent species that differ significantly in life-span. An important factor under investigation in this animal study is the influence of time on normal metabolic and physiologic processes.

There are three programs in the second category concerned with populations exposed to known or potentially hazardous agents. (1) The anticipated human health costs of the effluents from coal combustion and cleaning procedures are being evaluated in collaboration with the Energy and Environmental Systems Division and the Environmental Impact Studies Division of Argonne National Laboratory. (2) The influence of environment on life-span and

pathology as a function of genetic constitution is being examined in a diallel design using all F_1 crosses of five inbred mouse strains. Environmental factors include lower than normal temperature and the presence of gamma radiation or other energy-related pollutants. (3) The influence of both radiation and animal characteristics on the pathologic consequences of exposure to internal and external radiation is under investigation.

In the third category are three programs concerned with answering specific questions regarding dosage and mechanisms. (1) Bone cancer induction is examined when the physical, biological, and temporal parameters of dosage are altered in order to answer questions related to dose, dose rate, latent period, and animal sensitivity. (2) The effects of chemical carcinogens on the developing immune system are being examined by injecting the materials into prenatal and postnatal mice of various ages and then testing their capacity to give humoral and cell-mediated immune responses 2 weeks to 2 years later. (3) Dominant lethal mutations, spermatogonial chromosome translocations, and sperm morphology are being examined in mice treated with ^{239}Pu, neutrons, and gamma rays. Determinations of the distribution, retention, and dosimetry of plutonium in the testis are important aspects of these studies.

The highlights of the year's research in the programs are summarized in the following paragraphs.

The effort to characterize normal human populations has shown that life expectancy can be predicted with some reliability on the basis of ethnicity, labor-force composition, income, education, and urbanization. A computer program has been developed that takes a given population at a particular time, with its existing mortality and fertility rates, and applies dose-response coefficients and estimated increments of exposure to environmental factors in order to derive excess deaths by age and sex for 5-year intervals. The health effects of coal combustion effluents have been qualitatively summarized for all components, and the available quantitative analyses for sulfur dioxide and total suspended particulates have been re-examined and extended. A new analysis for hydrocarbons is undergoing development.

Characterization of normal laboratory mice has shown that they have a daily cycle of metabolic rate, and that, with increasing age, both the average rate and the cyclic amplitude decrease.

It has been determined that plutonium is deposited along the basement membrane of spermatogenic tubules, where it is tenaciously retained. Although only 0.05% of an initial injected body burden of monomeric Pu citrate is present in the gonads, its concentration near spermatogonia stresses the importance of considering the genetic risks of exposure to this radionuclide. An assessment of the possible genetic costs of exposure to mutagens resulting from coal and nuclear energy sources indicated that costs from coal were negligible but that costs per man-rem for nuclear energy would be about $100. Pollution abatement costs for the nuclear cycle would be in the range of from several hundred to many thousand dollars per man-rem-reduction.

The staff of the Pathology and Risk Assessment Group is also active in pursuing the research goals of other groups in the Division. The contributions of P. C. Brennan, S. P. Stearner, and L. S. Lombard and their co-workers are incorporated in the reports of the Neutron and Gamma-Ray Toxicity Group

(Section 2) and the Radiation Pathology in Dogs Group (Section 3). In addition, other members of the Pathology and Risk Assessment Group staff are involved in the research programs of the Carcinogenesis Group.

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*Supported in part by the Energy and Environmental Systems Division.

†Transferred to Environmental Impact Studies Division in 1977.

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HEALTH EFFECTS OF ENERGY GENERATION

*Douglas Grahn, Robert T. Lundy, Jane R. Benson, Diana K. Dixon-Davis, Charles D. Brown, and Phyllis M. Fuja**

Assessments of the potential health effects of energy generation are being approached from several directions concurrently. There are many cultural, economic, and demographic factors that significantly influence the health of individuals and populations. Therefore, an important first approach is to evaluate these variables and to develop prediction equations to standardize any population with respect to expected causes and levels of mortality. The second approach makes use of the present information on the effects of air and water pollution on human health. The available data, especially those relating to coal combustion effluents, are being assembled to develop and/or confirm dose-response relationships. The third approach focuses on generating demographically and actuarially correct models that predict energy or socioeconomically related causes of mortality as changes in individual life expectancy and as mortality rates.

A. SOCIOECONOMIC VARIABLES AND HUMAN HEALTH

Fifteen regions surrounding energy facility sites, encompassing 191 counties, were selected for a generic study of socioeconomic variables and human health. The following items have been incorporated in the master data file: Characterizations of (1) 1970 mortality data, (2) 1970 census data, (3) 1969 and 1971 mortality data (in progress), (4) 1970 census data on urban residence, as reclassified, and (5) cost of living index.

An analysis of "housing quality" as a predictive variable has been made, and the results indicate that variation in housing quality can be accounted for by other basic socioeconomic factors. Preliminary regression analyses of the socioeconomic variables have also been performed with life expectancy as the dependent variable. The expected strong relationship with income, education, and urbanization was found, and good preditability for life expectancy was observed for ethnicity and labor-force composition. An informational summary of factors influencing human health is well advanced; Table 7.1 presents a qualitiative survey of over two dozen environmental, cultural, economic, or personal factors that have an identifiable effect upon human mortality.

B. STATISTICAL AND ACTUARIAL MODELS FOR RISK ANALYSIS

The parameters to be constructed from mortality data fall into two categories: (1) those that predict the overall level of mortality, and

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*Energy and Environmental Systems Division.

Table 7.1. Summary of Factors Influencing Human Health as Measured by Mortality; Synthesized from Various Sources

Factor	General Effect[a] Direction	Relative Significance
Infant Mortality:		
Parental Factors:		
Age	Var	+++
Birth order	Pos	+++
Maternal history	Pos	+++
Socioeconomic class	Neg	+++
Other Factors:		
Birth weight	Var	+++
Season	Var	++
Geography	Var	+
Race	Var	++
Urban residence	Pos	+
Adult Mortality:		
Economic Factors:		
Education	Neg	+++
Income	Neg	++
Occupation class	Neg	+++
Housing quality	Neg	±
Urban residence	Pos	++
Social/Cultural Factors:		
Marital status	Var	++
Ethnicity	Var	+
Religion	Var	+++
Occupation	Var	+++
Geography	Var	±
Nativity	Var	+
Race	Var	++
Sex	Var	+++
Personal Habits:		
Alcohol	Pos	++
Diet	Var	+
Tobacco	Pos	+++
Sex	Var	+
Iatrogenic	Pos	+
Environmental Factors:		
Water quality	Neg	+
Air quality	Neg	++
Climate	Var	+

[a] A positive effect occurs when increases in the level, intensity, or frequency of the factor are accompanied by consistent linear or nonlinear increases in mortality. A negative relation indicates that lower mortality rates accompany increases in the level, intensity, or frequency of the associated factor. A variable relationship may identify a specific nonlinear (parabolic) association, an inconsistent association, or one that is not readily categorized by level, intensity, or frequency.

(2) those that predict mortality levels for different age groups. Measures of overall level are (1) the age-adjusted death rate, which reaggregates the age-specific mortality to give the total mortality rate for a population with a standard age distribution; and (2) the expectation of life, which uses age-specific mortality to determine the average years of life expectancy for a hypothetical cohort of people. A comprehensive set of computer programs has been written to generate measures of total mortality and mortality by cause of death.

Although the socioeconomic analyses described in Section A are needed before a complete predictive model of human mortality variation in the United States can be attempted, there is an immediate need for predictions of the health effects of energy-related effluents, all other variables assumed unchanged. The accomplishment of such projections requires a dose-response model for exposure to energy-related effluents and a demographic model through which the dose-response model can generate expected numbers of deaths. The dose-response model is a multiple linear equation specific for age and sex. The demographic model is a component projection model commonly used to project

populations forward through time and also to project numbers of deaths, births, and living persons.

A computer program has been developed that takes the existing mortality and fertility rates of a given population, applies dose-response coefficients and estimated increments of exposure, and derives the excess deaths by age and sex for 5-year intervals. This procedure not only predicts the magnitude of the expected effects but also estimates their timing and identifies the subgroups of the population most heavily affected. Preliminary results from the modeling project have been used in the analysis described in Section C.

C. ANALYSIS OF THE HEALTH EFFECTS OF AIRBORNE COAL COMBUSTION EFFLUENTS

The health effects of coal combustion effluents have been qualitatively summarized for all components, and the available quantitative analyses for sulfur dioxide and total suspended particulates have been re-examined and extended. A new analysis for hydrocarbons is undergoing development. These analyses have been applied to three studies: (1) Environmental Control Technology for Generation of Power from Coal (ANL/EES for ERDA), (2) Health and Ecological Effects of Coal Utilization (ANL/EIS for the Nuclear Regulatory Commission), and (3) A Preliminary Assessment of the Health and Environmental Impacts of Fluidized-Bed Combustion of Coal as Applied to Electrical Utility Systems (ANL for ERDA). A variety of coal combustion products are involved: gaseous oxides of carbon, sulfur, and nitrogen; vaporized volatile organic and inorganic materials; particulates of different sizes and derived from various sources. These products vary with the type of coal used, the combustion process, and the efficiency and types of pollution-control devices applied.

Under assumptions based on measurements of the effluent stream, and with use of appropriate atmospheric transport equations, it is possible to predict the most likely concentration of pollutants to be inhaled by the exposed population. Prolonged exposure may result in an increased incidence of chronic respiratory diseases and cancer. Predictions of health effects can be made through estimating increases in morbidity and mortality rates as a function of estimated dose. Use of mortality statistics is preferred because the records are readily accessible and easily analyzed. Premature deaths (those occurring in excess of the average age-specific rate expected for the population) are the health effect measured or predicted as a function of exposure to the specified pollutant.

Three independently derived models, based on different pollutants, are used in our preliminary analyses. The first model, Model A, is taken from Lave and Seskin as reported by Finch and Morris (Brookhaven National Laboratory Document 218081, 1976). This model relates total mortality to two independent pollutant measures simultaneously: sulfate ion ($\mu g/m$, $^3SO_4^=$) and total suspended particulates ($\mu g/m^3$, TSP). Model B is based on data from Winkelstein's study of mortality related to total suspended particles in Buffalo, New York. These data were also analyzed by Morris (Brookhaven National Laboratory Document 218081, 1976). Model C, based on data analyzed by Carnow and Meier (Arch. Environ. Health 27, 207, 1973) associates the pollution index of benzo(a)pyrene ($\mu g/m^3$, $\overline{BaP}$) with lung cancer mortality. With appropriate adjustments, this index can be related to total mortality as well.

The projection models all take the form of "absolute risk." A mortality schedule of sex and age-specific death rates (${}^{s}_{n}M_{x}$) is projected to the rates to be expected from an increment of population by a simple linear equation of the form

$$ {}^{s}_{n}M^{*}_{x} = {}^{s}_{n}M_{x} + B_i P_i + \ldots + B_k P_k, $$

where the final adjusted death rate is ${}^{s}_{n}M^{*}_{4}$, P_i is the increment in ambient pollutant concentration to be expected, and B_i is the slope of a regression line defining the effect that pollutant i will have on mortality. Width of age group, initial age, and sex are specified by n, x, and s, respectively. Two measures are generated: the expectation of life at birth (e_0) and the change in the annual death rate per million population that would be observed over a 25-year period. Calculations have been made for two limiting conditions. In the first, the population is distributed evenly throughout the impact area; in the second, the entire population lives in the region of higher pollution concentration. The latter can be taken as an upper limit. Such areas, it should be noted, are usually valleys or other flat areas partly surrounded by physical barriers to air movement, a characteristic that is likely to make them particularly desirable as locations for human activities. In this latter case, an excess of 15 to 65 deaths per million persons exposed per year is estimated to occur in the 50-mile radius area surrounding a 1000 MWe coal-fired plant meeting EPA's New Source Performance Standards of SO_2 and total suspended particulates. Excess mortality is only about $6/10^6$/year for the lower limiting condition.

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INFLUENCE OF TIME, ENVIRONMENT, AND GENETIC CONSTITUTION

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THE DAILY CYCLE OF ACTIVITY AND METABOLISM

The products of fossil fuel combustion may have their most serious effects in terms of impaired performance and disruption of normal patterns of working and living. However, the experimental tools for assessing the deterioration

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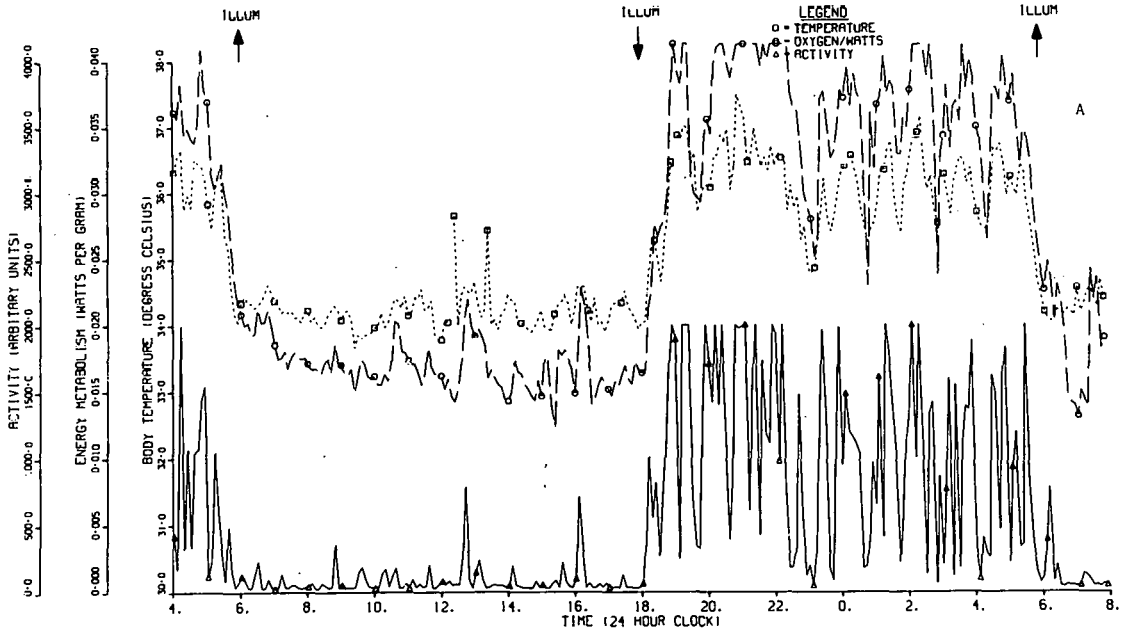
of the vital performances of populations have not yet been adequately developed so that, at present, we can say very little on the matter. Nevertheless, a responsible assessment of the impacts of energy production by fossil fuels must include an evaluation of the effects of pollutants on psychophysiological performances.

The research described below is intended to provide an experimental model that will make it possible to: (1) characterize the spontaneous activity patterns and daily (diel) metabolic cycles of small rodents, (2) measure the total amount of motor activity and energy consumption in any age period or over the entire lifetime, (3) assess the effect of environmental pollutants and stresses on these aspects of productivity, and (4) compare these effects of pollutants with the excess morbidity and mortality measured in epidemiological studies.

The experimental system allows monitoring of three physiological variables, motor activity, oxygen consumption, and deep body temperature, on four mice at a time for periods up to 3 weeks. Each mouse is in a separate small module within an environmental chamber that allows programmed control of illumination and ambient temperature. The data are sampled (typically) at 3-minute intervals, digitized, and recorded on punched paper tape for subsequent computer analysis.

Two 28-hour records of the three variables, produced by the computer graphics program, are given in Figure 7.1. Figure 7.1A shows the strong diel cycle that is characteristic for young mice, and Figure 7.1B shows the lower, flatter cycle that is typical for old mice. The decrease in average metabolic rate and amplitude of the daily cycle progresses at an almost constant rate throughout adult life. We have only recently begun to acquire data on motor activity and body temperature, but it is already evident that each of these variables has a distinctive aging pattern.



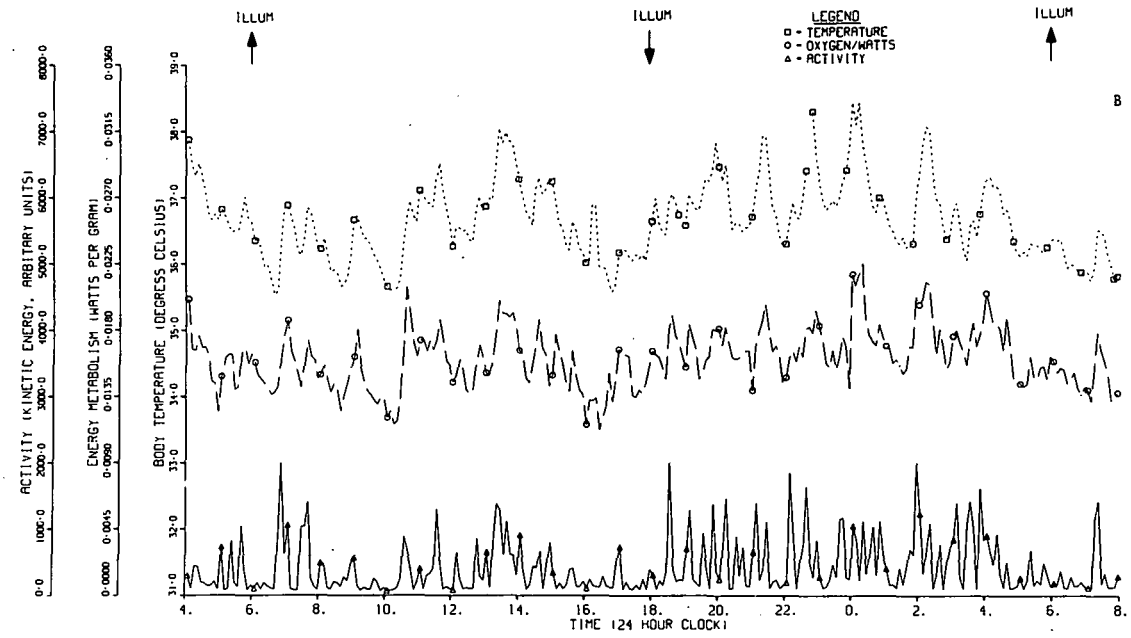


Fig. 7.1. Diel patterns of motor activity (bottom line), energy metabolism (center line), and deep body temperature (top line) for young and old *Peromyscus*. Data registered every 6 minutes for 28 hours, beginning at 0400 hours. Animals kept at 29°C in a 12:12 light-dark cycle, with light on at 0600 hours and off at 1800 hours. A. Male, age 6 months; B. Male, age 60 months. Note the almost complete disappearance of the 24-hour rhythm in the old *Peromyscus*. The same progression occurs in *Mus* in 30 months, i.e., in an equivalent fraction of the life-span.

In this continuing research program, the first priority is given to characterizating the age changes in the diel cycles of activity and metabolism. Following that, two lines of inquiry will be pursued. One is to determine how exposure to atmospheric pollutants, and specifically to the effluents from fluidized-bed combustion, affect the diel cycles and their aging trends. In order to interpret these effects, related studies will investigate how the aging of the activity and metabolism cycles is affected by added metabolic loads (low ambient temperature and forced exercise) and by drugs that modify the nervous system and behavior. The experimental approach will be based on earlier findings that ℓ-dopa, a precursor of the catecholamine neurotransmitters, administered to mice throughout adult life, results in prolongation of life (Cotzias, G. C., et al., Proc. Nat. Acad. Sci. U.S.A. 71, 2466, 1974). We are interested in learning how ℓ-dopa and other neurochemical substances affect the course of aging of the daily metabolic cycle, because this information should improve our understanding of how noxious agents alter activity patterns.

ENVIRONMENTAL INTERACTIONS WITH THE AGING PROCESS

The conventional experiment paradigm for assessing toxicological dose-effect relationships can be called a "one-treatment, one-genotype, one-environment" design. Such an approach, although appropriate for some experimental situations, has serious shortcomings for the assessment of the health effects of low levels of environmental pollutants produced by fossil fuel combustion. Such substances can be expected to affect many body systems, involving numerous genetically determined mechanisms, so that the consequences in functional deterioration, morbidity, and life shortening may be strongly influenced by the genetic constitution of the treated population as well as by other environmental factors present in the experimental situation.

This program is intended to evaluate the importance of genetic and environmental factors as influences on the long-term toxic effects of energy by-products. If such factors should be found to be important, new experimental designs will be required that incorporate genetic and environmental variance in the treatment group.

The experimental procedure is to employ the set of genotyes obtained by making all 25 matings of 5 inbred mouse strains. This yields 20 F_1 hybrids (including reciprocal crosses) and the 5 inbred genotypes. Both sexes of progeny are used. This is one form of diallel design, which is widely used in genetic studies.

Experimental populations constituted according to this diallel design are exposed to various environments for all or a major part of adult life. The end points to be examined are length of life and disease incidence. Because of the number of genetic degrees of freedom (24 for each sex), it is possible to carry out a genetic analysis of variance for each environmental condition separately. This has already been done for three environments for which survival studies have been completed, namely: (1) the conventional animal room environment, (2) duration-of-life (D.O.L.) gamma-ray exposure at a level (43 R/day) that causes about 50% life shortening, and (3) D.O.L. gamma-ray exposure at 125 R/day.

An analysis of survival in the conventional environment revealed that there is significant genetic variance, a large part of which is in nonadditive and interactive genetic components. In other words, the genetic factors governing length of life in a relatively unstressed environment are complex. On the other hand, genetic analysis of survival under D.O.L. exposure to 125 R/day revealed a simpler pattern of genetic determination, with a larger component of additive genetic variance that was found for the conventional environment. The 43 R/day D.O.L. exposure had a genetic pattern that could be accounted for as a linear combination of the genetic terms for the 125 R/day and the conventional environment.

Anatomical and physiological measurements have also been made on all the genotypes in the diallel design, and it has been possible to show that longevity in the conventional environment has a significant genetic correlation with certain of these variables.

At present a sample of about 100 mice of the diallel genotypes is being kept in a cool environment (12°C) to determine the genetic factors related to survival at a moderately elevated metabolic rate. This is a critically important step, because many organic carcinogens are activated or inactivated in the liver. Since the liver bears the brunt of the increased metabolism in the cold, the interaction of carcinogen effects with environmental temperature will be a key factor in our decision about the importance of genetic and environmental interaction terms in the toxic effects of pollutants.

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RADIATION PATHOLOGY AND ONCOLOGY

Miriam P. Finkel

The radiation pathology and oncology program is directed toward providing a firm foundation for estimating cancer risks to human populations in the event of accidental incorporation of radionuclides. Historically, the first approaches to this goal centered around accumulating information on radiation dose-response relationships in animals and extrapolating the animal data to man, the induction of bone cancer being the major response under investigation because most fission products and transuranic elements of particular concern localize in bone. After bone-cancer-incidence data had been obtained for a number of radionuclides under standard conditions in CF#1 mice, incidence was examined when age, sex, strain, species, and exposure patterns were varied. Unfortunately, estimations of human hazard from very low levels of radionuclide contamination that are based on moderate and high-level exposures of animal populations are not reliable because too many assumptions must be made. Sound, unequivocal, universally acceptable estimates require knowledge of mechanisms of radiooncogenesis. Accordingly, the present primary concern of the program is to determine how radiation causes cancer.

The purposes of the radionuclide experiments have been to determine the relative toxicities of different radionuclides, the toxicity of selected radionuclides in different species, and the dependence of oncogenicity upon exposure situation. Three experimental plans have been used: (1) A variety of radionuclides are tested under a set of standard conditions, which includes the effect on longevity and disease of a single intravenous injection into 70-day-old CF#1 female mice. With the same standard procedures in all other respects, mice receive single exposures to fission neutrons or gamma rays so as to permit comparison of internal and external exposure. (2) The same radionuclide is tested in several species that differ in body size and life-span. (3) Different exposure situations are compared, such as ingestion versus injection, chronic versus acute exposure, and treatment of prenatal or neonatal animals versus adults. To investigate mechanisms of radiation oncogenesis, the test system has been the induction of bone cancer by radionuclides that localize in the skeleton or by X-ray-exposure of hind limbs only. Objectives have been: (1) to define the true latent period, or time to irreversible neoplastic change, and determine whether it varies with sex,

age, dose, nuclide, radiation quality, or animal species, and (2) to locate the microscopic site of neoplastic change, estimate the actual amount of energy delivered to that site, and assess the influence of dose rate and exposure pattern on the oncogenic response.

Two experiments in this program are active at the present time. One concerns the toxicity of ^{90}Sr in beagles and the other the consequences of the exposure of mice to fission neutrons and gamma rays from the CP-5 reactor.

In the beagle experiment, exposure to ^{90}Sr was either by a single injection or multiple injections throughout the course of a year; age at the time of exposure was prenatal, neonatal, juvenile, or adult. The objectives are to compare (1) uniform versus nonuniform distribution of the dose both in time and in space, and (2) sensitivity as a function of age. Seven beagles in this study, which began in 1953, died since July 1, 1975. All were controls, 13.4 to 17.5 years of age. Their deaths were associated with heart and kidney disease, osteoarthritis, and neoplasms of stomach, mammary glands, and clitoris. Three controls are still alive. Since they are now 14 years old, all should come to autopsy within the next few years. Histopathologic study of the 143 beagles in the experiment is continuing. Present data indicate that ^{90}Sr is equally oncogenic in very young and adult animals when retention of the radionuclide is taken into account, that lymphosarcoma and myeloid leukemia can result from appropriate dosage regimens, and that, although bone cancer is the primary pathologic consequence, tumors of the central nervous system may be a more sensitive indicator of toxicity at low doses and after long periods of time.

The mouse experiment, initiated in 1957, was designed to compare the consequences of exposure to fission neutrons and gamma rays from Argonne's CP-5 reactor, a prototype of the present commercial reactors, with the effects of irradiation from internally deposited radionuclides. This study has remained dormant because other experiments had higher priority and personnel were not available to study the histopathologic material. The study has now been resumed, and analysis of the data is underway.

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EARLY AND LATE EFFECTS OF ENERGY-RELATED POLLUTANTS ON DEVELOPMENT OF THE IMMUNE RESPONSE

Bernard N. Jaroslow, Suzanne S. Dornfeld, and Katherine M. Suhrbier

This research program is directed toward detecting and describing functional derangements of immunologically reactive cells that may be induced by energy-related pollutants, and determining the developmental stage at which such derangements occur. Mice are exposed to the pollutant under investigation at immunologically important developmental stages. Cell-mediated and

humoral immunity are measured *in vivo* and *in vitro*, and the effects of the test material on the appearance and disappearance of developmental subsets of immunocytes are determined.

Polycyclic hydrocarbons, such as benzo(a)pyrene (BaP) and methylcholanthrene (MCA), are common carcinogenic energy-related pollutants. More than 1300 tons of BaP are released each year by industrial combustion processes (Particulate Polycyclic Organic Matter, Committee Report to NRC, National Academy of Sciences, Wash., 1972). These carcinogens are well-known immunodepressants (Baldwin, R. W., Adv. Cancer Res. 18, 1, 1973). Although their immunosuppressant action is known to occur soon after exposure, nothing is known of their late effects on the immune response, nor is it known whether the immunosuppressive and carcinogenic roles of these pollutants are related. Animals that are immunologically suppressed by MCA are more likely to accept tumor transplants than nonsuppressed animals (Stjernswärd, J., J. Nat. Cancer Inst. 37, 505, 1966), but this observation does not demonstrate a causal relationship between immunologic suppression and spontaneous tumor induction.

We are studying the effects of carcinogenic polycyclic hydrocarbons on the immune competence of mice treated at defined developmental stages and assayed for immunologic responsiveness at later stages throughout life. This study should identify the developmental stages that are most susceptible to the immunotoxic effects of the pollutants and thereby contribute to the basic information required for setting standards for human exposure.

Two assays of specific immune competence are being used: (1) Humoral immunity: Spleen cells from C57Bl/6 mice immunized with sheep red cells are tested for antibody-producing cells by plaque assay (Jerne, M., and A. Nordin, Science 140, 405, 1963). (2) Cell-mediated immunity: Spleen cells from C57Bl/6 mice immunized with DBA/2 mastocytoma cells are tested for their ability to kill ^{51}Cr-labeled mastocytoma cells.

Mice are treated with a wide dose range of either BaP or MCA by different routes of administration and in different vehicles. Some results are now available for mice that were immunized within 30 days of carcinogen treatment.

Humoral immunity was suppressed by BaP and MCA when these carcinogens were dissolved in oil and injected intraperitoneally or subcutaneously. However, it was not suppressed when they were injected by these two routes as an aqueous slurry, or when they were injected intravenously either in colloidal suspension in serum or enclosed in liposomes. Maximum suppression was observed 4 to 8 days after mice had been given an intraperitoneal injection of MCA dissolved in oil. The lowest dose to result in significant suppression was 2 to 10 times less than that commonly used to cause cancer. These results show that spleen cells from mice treated with MCA by intraperitoneal or subcutaneous injection in oil do not respond to antigenic stimulation as well as spleen cells from control mice.

Cell-mediated immunity was not affected by the test drugs in the same way as humoral immunity. The production of cytotoxic cells in C57Bl/6 spleens, in response to antigenic stimulation by DBA/2 mastocytoma cells, was suppressed by MCA given subcutaneously but enhanced by MCA given intraperitoneally. As with humoral immunity, the administration of the two polycyclic hydrocarbons in aqueous or liposomal suspensions had no effect, whatever the route of injection, or whether immunization was *in vivo* or *in vitro*.

As others have shown (Baldwin, R. W., Adv. Cancer Res. 18, 1, 1973), carcinogenic polycyclic hydrocarbons suppress humoral immunity when immunization is initiated shortly after hydrocarbon administration. Our results show that the route of injection and the medium in which the drug is given determine whether there is suppression, no effect, or enhancement of the two immunological assays. These investigations will ultimately help to pinpoint the particularly sensitive stages in immunologic development and will help to discriminate between the hazardous and relatively nonhazardous conditions of environmental exposure to carcinogenic pollutants.

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MAMMALIAN GENETICS

Douglas Grahn, Barbara H. Frystak, Chung H. Lee, John J. Russell, and Arthur Lindenbaum**

The expanding light-water-reactor nuclear power industry has raised concern over the potential genetic hazards of ^{239}Pu and the other transuranic elements. Plutonium is now ubiquitous as a consequence of several decades of weapons testing, an activity that has resulted in an average body burden in the United States of 5 picocuries or less (Bennett, B. F., Health and Safety Laboratory Report HASL 278, 41, 1974). Small amounts have been detected in the human gonads. Existing data from animal experiments show a moderately consistent testicular burden of about 3×10^{-4} of the initial body burden (Richmond, C., and R. L. Thomas, Health Phys. 29, 241, 1975). There are recent reports that plutonium retained in the testes will induce dominant lethal mutations, chromosome aberrations, and associated testicular damage (Searle, A. G., et al., Mutat. Res. 41, 297, 1976; Lüning, K. G., et al., Mutat. Res. 34, 539, 1976). Searle's report compared plutonium alpha particles with ^{60}Co gamma rays and gave an RBE of 22-24.

The present study directly compares the effects on several selected genetic end points of ^{60}Co gamma rays, fission neutrons ($\sim$ 1 MeV neutrons from the JANUS reactor), and alpha particles from ^{239}Pu. Evaluation of testicular uptake, retention, and alpha particle dose distribution is being done concurrently with the genetic testing. Monomeric plutonium citrate was injected intravenously into 100- to 120-day-old male $B6CF_1$/Anl mice at dose levels of 5 and 10 μCi/kg. Radiochemical and genetic assays have been performed at intervals up to 350 days after injection. Genetic tests have included the measurement of dominant lethal mutation rates, frequency of reciprocal translocations induced in spermatogonia, frequency of abnormal sperm, and changes in testis weight.

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*Therapy of Metal Poisoning Group.

DOMINANT LETHAL MUTATIONS

The frequency of prenatal mortality is detected by dissection of the pregnant uterus on the 12th to the 17th day of gestation. As 95-96% survival rate for implanted embryos is normal, reductions from this value are attributed to dominant lethal mutations. The mutation rate, μ, is estimated from the first term of the Poisson distribution, $e^{-\mu}$, which is set equal to the probability of survival (i.e., no lethal genetic damage), namely:

$$\mu = -\ln \left[\frac{\text{live fetuses/total implants (irradiated)}}{\text{live fetuses/total implants (control)}} \right].$$

Estimates of number of lethal mutations per gamete per rad for single exposures to ^{60}Co gamma rays and neutrons, equal weekly fractions (up to 24 weeks) of gamma rays and neutrons, continuous (22.5 hour/day) gamma irradiation, and continuous plutonium alpha irradition have been made. The mutation rate for cells in premeiotic stages at the time of irradiation is related to total accumulated dose, whereas the rate for postmeiotic cells is related to the dose accumulated during the last 4 weeks before mating. The dominant lethal mutation rate data are summarized in Table 7.2.

Table 7.2. Dominant Lethal Mutation Rates per Gamete per Rad (D)[a]

Exposure Condition	Postmeiotic		Mixed	Premeiotic	
	γ-rays	neutrons	Pu-α	γ-rays	neutrons
	($\times 10^{-4}$)		($\times 10^{-4}$)	($\times 10^{-4}$)	
Single	10 ± 1	54 ± 4	-	1.0 ± 0.3	3.7 ± 1.1
Weekly	11 ± 1	103 ± 22	-	0.36 ± 0.10	3.0 ± 1.1
Continuous	$2.1D + .02D^2$	-	45 ± ?	0.33 ± 0.19	-

[a]All mutation rates are linear functions of dose except for the postmeiotic germ cells under continuous gamma irradiation.

Although the mutation rate for plutonium is the result of irradiating cells in various stages of differentiation, most of the mutation rate is probably due to the irradiation of postmeiotic cells. The rate for plutonium approximates the rate for fission neutrons. The RBE for plutonium compared to single or fractionated gamma-ray exposures is about 5 to 1, and, compared to continuous gamma-ray exposure, is about 20 to 1. Dose rates for the compared conditions are: single exposure gamma rays, 45 rad/min; fractionated exposure gamma rays, 0.2-2 rad/min; continuous exposure gamma rays, 0.003-0.005 rad/min; plutonium alpha particles, 0.001 rad/min.

CYTOGENETIC ANALYSES

Suspensions of the spermatogenic elements were prepared according to the method of E. P. Evans et al. (Cytogenetics 3, 289, 1964), and the stained slides were examined for chromosome aberrations in the first meiotic metaphase plates. The frequency of cells with reciprocal translocations increased linearly after single, fractionated, or continuous gamma radiation at the approximate rates per rad of 18×10^{-5}, 7×10^{-5}, and 1×10^{-5}, respectively. Neutron-induced aberrations increased linearly to about 40 rad of single exposure, then remained at a roughly constant level up to 160 rad. Following fractionation exposures during 6 weeks or more, the frequency of cells with translocations increased linearly with a slope of 64 ± 5 ($\times 10^{-5}$) up to a dose of 120 rad. Mice carrying plutonium burdens showed a rate of about 60×10^{-5} for doses up to 20 rad, a result comparable to that of fission neutrons. One datum point at about 50 rad fell far below linear expectations. The RBE values for alpha particles to gamma rays vary with dose rate, and the maximum ratio may be greater than 50 at the lowest doses and dose rates tested.

DOSIMETRY STUDIES

Routine autoradiographic and histological techniques have been employed for the microdistribution, retention, and dose estimate studies. Intravenously injected monomeric ^{239}Pu citrate rapidly becomes located in the interstitial tissues of the male gonad and is concentrated in part along the basement membrane of the spermatogenic tubules. The total amount in the testis was approximately 0.05% of the whole body burden 6 days after injection, and remained constant over a 1-year period. As testis weight declined steadily during the year, the average dose to the whole gonad rose steadily from about 0.14 rad/day to about 0.2 rad/day.

About one half of the alpha tracks were in the interstitial tissue and the rest were in the tubules, mostly along the basement membrane. About 85% of the gonad was free of radiation exposure. The dose to spermatogonial stem cells was estimated to be 2.5 to 3 times higher than the average testicular dose, and the dose to interstitial tissue was about 15 times higher.

At 1 year there appeared to be some aggregation of plutonium into the glycolipid droplets commonly seen in interstitial cells. This type of relocation would reduce the irradiation of spermatogonial elements.

A collaborative experiment was initiated in December 1976, with W. L. Russell of Oak Ridge National Laboratory; 10 μCi/kg monomeric plutonium citrate, prepared by A. Lindenbaum, was injected into Oak Ridge C3H × 101 F_1 male mice in order to carry out a specific locus mutation-rate study. Several dozen untreated Oak Ridge mice were brought to ANL so that their response to 10 μCi/kg can be compared to that of B6CF_1/Anl mice. Distribution and retention studies will be done along with limited genetic testing.

AGE DEPENDENCE OF BODY WEIGHT AND LINEAR DIMENSIONS IN ADULT *Mus* AND *Peromyscus*[*]

P. H. Duffy and G. A. Sacher

Head plus body length (HBL), tail length (TL) and body weight (BW) were measured on two species of small myomorph rodents, *Mus musculus* and *Peromyscus leucopus*, throughout adult life. Both sexes of *Mus* show about a 15 per cent increase of HBL and TL between 5 and 24 months of age, and a BW increase of about 47 per cent. There is no age-trend of HBL in either sex of *Peromyscus* between 5 and 67 months of age, while TL increases about 7 per cent. In both species, cessation of skeletal growth is followed by a steady decrement of body mass, which averages about 0.23 per cent per month throughout adult life for the two sexes of *Peromyscus*, and over two per cent per month after cessation of skeletal growth in *Mus*. The weight loss in *Mus* in the last half of the lifespan is shown to be associated with age, rather than with the duration of subsequent survival. This is evidence against the hypothesis that the late weight loss in *Mus* is due to terminal disease.

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[*]Abstract of a paper published in Growth 40, 19 (1976).

DUAL CHANNEL TEMPERATURE RECORDER[*]

William J. Eisler and Donald A. LeBuis

A dual channel temperature recorder is described which can measure temperatures linearly over a range of 0 to 80 C. The sensitivity can be adjusted so that any temperature span, as small as 3°, will cover recorder full scale, and temperatures can be read to 0.1°. The recorder provides two independent, permanent records of temperature variations which can be related directly to the time of day.

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[*]Abstract of a paper published in Appl. Microbiol. 30, 746 (1975).

PLUTONIUM INCORPORATION THROUGH INGESTION BY YOUNG ANIMALS*

Miriam P. Finkel and W. E. Kisieleski

Studies to determine whether animals nursed by dams with a ^{239}Pu burden would themselves acquire plutonium showed that rats incorporated about 0.019% of the amount injected into the dam, mice incorporated about 0.11%, and cats about 0.28%. Plutonium obtained in this fashion was avidly retained by bone and resulted in the appearance of two osteogenic sarcomas in a seven and one-half-year-old cat with an estimated terminal body burden of 0.23-0.27 μCi. In comparing the incorporation of ingested Pu-milk and Pu-citrate by rats of different ages, it was found that nurslings incorporated more than weanlings and weanlings more than adults. Also, 1.6 to 3 times as much plutonium was incorporated from ingested Pu-milk as from ingested Pu-citrate.

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*Abstract of a paper published in The Health Effects of Plutonium and Radium, Ed. W. S. S. Jee. The J. W. Press, Salt Lake City, pp. 57-69, 1976.

PATHOGENESIS OF RADIATION AND VIRUS-INDUCED BONE TUMORS*

M. P. Finkel, C. A. Reilly, Jr., and B. O. Biskis

Bone cancer can be induced by radionuclides that localize in the skeleton. Histologically, these experimentally induced tumors resemble those found naturally in man; they range from densely ossified osteogenic sarcomas to osteolytic tumors with giant cells and only a small osteoid component. Fibrosarcomas and hemangiosarcomas also can occur in some species. It has not been possible to determine the dose in terms of absorbed energy necessary for bone-tumor induction because radionuclides are not deposited uniformly, and they diminish in amount with time. Also the precise time when irreversible neoplastic change occurs is not known. With X-rays, however, 500 rads delivered to the endosteal surface of a mouse femur has been shown to cause osteogenic sarcoma.

Bone tumors can be induced in mice by viruses. FBJ osteosarcoma virus and RFB osteoma virus were obtained from spontaneous tumors; FBR osteosarcoma virus came from a radiation-induced tumor. All three are RNA viruses with C-type particle morphology, and they are propagated by injecting cell-free extracts of virus-induced tumor into newborn mice. Interaction studies with bone-seeking radionuclides and these viruses have led to the hypothesis that radiation produces cancer by inactivating a viral inhibitor.

There is also evidence of a bone tumor virus in the human disease. The injection of cell-free extracts of human bone cancer into newborn Syrian

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*Summary of a paper published in Recent Results in Cancer Research, Vol. 54, Ed. E. Grundmann. Springer-Verlag, Berlin, pp. 92-103, 1976.

hamsters has induced a variety of mesenchymal tumors at a rate significantly higher than in the control hamsters. Sixty tumors of this type, including 20 osteosarcomas, 11 fibrosarcomas, and 9 osteomas, have been diagnosed so far in experimental animals; in control hamsters there has been only one, a fibrosarcoma. Immunofluorescence assays and cytotoxicity studies indicated that these hamster tumors carried a human antigen.

COST-BENEFIT AS WEIGHED ON GENETIC SCALES*

Douglas Grahn

The genetic cost that may be incurred by exposure to mutagenic agents in the coal and nuclear fuel cycles is assessed, using as a point of departure the presently estimated burden of spontaneously occurring genetic defects in human populations. Risk estimates are necessarily derived from radiation studies, but chemical mutagenic hazards can probably be evaluated relative to the known dose-response relationships of radiation exposure. Cost-benefit analyses for the coal and nuclear fuel cycles are discussed and translated into monetary terms. Coal-associated risks are almost entirely somatic while nuclear risks are somatic and genetic in equal proportions. Dollar costs per man-rem are concluded to be in the $100 range. Pollution abatement costs for the nuclear cycle lie in the range of several hundreds to many thousands of dollars per man-rem reduction. It is considered appropriate to incur such costs, because genetic risks to future generations involve primarily societal and ethical issues rather than economic considerations.

*Abstract of a paper published in Energy and the Environment - Cost-Benefit Analysis, Eds. R. A. Karam and K. Z. Morgan. Pergamon Press, New York, pp. 371-386, 1976.

RADIOSENSITIVITY OF ILEUM CRYPT CELLS IN HIBERNATING, AROUSING, AND AWAKE GROUND SQUIRRELS (*Citellus tridecemlineatus*)*

Bernard N. Jaroslow, R. J. Michael Fry, Katherine M. Suhrbier, and Anthony R. Sallese

Radiosensitivity of ileal crypt cells, to ^{60}Co gamma-radiation, was studied in ground squirrels (*Citellus tridecemlineatus*) during hibernation, arousal, and the euthermic state. Survival of ileal crypt cells, assayed by the microcolony technique from stained transverse sections of ileum, was greater in animals irradiated in hibernation or 1 hr after initiation of

*Abstract of a paper published in Radiat. Res. 66, 566 (1976).

arousal from hibernation. Crypt survival returned to the level of irradiated nonhibernating controls in animals irradiated 3-7 hr after initiation of arousal. Over the exposure range of 1500 to 2400 R, the survival of crypt cells for euthermic controls gave a D_0 = 133 ± 12 R and for animals irradiated in hibernation it gave a D_0 = 487 ± 92 R. In animals irradiated 1 hr after initiation of arousal, when core temperature is within the range of euthermic controls, crypt survival was almost as high as in the hibernators. These results suggest that the increased resistance of ileal crypt cells in hibernating animals could be due to hypoxia, although no direct evidence for hypoxia in hibernation was established. The changes in mitotic index of ileal crypt cells during hibernation and arousal indicate an alteration in the distribution of cells in the phases of the cycle. This change in distribution may also have contributed to the increased radioresistance of hibernators.

SUPPRESSION AND ENHANCEMENT OF THE IMMUNE RESPONSE IN CULTURE BY PRODUCTS OF A LYMPHOMA*

Bernard N. Jaroslow and Katherine M. Suhrbier

Several tumors produce separate factors that suppress or enhance production of antibody-forming cells in mouse spleen cell cultures stimulated with sheep red cell antigens. One million L-1V lymphoma cells, added to a culture of 20 million spleen cells on the first day, suppresses the response to sheep red cells. If they are added 24 or 48 hours later, when induction is complete, the production of antibody-forming cells is normal. An aqueous extract of these cells enhances the immune response whether it is added before, with, or as late as 72 hours after antigen. The active factor appears to be a nondialyzable protein. Immunoenhancement is also seen when one million mitomycin C-treated L-1V lymphoma and P815 mastocytoma cells are added to cultures of allo- as well as isogenic spleen cells.

*Abstract of a paper submitted for publication.

DOSE, DOSE RATE, RADIATION QUALITY, AND HOST FACTORS FOR RADIATION-INDUCED LIFE SHORTENING*

George A. Sacher

The salient phenomena of life shortening in mammals due to ionizing radiations can be summarized as follows:

*Summary of a paper published in Aging, Carcinogenesis, and Radiation Biology, Ed. K. C. Smith. Plenum Press, New York, pp. 493-517, 1976.

i. Actuarial analysis of the effects of single exposure and lifetime exposure to gamma rays and fast neutrons leads to the conclusion that radiation injury does not "accelerate" the aging process, but rather is superimposed additively on the aging injury, which continues to accumulate much as it does in the untreated population.

ii. Ionizing radiation leaves a residue of irreparable tissue damage, so that each increment of dose causes a permanent increase by a constant factor in the death rate.

iii. The life shortening, for mice and rats given single doses of low-LET radiation, estimated either by the decrease of life expectation, $\Delta E(D)$, or by the increase of Gompertz function intercept, $\Delta G(D)$, is a quadratic function of dose.

iv. In mammals given daily duration-of-life exposure to low-LET radiations the increase of Gompertz function slope or increase of mean radiation-specific mortality rate is proportional to the daily dose at low daily doses, then shifts to the square of daily dose at higher daily doses, and decreases again toward a first-power dependence on daily dose for daily doses in the range of hundreds of rad/day. This means that the effectiveness per rad is constant at low daily doses, increases over an intermediate range, then approaches constancy again at very high daily doses.

v. The effectiveness per rad for fast neutrons is almost constant, but has a weak dependence on dose and fractionation.

vi. The effectiveness per rad for life shortening by fractionated exposures to a fixed total dose of low-LET radiation decreases with increase in the number of fractions, or interval between fractions, or total elapsed exposure time.

These phenomena can be accounted for by a simple and consistent mathematical model. It is noteworthy that the kinetics of life shortening consistently show a dependence of effect on the square of the dose, whereas the data on killing of cultured mammalian cells show a considerably greater degree of variability in dose-dependence. The reason for this difference is not known, but the facts justify serious consideration for the hypothesis that the quadratic dose dependence for irreparable injury arises from chromosome aberrations.

EVALUATION OF THE ENTROPY AND INFORMATION TERMS GOVERNING MAMMALIAN LONGEVITY*

George A. Sacher

Investigation of the constitutional factors in mammalian longevity was initiated by Rubner and Friedenthal, and taken up again by Sacher. The most recently completed phase of this research, which examines the dependence of life span on four constitutional variables, body weight, brain weight, specific metabolic rate and deep body temperature, is reported here. The analysis of these relations leads to theoretical considerations that open a new approach to the thermodynamic analysis of the living state, and that have implications for the direction of research in molecular gerontology.

The relationships established here contradict some current theories of the mechanisms of aging, and should, therefore, lead to the abandonment of some unprofitable research. As the interdependence among the molar parameters becomes progressively better known, the constraints on admissable mechanistic theories will increase, and the goal of a unified molecular-molar theory of longevity and aging will be brought nearer.

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*Summary of a paper published in Interdiscip. Top. Gerontol. 9, 69 (1976).

LONGEVITY IN VERTEBRATES: A FURTHER COMMENT*

George A. Sacher

It is possible that the prolongation of life that is required by increase of brain size is achieved by mechanisms not related to brain function. Genetic selection for increased activity of enzymes for DNA repair is one possible mechanism. This hypothesis is supported by the demonstration of a high correlation of the life-span of mammalian species from shrew to man with the activity of the enzyme system for excision repair of thymidine dimers. Other genetically controlled mechanisms for determining the different aging rates of the cells of mammalian species will assuredly be discovered when biogerontologists take up Hayflick's suggestion for comparative analysis of the aging processes in cultured cells from species with different somatic life-spans.

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*Conclusion of a Letter published in Fed. Proc. 35, 1112 (1976).

POSITIVE ASSOCIATION BETWEEN TUMOR INCIDENCE AND SURVIVAL TIME IN MICE AND RATS GIVEN LIFETIME EXPOSURE TO VARIOUS TRACE ELEMENTS: A REVIEW OF PUBLISHED DATA*

George A. Sacher

Published data of Schroeder and his colleagues on the effects of low-level lifetime administration of 31 different trace elements or valence states on survival and tumor incidence in male and female mice and rats is examined synoptically. Significant increase of tumor incidence was observed in several of the 95 treatment groups, but significant decreases were observed also, and increase as well as decrease of survival time was noted. There is an apparent opposition between mortality from tumors and mortality from other causes: increased tumor incidence is significantly associated with increased survival time, and decreased tumor incidence with decreased survival time.

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*Summary of a paper published in The Biomedical Role of Trace Elements in Aging, Eds. J. M. Hsu, R. L. Davis, and R. W. Neithamer. Eckerd College Gerontology Center, pp. 237-248, 1976.

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8. THERAPY OF METAL POISONING

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ANL 64100
ANL 64102

GROUP LEADER'S INTRODUCTION

Arthur Lindenbaum, Group Leader

The overall objective of this program is the development of methods for decorporation of toxic metals whose compounds deposit primarily in the skeleton and liver. In the prosecution of this work it is recognized as a fundamental principle that the development of effective therapeutic procedures is dependent upon results from parallel physiological experiments and morphological observations designed to elucidate the interactions between mammalian tissues and the toxic metal compounds under study. Accordingly, three secondary research objectives are presently being pursued: (1) Biochemical and physicochemical studies *in vitro*, related to the interactions of toxic metals and/or therapeutic substances with tissue components. (2) Morphological studies, including autoradiography and histology, designed to demonstrate (a) gross and microscopic localization of toxic metals in living tissues, (b) the pathological sequelae of toxic metal retention, and (c) the possible reversal of such sequelae by treatment. (3) Generation of metabolic and therapeutic data regarding the interactions of toxic metals with small living animals, with special attention toward applicability to man. Because of the high radiotoxicity of plutonium, which in many respects is prototypical of other actinides and nonradioactive toxic heavy metals, our present research efforts are mainly, but not exclusively, directed toward this radionuclide.

Significant advances toward the above research goals over the past year are described in the following section. The results may be summarized as follows:

1) One of a new group of therapeutic compounds, EMH-217, has been shown, in mice, to be the most effective adjunct substance yet tested with diethylenetriaminepentaacetic acid (DTPA) for removal of long-retained polymeric plutonium-239 from the liver.

2) We have shown in the mouse, by means of plutonium isotopes of widely differing masses, but injected at equal radioactivity levels, that metabolic results obtained with relatively high levels of plutonium, as used in

experimental animals, are probably valid for cases (contamination in man) in which the plutonium burden may be around the level of the maximum permissible body burden.

3) We have presented evidence for the applicability of a new method for estimating the burden of plutonium in the liver and skeleton in a variety of mammalian species. The method also may be applicable to man.

4) In determining that DTPA-induced removal of monomeric plutonium from the mouse and dog liver is mainly *via* the bile, we have determined that DTPA also undergoes biliary excretion in intact form, probably in part as the plutonium complex.

5) A new cooperative program on genetic effects of high-LET radiation is now underway (see Section 7 on Pathology and Risk Assessment). We are presently investigating the effects of gonadal retention of plutonium and other actinides in mice. Early results show monomeric plutonium to undergo early and prolonged retention at the basement membrane of the seminiferous tubules of the testis; sperm abnormalities and reduction in sperm numbers have been noted.

One less positive development this year was the loss of financial support for a new program directed toward nonradioactive toxic metals of environmental interest. Some of this work (on lead) has been incorporated into the present program.

THERAPY OF METAL POISONING STAFF

REGULAR STAFF

Bhattacharyya, Maryka H. (Assistant Biochemist)
Lindenbaum, Arthur (Biochemist)
Moretti, Elizabeth S. (Scientific Assistant)
Peterson, David P. (Scientific Assistant)
Russell, John J. (Scientific Associate)

TEMPORARY STAFF DURING 1976

Guilmette, Raymond A. (Postdoctoral Appointee)
Sorensen, Elsie M. (Postdoctoral Appointee)

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ERDA RT-01-03
ANL 64100
ANL 64102

THERAPY OF POISONING BY RADIOACTIVE AND NONRADIOACTIVE METALS

Arthur Lindenbaum, Maryka H. Bhattacharyya, Raymond A. Guilmette, Elsie M. Sorensen, Elizabeth S. Moretti, David P. Peterson, John J. Russell, Marian H. Badorski, Frank Chao, and Valerie Riotte†*

DECORPORATION OF POLYMERIC PLUTONIUM

Polyanionic copolymers, used as adjuncts to the chelating agent diethylenetriaminepentaacetic acid (DTPA), continue to show promise for decorporation of long-retained deposits of plutonium not removable by chelation alone. Two such compounds have been shown to be particularly effective, when given with DTPA, in removing polymeric plutonium from mouse liver. One of these is the divinyl ether-maleic anhydride pyran copolymer XA 146-85-2 (now being used clinically as an antitumor, antiviral drug); the other is an acrylic acid-isobutyl vinyl ether copolymer, designated EMH-217 as prepared for us by Prof. E. M. Hodnett, Department of Chemistry, Oklahoma State University.

Other recent studies have aimed at identification of the structural characteristics relevant to the therapeutic action of the polyanionic copolymers with respect to plutonium. Preliminary results indicate the need for a rather large molecule. The most effective compound to date, EMH-217, has a molecular weight of 250,000, whereas compounds of mol wt $\leq$ 5200 were ineffective. Within limits, the effectiveness of the copolymers so far tested tends to increase as the degree of lipid solubility increases. However, the toxicity of the compound also increases with lipophilicity.

EFFECT OF PLUTONIUM MASS ON ITS DISTRIBUTION IN MOUSE TISSUES

In an attempt to evaluate the role of injected mass of soluble plutonium on its initial distribution and subsequent retention in the mouse, we have used four alpha-emitting isotopes of plutonium--236, 239, 242, 244--whose specific activities differ by more than 10^7. (The ^{236}Pu, ^{242}Pu, and ^{244}Pu were supplied through the courtesy of A. M. Friedman of the Chemistry Division.) The injected masses of these isotopes, which were administered at approximately equal activity levels, ranged from 0.2 to 10^5 times the maximum permissible body burden (MPBB) of ^{239}Pu set for man (9 ng/kg). Up to 100 days, values for the initial deposition and retention of all plutonium isotopes were similar for liver and nearly so for kidneys. The initial deposition of

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*Summer 1976 participant in the Undergraduate Honors Research Participation Program, University of Illinois, Urbana.

†Fall 1976 participant in the Undergraduate Honors Research Participation Program, Rosary College.

the isotopes with the highest injected mass, $^{242,244}Pu$, however, was consistently higher in other soft tissues (lymph nodes, lung, spleen, bone marrow) than the isotopes of lowest mass, ^{236}Pu and ^{239}Pu. The similarities in tissue distribution of the isotopes ^{236}Pu and ^{239}Pu (injected mass equivalents of 0.2 x MPBB and 1800 x MPBB, respectively) indicate that metabolic and therapeutic results obtained in the laboratory with soluble plutonium at levels far in excess of the MPBB probably are applicable to cases of actual actinide contamination in man. In contrast, the results obtained with ^{242}Pu (28,000 x MPBB) and ^{244}Pu (100,000 x MPBB) suggest that extrapolation upward to very large masses of plutonium may not be similarly valid. The implications of these studies for other physical and chemical forms of plutonium, as well as for isotopic mixtures (as in reactor grade plutonium), remain to be studied.

INTERACTIONS OF MONOMERIC PLUTONIUM WITH THE LIVER AND SKELETON AS RELATED TO DECORPORATION

The objectives of these studies are (1) to study the biochemical interactions of monomeric plutonium with the two main organs of the body that take up plutonium, liver and skeleton, and (2) to make use of the knowledge thus obtained to aid in better diagnosis and treatment of cases of accidental exposure to actinides in man.

Experiments were carried out to establish a new method for estimation of skeletal plutonium levels. The method is based on the finding that, in the mouse, the amount of plutonium appearing in the feces following DTPA treatment equals the amount of plutonium lost from the liver during that time (Schubert, J., et al., Radiat. Res. 15, 220, 1961). Using the assumptions (1) that the burdens of plutonium in liver and skeleton occur in predictable ratios, and (2) that fecal excretion of plutonium following DTPA therapy reflects the amount of plutonium in the liver at the start of therapy, it should be possible to estimate the skeletal burden of plutonium in mouse from the amount in the feces after DTPA treatment. In the case of DTPA treatment begun at 1 hour, the ratio of plutonium in the skeleton at 1 hour, S, to DTPA-induced fecal excretion of plutonium, F, was found to be 1.08 ± 0.03. When DTPA treatment was begun at 24 hours, the corresponding S/F ratio was 1.68 ± 0.08. In the mouse, therefore, a reasonable estimate of plutonium in the skeleton just prior to treatment can be determined from the cumulative amount measured in the feces for 6 days following DTPA therapy. Application of this estimation method to other species and to cases of accidental exposure in man is being investigated.

In addition, the mechanism of removal of plutonium from the liver in response to DTPA treatment was studied. The amount of plutonium measured in the bile of bile duct-cannulated rats following DTPA injection was 98% of the amount of plutonium lost from the liver following DTPA treatment. Thus, DTPA brings about the excretion of plutonium from the liver into the bile, accounting for the appearance of the actinide in the feces during DTPA therapy. Additional results obtained using ^{14}C-labeled DTPA support this biliary mechanism for DTPA-induced plutonium removal from the liver: When ^{14}C-labeled DTPA was injected into bile duct-cannulated rats, a small fraction of the injected radioactivity was excreted into the bile and was

identified by anion exchange column chromatography to be unchanged DTPA. Thus, the plutonium leaving the liver appears to be removed in the form of the DTPA complex.

Experiments were also conducted to determine whether diet-induced loss of bone mineral from the skeleton would result in concomitant removal of skeletal plutonium. Young-adult plutonium-injected mice were subjected to a diet that was low in phosphate. By 6 weeks the mineral content of the lumbar vertebrae (L1-L5) was 15% lower than the mineral content of lumbar vertebrae from control mice fed the same diet plus added phosphate. In addition, the mean plutonium content of these vertebrae was 14% lower than in the control mice. Thus, the loss of bone mineral appears to have produced a concomitant loss of skeletal plutonium. These positive results in mice suggest the need for application of this treatment procedure to another species, preferably a primate.

TISSUE DISTRIBUTION AND TOXICITY OF LEAD COMPOUNDS

The purposes of this research are (1) to produce consistently elevated organ burdens of toxic nonradioactive metals in mice; (2) to identify toxicologic responses (e.g., functional, hematological, histological, and ultrastructural) to these organ burdens; and (3) to establish the efficacy of therapeutic procedures and/or substances for toxic metal decorporation and reversal of toxicological responses.

In an effort to establish the therapeutic efficacy of $CaNa_3DTPA$ in removal of lead injected intraperitoneally as the acetate (1 mg/kg), three daily intraperitoneal injections of 125 mg/kg $CaNa_3DTPA$ were initiated, beginning 1 day following lead injection. Despite the low organ uptakes of lead, the production of peritoneal adhesions, and high biological variability inherent in this experimental model, mean lead burdens in the kidney, brain, and skeleton were lower than in untreated mice.

Optical and electron microscopic observations of mouse liver have demonstrated the presence of nuclear lead inclusions characteristic of lead poisoning. Low resolution autoradiographic data and X-ray energy dispersive analysis data are being used in localization and transport studies. Although no hind limb paralysis, neuromuscular impairment, or encephalopathy-like symptoms were observed, a gamut of somewhat variable hematological responses was found; these included basophilic stippling, polychromasia, and morphological abnormalities of erythrocytes. Such findings indicate that this mouse strain ($B6CF_1$) can be used primarily for cytological (and possibly hematological) studies, but not for functional tests of neuromuscular involvement. These data will be used to provide baselines for study of the efficacy of other therapeutic agents.

GENETIC EFFECTS OF PLUTONIUM DEPOSITION IN MOUSE TESTIS

New cooperative studies of genetic effects of high LET irradiation are being carried out with D. Grahn of the Pathology and Risk Assessment Group (dominant lethals) and with W. L. Russell of Oak Ridge National Laboratory (specific locus studies). To ensure carryover of genetic information

obtained with different experimental approaches, we are comparing the tissue deposition of monomeric plutonium, the gonadal microdeposition of plutonium, and the consequent production of cytogenetic abnormalities in the two strains of mice ($B6CF_1$/Anl and C3H × 101) being used for these studies. (For additional descriptions of this work see Section 7 of this report.)

New results with the $B6CF_1$/Anl strain include: (1) a 30% reduction in testis weight 1 year after intravenous injection of 10 μCi/kg of monomeric ^{239}Pu; (2) some evidence of aggregation of plutonium over this time span, e.g., autoradiographic appearance of "hot-spots" associated with glycolipid-positive areas in the testis.

Based on our previous evidence that two effects of retained plutonium in mice (life shortening and production of osteogenic sarcoma) can be reversed by DTPA-induced decorporation of plutonium, an experiment now in progress is designed to test whether the testicular burden of plutonium can be reduced by DTPA, and whether this would provide a concomitant reduction in the genetic risk. This work is a radiochemical and autoradiographic interspecies comparison in the mouse and dog of the effects of twice weekly treatments with DTPA for 3 months on the testicular burdens of plutonium when treatment is begun early (6 hours after plutonium administration) or is delayed for 6 days.

OTHER AUTORADIOGRAPHIC AND HISTOLOGIC STUDIES

Quantitative autoradiography was used to compare the deposition patterns in mouse liver of four plutonium isotopes, ^{236}Pu, ^{239}Pu, ^{242}Pu, and ^{244}Pu, of widely differing masses (*vide supra*). Confirming the radiochemical results, the alpha track distribution at both 4 and 40 days was essentially the same for all four isotopes. Nearly 60% of the tracks were associated with liver parenchymal cells.

Other studies were carried out with livers and spleens of plutonium-injected mice that subsequently received adjunct therapy with pyran copolymers or their analogs, the EMH compounds (*vide supra*). After pyran treatment, the livers contained numerous foci of reticuloendothelial (RE) cells, whereas there was less RE proliferation after administration of EMH compounds. In contrast, the spleens of pyran-treated mice contained very few deposits of hemosiderin, whereas after treatment with EMH compounds (except EMH-250) the spleens contained numerous hemosiderin deposits; these were associated with alpha tracks.

DIFFERENCES IN EARLY RETENTION OF LEAD ACETATE AND LEAD CITRATE IN MOUSE TISSUES*

David W. Baxter, Nancy G. Doan, and Arthur Lindenbaum

Lead acetate (73% ultrafilterable) or lead citrate (93% ultrafilterable) containing tracer amounts of ^{210}Pb was intravenously injected (1 mg/kg at pH 5.1) into 85 day old $B6CF_1/Anl$ female mice, and a comparison of lead accumulation and rate of loss from liver, spleen, kidney, femur, lung, brain, and blood was made at intervals up to 14 days. At 1 hr, mice injected with lead acetate accumulated 51.1 percent of the injected dose per gram (% ID/g) in the liver and 18.4% ID/g in the spleen, while mice injected with lead citrate contained 17.6% ID/g in the liver and 3.6% ID/g in the spleen. In contrast, animals injected with lead acetate accumulated lower levels of lead in kidney and femur at 1 hr than were found in animals injected with lead citrate: kidneys contained 35.3% ID/g following lead acetate injection and 63.4% ID/g following lead citrate injection, while femurs contained 11.3% ID/g after lead acetate and 22.5% ID/g after lead citrate. Animals given lead acetate tended to accumulate more lead in lung and less in brain and blood at 1 hr than those given lead citrate. Between 1 hr and 14 days, the loss of lead was greater from liver and spleen following lead acetate, but greater from kidney following lead citrate. These data, plus additional ultrafiltration results, indicate that the distribution and kinetics of lead in living tissues are influenced by physical factors such as particulate size of the administered substance. The data also suggest that lead acetate undergoes hydrolysis in blood and is more rapidly converted to the particulate state than lead which is administered as the more stable citrate complex.

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*Abstract of a paper to be published in the Proceedings of the 15th Annual Hanford Life Science Symposium, Biological Implications of Metals in the Environment, Richland, WA, Sept. 29-Oct. 1, 1975.

ASSOCIATION OF PLUTONIUM WITH ISOLATED LIVER PARENCHYMAL CELLS FOLLOWING INJECTION OF MONOMERIC PLUTONIUM INTO MICE*

Maryka H. Bhattacharyya and Arthur Lindenbaum

The early kinetics of the association of plutonium with liver parenchymal cells was measured after intravenous injection of monomeric plutonium [^{239}Pu(IV)citrate] into CF 1 female mice. The amount of plutonium associated with isolated liver parenchymal cells and the total liver plutonium concentration were determined at times ranging from 1 hr to 6 days after plutonium administration. The number of parenchymal cells per gram mouse liver as well as the ratios of parenchymal cell-associated plutonium (DPM/isolated cell) to

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*Abstract of a paper published in Radiat. Res. 66, 552 (1976).

total plutonium concentration (DPM/g liver) were calculated in order to determine the percentage of the liver burden associated with parenchymal cells. Up to 6 days, the concentration of monomeric plutonium associated with isolated mouse liver parenchymal cells increased more slowly than the total liver plutonium concentration. The latter reached a maximum by 5 hr and decreased only slightly by 6 days. In contrast, the fraction of the liver burden associated with parenchymal cells increased from 29% at 5 hr to 70% at 6 days after plutonium administration.

MONOMERIC PLUTONIUM AND MOUSE LIVER PARENCHYMAL CELLS: DEPOSITION AND DTPA-INDUCED REMOVAL*

Maryka H. Bhattacharyya and A. Lindenbaum

The amount of plutonium associated with isolated liver parenchymal cells and the total liver plutonium concentration were determined at times ranging from one hour to six days after a single intravenous injection of 4.8 μCi/kg of monomeric ^{239}Pu(IV)-citrate (Pu-M) into ∿ 100-day-old CF 1 female mice. Pu-M gradually accumulated in the parenchymal cells of the liver; over this time the parenchymal cell plutonium accounted for an increasing proportion of the liver plutonium burden. The fraction of the liver burden associated with parenchymal cells increased from about 29% at five hours to about 70% at six days after plutonium administration.

In addition, the removal of plutonium from the whole liver and liver parenchymal cells was measured 6 and 24 hours after intravenous injection of DTPA (diethylenetriaminepentaacetic acid). The DTPA (250 μmole/kg) was administered 24 hours following Pu-M injection. By six hours after DTPA administration, the plutonium concentration in the intact liver had not yet decreased significantly, but there was a 25% reduction in plutonium associated with the parenchymal cells. By 24 hours, the plutonium concentration in the intact liver was reduced by 46%, as compared to a reduction of 69% in the parenchymal cells. Thus, DTPA, in stimulating the removal of Pu-M from mouse liver during the first 24 hours after injection, acted preferentially on the parenchymal cell-associated plutonium.

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*Abstract of a paper published in The Health Effects of Plutonium and Radium, Ed. W. S. S. Jee. The J. W. Press, Salt Lake City, pp. 233-243, 1976.

RAPID SPECTROPHOTOMETRIC DETERMINATION OF DIETHYLENETRIAMINEPENTAACETIC ACID (DTPA) IN URINE*

N. G. Doan, J. E. Parks, and A. Lindenbaum

A method for the quantitative determination of DTPA in urine at concentrations as low as 10^{-4} M is described. The procedure involves spectrophotometric determination of Fe(III) as ferric thiocyanate after complexation of a known excess quantity of ferric ion by DTPA. The absorbance of the ferric thiocyanate complex decreases linearly with increasing DTPA concentration. Pretreatment of urine samples with excess $BaCl_2$ and $(NH_4)_2SO_4$ removes interfering anions such as oxalate and orthophosphate without cosedimentation of DTPA. The effects of other interfering substances are discussed and a modified procedure is described for samples containing iron.

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*Summary of a paper published in Biochem. Med. 14, 220 (1975).

STUDIES OF TUMOR METABOLISM I: BY USE OF MOSSBAUER SPECTROSCOPY AND AUTORADIOGRAPHY OF ^{153}Sm*

*A. M. Friedman,† J. C. Sullivan,† S. L. Ruby,‡ A. Lindenbaum, J. J. Russell, B. J. Zabransky,‡ and G. V. S. Rayudu***

Recently, several authors have noted that a variety of metallic tracers concentrate in tumors and may be used as tumor specific diagnostic tools. The work described in this paper was performed as an initial attempt to understand the metabolic pathways (mechanism) for this concentration. Studies of the Mossbauer spectra from ^{153}Sm sources in tumor tissue show that these nuclei are in aqueous, ionic trivalent atoms probably bonded to serum proteins. Autoradiography shows that the Sm is concentrated in the viable outer layers of the tumor.

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*Abstract of a paper published in Int. J. Nucl. Med. Biol. 3, 37 (1976).

†Chemistry Division, Argonne National Laboratory.

‡Physics Division, Argonne National Laboratory.

**Rush University Medical Center, Chicago.

PROGRESS IN THE USE OF PYRAN COPOLYMERS FOR DECORPORATION OF POLYMERIC PLUTONIUM*

R. A. Guilmette and A. Lindenbaum

Previous studies have shown that polymeric forms of plutonium (Pu-P) are removed from the liver with difficulty by diethylenetriaminepentaacetic acid (DTPA). We have been able to decrease liver burdens below those attainable with DTPA alone with compounds known to alter the activity of reticuloendothelial tissues. Of special interest among the agents used are glucan, a yeast cell wall polysaccharide, and the soluble, antiviral, polyanionic pyran copolymers, the latter being condensation products of divinyl ether and maleic anhydride. Selected results are presented which show the efficacy of the pyran copolymers used as adjuncts to DTPA and some of the problems associated with their use. Differences between the action of pyran copolymers and glucan are discussed in terms of possible mechanisms of action and toxicity. The effectiveness of pyran copolymers, used with DTPA, in accelerating removal of hepatic Pu is considered in light of some of their pharmacological properties.

*Abstract of a paper published in The Health Effects of Plutonium and Radium, Ed. W. S. S. Jee. The J. W. Press, Salt Lake City, pp. 223-231, 1976.

RETENTION OF PLUTONIUM IN MOUSE TISSUES AS AFFECTED BY ANTIVIRAL COMPOUNDS AND THEIR ANALOGS*

A. Lindenbaum, M. W. Rosenthal, and R. A. Guilmette

The chelating agent DTPA (diethylenetriaminepentaacetic acid) is an effective therapeutic substance for decorporation of extracellular monomeric plutonium in the mouse and dog, but is much less effective in removing intracellular polymeric plutonium (Pu-P). In the absence of effective therapy, this intracellular plutonium is long retained in the body, particularly in reticuloendothelial tissues like the liver. Our interest, therefore, turned to the development of adjunct substances capable of removing additional plutonium from the liver beyond that removable by DTPA alone. We showed that glucan, a yeast cell wall polysaccharide, is a useful adjunct to DTPA for removal of Pu-P from the mouse liver. Its toxicity, however, makes it a less than desirable drug for potential human use. Therefore, we initiated a search for more soluble (and presumably less hazardous) therapeutic agents similar to glucan, i.e., capable of adjunct action with DTPA. Of over 20 substances tested the most successful results were obtained with two antiviral, antitumor compounds, the pyran copolymers XA-124-177 and XA-146-85-2. These are

*Abstract of a paper published in Diagnosis and Treatment of Incorporated Radionuclides. IAEA International Seminar on Diagnosis and Treatment of Incorporated Radionuclides, Vienna, Austria, Dec. 8-12, 1975. International Atomic Energy Agency, Vienna, pp. 375-372, 1976.

condensation products of divinyl ether and maleic anhydride. Another analog, EMH-227, prepared by condensation of acrylic acid and itaconic acid, was similarly successful. Maximal removal of plutonium from mouse liver was obtained with a single intravenous (I.V.) injection of 10-90 mg/kg of pyran copolymer given 5 days after I.V. Pu-P administration. Although these doses increased splenic uptake of plutonium, a dose of 10 mg/kg produced a minimal increase in the splenic burden while producing maximal removal of hepatic plutonium.

INDUCTION OF OSTEOGENIC SARCOMA BY POLYMERIC PLUTONIUM (^{239}PuIV)*

W. Stevens,† *D. R. Atherton,*† *W. S. S. Jee,*† *D. S. Buster,*† *B. J. Grube,*† *F. W. Bruenger,*† *and A. Lindenbaum*

Previous studies have demonstrated that a single I.V. injection of ^{239}PuIV in monomeric form (Pu-Cit) will induce osteogenic sarcomas in beagles. The latent period for tumor induction decreased as the injected dose increased from 0.016 to 0.9 μCi/kg ^{239}PuIV. The effect of the chemical form of the injected ^{239}Pu on its distribution within the dogs has been studied. Two dogs were injected with 0.9 μCi/kg of polymeric ^{239}Pu (Pu-P) for lifespan studies. Both of those dogs developed osteogenic sarcomas with latent periods somewhat less than those observed in animals which received 0.9 μCi ^{239}Pu/kg of monomeric plutonium. The fraction of the injected polymeric plutonium retained in the skeleton increased from 2.2% at 14 days post injection (P.I.) to 24.4% at death (1166 days average). Plutonium retention in organs with a high percentage of mononuclear phagocytes showed a significant loss of Pu-P from these organs during this period.

Dose calculations indicate that the slow release of plutonium, primarily from the liver, and the subsequent protracted and continuous deposition of the nuclide on the skeletal surfaces was much more efficient in producing osteogenic sarcoma than the deposition resulting from a single injection of monomeric plutonium.

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*Abstract of a paper published in The Health Effects of Plutonium and Radium, Ed. W. S. S. Jee. The J. W. Press, Salt Lake City, pp. 81-95, 1976. This work was also described in the Annual Report of Work in Progress in the Internal Radiation Program, Radiobiology Division of the Department of Anatomy, University of Utah College of Medicine. COO-119-250, March 31, 1975, pp. 128-137.

†University of Utah.

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9. LIPOSOMES AS BIOLOGICAL CARRIERS

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ANL 64101

GROUP LEADER'S INTRODUCTION

Yueh-Erh Rahman, Group Leader

The program concerned with liposomes as biological carriers, formerly a project within the Biochemistry Group, was transferred to the Diagnosis and Therapy Group early in 1976. In recognition of the increasing applications of liposomes, as demonstrated by the significantly increased number of publications from many laboratories during the last two years, the present group was created in October, 1976.

The delivery of a given drug specifically to a target tissue is one of the most challenging problems in the field of pharmacology. We propose to use biological carriers, namely liposomes, to achieve this selective delivery. The foundation of this delivery system is cell biology, particularly in the areas of membrane chemistry, membrane movement, and cellular enzymology. The significant progress made during recent years in these basic areas of cell biology has made possible the method we propose.

Liposomes are microscopic lipid spherules that are formed by mixing aqueous solutions of various electrolytes with lipid materials under appropriate conditions. The technique has been used in our laboratory to encapsulate within the spherules a variety of organic compounds, primarily chelating agents and antitumor drugs. Encapsulation profoundly alters the distribution, metabolism, and physiological action of these compounds.

The now existing drugs used for treatment of metal poisoning and their delivery systems have many undesirable features. These features can be classified into two major categories: (1) the high toxicity of many drugs, which limits their common use; and (2) the failure of most chelating agents to reach the toxic metals that are located inside tissue cells, so that efficient removal of the toxic metals cannot be satisfactorily obtained. Antitumor drugs used in cancer treatment are usually toxic not only to the tumor cells, but also to the fast-dividing normal cells. e.g., those in the bone marrow and the gastrointestinal mucosa.

The use of liposome encapsulation for both the chelating agents and the antitumor drugs can be expected to achieve

1) selective transport of drugs to target organs;

2) reduction of drug toxicity to normal tissues of animals; and

3) increase of therapeutic index of the drug.

In addition, liposomes used as biological carriers have the following advantages:

1) The encapsulation of a drug within liposomes protects the drug from enzymatic degradation before it reaches the critical organs.

2) Since liposomes are made of lipids, immunological reactions are not likely to be induced after repeated injections into animals.

3) Since phospholipids, usually used in liposome preparation, are common constituents of all living cells, liposomes are readily assimilable.

4) Surface characteristics of liposomes can be modified to achieve a specific tissue delivery of a given drug by varying the lipid constituents. For example, surface charge of the liposomes can be modified or specific reactive groups can be inserted on the surface of the liposomes.

5) Drugs soluble either in aqueous or in lipid media can be incorporated into liposomes. Water soluble drugs are incorporated in the center and between the lipid bilayers of the liposomes, and lipid soluble drugs as part of the liposomal membrane.

There are two major mechanisms by which liposomes interact with cells: by fusion with the plasma membrane of the tissue cells that have phagocytic capacity, and by endocytosis. The physical state of lipids in the liposomes is the major factor in determining which of the above two mechanisms would predominate. The use of liposomes as biological carriers can therefore achieve the following two objectives: (1) modification of a tissue cell surface by inserting portions of a liposomal lipid; and (2) introduction of materials entrapped within liposomes into a target cell. Our program so far has emphasized the latter objective; in the future, however, we plan to include studies on cell surface modification by fusion between cells and liposomes.

We have used liposomes to encapsulate metal-chelating agents, specifically ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). Liposome-encapsulated DTPA has been successful in mice in removing various intracellularly deposited toxic metals (e.g., plutonium, lead, and inorganic mercury) that are not readily removable by the usual nonencapsulated DTPA, which cannot cross cellular membranes. The liposome-encapsulated chelating agents also have a remarkably different tissue disposition compared to that of the nonencapsulated, free form of the chelating agent. Electron microscopic studies have shown that liposomes containing EDTA are rapidly taken up not only by Kupffer cells, but also by liver parenchymal cells. Liposomes containing a physiological electrolyte such as KCl or NaCl, injected either intravenously or intraperitoneally, have also been found to be nontoxic to animals.

LIPOSOMES AS BIOLOGICAL CARRIERS STAFF

REGULAR STAFF

Cerny, Elizabeth A. (Scientific Assistant)
Dainko, Julia L. (Scientific Assistant)
Rahman, Yueh-Erh (Biologist)

TEMPORARY STAFF DURING 1976

*Jonah, Margaret M. (Research Associate)

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*Terminated during 1976.

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THERAPEUTIC APPLICATIONS OF LIPOSOME-ENCAPSULATED DRUGS

Yueh-Erh Rahman, Elizabeth A. Cerny, Betty Jean Wright, Margaret M. Jonah, Julia L. Dainko, Avrom M. Brendzel,† and Gordon L. Jendrasiak‡*

This program has two specific objectives: (1) to develop the delivery of drugs to specific target tissues by using liposomes with different physical and chemical properties--for example, liposomes varying in surface charge and particle size, and with specific chemical groups, including antibodies, attached to the surface; and (2) to define and solve possible problems before attempting to use this drug delivery system for clinical applications. Results during the year follow.

These chelating agents have been successfully encapsulated within liposomes: (1) D-penicillamine, a drug now in clinical use for removal of lead, copper, and gold; (2) desferrioxamine, specific for iron, and in clinical use for cases of iron overload (e.g., that associated with transfusion treatment of Cooley's anemia); and (3) phthalyltetrathioacetic acid. The latter compound, a gift from Dr. M. M. Jones of Vanderbilt University, has been reported to have a very high stability constant for mercury.

Methods for size determination of liposomes have been satisfactorily worked out: (1) The sizes of unilamellar liposomes are determined by direct measurement on electron micrographs after negative staining; their diameter was found to be in the range of 300-500 Å. (2) Multilamellar liposomes are successfully measured by the electrical resistance method in a Coulter transducer equipped with a 400-channel analyzer, by the use of very small apertures (11-15 μm). The diameter of the majority of neutrally charged multilamellar liposomes, prepared with dipalmitoylphosphatidylcholine, varies from 0.50 to 0.55 μm.

Liposomes containing either a common electrolyte (i.e., KCl) or the calcium trisodium salt of diethylenetriaminepentaacetic acid (DTPA) were given to mice, and the toxicity in various tissues was studied from 1 day to 7 days after liposome administration. At the tissue level, no significant weight changes in the major organs such as liver, spleen, kidney, lung, and heart have been observed after KCl-liposome administration. However, a consistent 1.3- to 3-fold increase in the wet weight of the spleen has been obtained after administration of liposomes containing DTPA. Possible toxicity by removal of essential metals from cells by the DTPA introduced into cells by the liposomes has been tested by the analysis of two zinc-requiring enzymes (glutamic dehydrogenase and alkaline phosphatase) in the liver, kidney, lung,

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*Electron Microscope Center.

†Guest Graduate Student, University of Illinois, Circle Campus.

‡Faculty Research Participant, University of Illinois, Urbana.

and spleen. Glutamic dehydrogenase activity shows no change in all four tissues after administration of liposomes containing either KCl or DTPA. However, a 6-fold increase in alkaline phosphatase occurred, in the liver only, 3 days after injection of mice with liposomes with DTPA. The significance of this increase is not clear at this time.

Partial selective uptake of ^{14}C-EDTA in the lung and brain has been obtained by use of liposomes made from the lipid mixture of phosphatidylcholine, ganglioside, and cholesterol.

Morphological identification of tissue cells that can take up liposomes containing chelating agents has recently been accomplished with electron microscopy of lung and bone marrow. In the lung, liposomes are found in epithelial cells (including the Type II alveolar cells), endothelial cells, and alveolar macrophages. In the bone marrow, they are found in the cells of the reticuloendothelial system, and in mature and immature white blood cells.

Single or multiple injections of liposomes containing DTPA, given to mice with lead burden, were significantly more effective for removal of lead from bone, liver, spleen, kidney, and lung, than the nonencapsulated, free form of DTPA.

A mouse model for iron overload disease has been worked out, in cooperation with Raymond A. Guilmette of the Therapy of Metal Poisoning Group. Mice are given a hypertransfusion of ^{59}Fe-labeled damaged red blood cells. This model will be used for testing the therapeutic efficacy of liposome-encapsulated desferrioxamine.

We have prepared unilamellar liposomes containing methotrexate, defined their size range, and found them to be stable and with minimal drug leakage for up to 2 weeks.

In studies to investigate the mechanisms by which the actinomycin D toxicity is reduced by liposome encapsulation, we observed pronounced protection of the bone marrow cells as well as the stem cells of the spleen and the femur by liposome encapsulation, and we also found a significant reduction of toxic effects to the regenerative capacity of the intestinal epithelium.

Liposomes containing ^{125}I-iododeoxyuridine (^{125}IUdR) have been studied in association with R. E. Krisch of the Genetics Group. This compound localizes specifically in the DNA where the decay of the ^{125}IUdR is lethal. ^{125}IUdR uptake was increased in liver, spleen, lung, and bone marrow by liposome encapsulation, while the uptake in the small intestine was decreased.

Cells in bone that take up liposomes containing actinomycin D have been identified as osteoclasts, osteocytes, and osteoblasts.

In studies of *in vitro* uptake of liposome-encapsulated actinomycin D in a human colon tumor cell line, LS174, done in collaboration with B. H. Tom and C. M. Macek of the Department of Surgery at Northwestern University Medical School, we have shown that uptake of liposomes is higher when cells are incubated in suspension than in monolayer. After neuraminidase treatment to remove the surface sialic acids of the tumor cells, the liposome uptake by these tumor cells was significantly decreased.

NMR studies of liposomes made with phosphatidylcholine and cholesterol and containing actinomycin D, either in the aqueous or the lipid phase, suggest that the actinomycin D is trapped within the liposomes, and that no specific complex between the drug and the lipids exists.

REMOVAL OF LEAD BURDEN FROM MOUSE TISSUES BY LIPOSOME-ENCAPSULATED CHELATING AGENT*

Yueh-Erh Rahman and Elizabeth A. Cerny

Liposomes were used as biological carriers to transport a chelating agent, diethylenetriaminepentaacetic acid (DTPA), into cells for treatment of lead poisoning. Mice were injected with radioactive lead in the form of $^{210}Pb(NO_3)_2$. The highest lead burden was found in the skeleton. Single or multiple treatments with liposome-encapsulated DTPA, given three days after ^{210}Pb injection, were shown to be significantly more effective for removal of lead from the skeleton than the nonencapsulated free form of DTPA. Liposome-encapsulated DTPA also removed more lead from liver, spleen, kidney, and lung.

*Abstract of a paper submitted for publication.

LIPOSOMES CONTAINING ^{3}H-ACTINOMYCIN D. DIFFERENTIAL TISSUE DISTRIBUTION BY VARYING THE MODE OF DRUG INCORPORATION*

Yueh-Erh Rahman, Walter E. Kisieleski, Evelyn M. Buess, and Elizabeth A. Cerny

Actinomycin D was encapsulated within liposomes in two different ways. When the drug was incorporated in the lipid phase, as part of the membrane bi-layers of the liposomes, they were called "lipid phase liposomes" (LPL); when the drug was incorporated in the aqueous solution in the center and between the lipid bi-layers of the liposomes, they were called "aqueous phase liposomes" (APL). Distributions of ^{3}H-actinomycin D in tissues of mice were determined from 15 minutes to 48 hours after a single intravenous injection of either LPL, APL, or nonencapsulated actinomycin D. Marked differences in tissue distribution were shown. ^{3}H-actinomycin D incorporated in LPL showed high concentrations in the lungs and low concentrations in the intestinal wall; whereas the reverse was found with APL. The spleen and bone marrow of mice receiving LPL showed an increase in ^{3}H-radioactivity between 3 and 24 hr after injection that was closely correlated with a concomitant decrease in activity in the liver and lungs. Mice receiving either APL or nonencapsulated actinomycin D had higher levels of the drug in the blood, kidneys and intestinal wall than did mice receiving LPL. *In vitro* studies showed that ^{3}H-actinomycin D leaked out from APL significantly faster than that from LPL. We have demonstrated that the tissue distribution of a drug can be modified not only by liposome encapsulation, but also by varying the way of incorporating the drug within liposomes, and thereby altering the surface properties. The two forms of incorporating drugs within liposomes are potentially useful to direct antitumor agents to specific tumor bearing tissues.

*Abstract of a paper published in Eur. J. Cancer 11, 883 (1975).

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10. DIAGNOSIS AND THERAPY

ERDA RT-02-01
ANL 61202
NIH AM 17862-01
NIH AM 15351-05
NIH AM 6-0040

GROUP LEADER'S INTRODUCTION

Peter D. Klein, Group Leader

This program, representing activities of the Argonne Bioanalytical Center, has the following objectives: (1) to develop instrumentation, labeled compounds, and analytical methodology for applications of stable isotopes to biomedical and clinical problems; (2) to conduct collaborative studies with clinicians in the areas of gastroenterology, pediatrics, pharmacology, and gynecology; (3) to provide an analytical resource for clinicians and scientists requiring bile acid identification, quantitation, resolution, and kinetic measurements; (4) to develop new areas of gas chromatography-mass spectrometry application that have clinical research and diagnostic potential; and (5) to maintain responsibility for convening the community of interest in stable isotopes at approximately 30-month intervals for the International Conference on Stable Isotopes.

The Argonne Bioanalytical Center is one of the recognized mass spectrometry centers in this country, not only by virtue of its physical facilities and equipment, but also for its emphasis on the measurement of isotope ratios in intact organic molecules, and the application of this measurement technique to stable isotopic tracer studies. The stable isotopes, which pose no radiation hazard to the patient, are particularly applicable in studies of premature and newborn infants, of young children, and of women at risk of childbearing. Consequently, the Center receives many requests for assistance and collaboration from clinicians all over the United States, Canada, and Central America. These requests, which range from single mass spectral identifications to extended clinical studies requiring several years, lead to a major portion of the Center's activities. In addition, developmental work to provide new, improved, and more sensitive instrumentation, to synthesize specific compounds required in metabolic studies or for use as internal standards, and to isolate metabolites from the bile, plasma, urine, feces, or tissue is constantly required.

During this year, new instrumentation for the automatic sampling, purification, and analysis of respiratory CO_2 for the $^{13}CO_2/^{12}CO_2$ ratio has been undergoing development, and completion of the prototype system is expected

during fiscal year 1977. It will be evaluated in a clinical setting to determine whether unattended operation and cost-effectiveness can be expected in measurement of clinical $^{13}CO_2$ samples.

An extensive series of organic syntheses related to the preparation of 24-^{13}C and 3β-d_1 labeled bile acids, their glycine and taurine conjugates, and their 3-, 7-, and 12-sulfate esters have been developed. These syntheses provide labeled bile acids of greater purity, in higher yield, and with less effort than previous syntheses. They are being used in clinical studies of bile acid kinetics, in measurements of bile acid deconjugation by intestinal bacteria, and in the estimation of bile acid levels in plasma by inverse isotopic dilution with deuterated internal standards.

The individual optical isomers of d_5-methadone have been tested for their pharmacokinetic behavior in patients on methadone maintenance therapy. The inactive (+) form of methadone was eliminated from the body almost twice as fast as the active (-) form. Significant levels of methadone could be detected in saliva, and initial studies on the feasibility of drug kinetic measurements using this noninvasive sampling technique have been conducted.

DIAGNOSIS AND THERAPY STAFF

REGULAR STAFF

Hachey, David L. (Assistant Biochemist)
Klein, Peter D. (Senior Biochemist)
Szczepanik, Patricia A. (Scientific Associate)

TEMPORARY STAFF DURING 1976

Mede, Karin A. (Postdoctoral Appointee)
*Schoeller, Dale A. (Postdoctoral Appointee)
Stellaard, Frans (Research Associate)
Tserng, Kou-Yi (Postdoctoral Appointee)

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*Terminated during 1976.

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NIH AM 17862-01
NIH AM 15351-05
NIH AM 6-0040

PROGRESS REPORT: HIGHLIGHTS OF THE ARGONNE BIOANALYTICAL CENTER

David L. Hachey, E. Roseland Klein, Peter D. Klein, Mary Jeanne Kreek, Karin A. Mede, Dale A. Schoeller, Frans Stellaard, Patricia A. Szczepanik, and Kou-Yi Tserng*

The best description of the progress made during the past year will be found in the abstracts of papers, published or submitted for publication, that accompany this report. Some selected topics are discussed below.

INSTRUMENTATION

A Nuclide 3-60-RMS magnetic sector mass spectrometer, designed to measure the abundance of $^{13}CO_2$ in CO_2 samples, is expected to be operational by June 1977, and will be used in the development of an automatic $^{13}CO_2$ analyzer for clinical studies. Considerable progress has been made on the design and construction of an automatic sampling system for withdrawal of gas from the Vacutainer storage capsule and for purification by cryogenic removal of water vapor and condensation of the CO_2 at liquid nitrogen temperatures. The work on mating this sampling system with the mass spectrometer will begin as soon as the latter is operational. The Nuclide instrument inlet system will accept samples from the purification stage and adjust the ion source pressure to a predetermined level. It is expected that this will provide the compensation necessary to obtain the precision required in isotopic ratio measurements in the absence of an operator. Overall control of the mass spectrometer, data collection, and report generation will be provided by a microprocessor unit already constructed and partially programmed.

High pressure liquid chromatography has been employed to separate and purify the deuterium-labeled peptide *p*-aminobenzoyl glutamate (PABA-GLU) arising from the cleavage of labeled folic acid, after isolation of folate from urine by an affinity column. This procedure has permitted the recovery of adequate amounts of purified PABA-GLU for isotopic analyses by gas chromatography-chemical ionization mass spectrometry. A high pressure liquid chromatography method for the resolution, quantitation, and preparative recovery of biliary bile acids has also been investigated. This method depends upon the formation of a bile acid derivative with UV absorption properties that permit it to be detected and quantitated by an appropriate flow detector. The phenacyl esters of individual bile acids were synthesized and were found to give highly linear extinction coefficients over a wide (10^4) concentration range. Elution conditions for baseline resolution of mixtures of all major bile acids were developed, and quantitation of standard mixtures has been satisfactorily achieved. The ultimate utility of this method will depend

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upon achieving quantitative derivatization of the biological sample and removal of interfering substances that react with the derivatizing reagent to form spurious compounds that absorb UV.

LABELED COMPOUNDS

Substantial progress has been made in the synthesis of labeled bile acids required in clinical studies of bile acid kinetics and metabolism and in the measurement of bile acid concentrations by inverse isotopic dilution techniques. An improved procedure for the synthesis of 24-^{13}C-labeled bile acids offers higher yield and less manipulation than previously required. In this procedure, bile acids are formylated to protect the hydroxyl groups, by a new quantitative process employing formic acid and acetic anhydride. The formyl bile acids are then degraded to the corresponding 23-chloronorcholane compounds with lithium chloride and lead tetraacetate in benzene. The 23-chloronorcholane is then reacted with sodium cyanide-^{13}C in dimethyl formamide. ydrolysis of the resulting 23-cyano norcholane yields the desired 24-^{13}C bile acid in an overall yield of 70% ^{13}C, as opposed to about 30% by previous methods.

The synthesis of 3β-d_1-labeled bile acids requires the availability of 3-keto bile acids. These are traditionally produced by Oppenhauer oxidation which typically results in yields of less than 50% and which requires extensive chromatographic purification of the reaction product. A new method employing silver carbonate on celite (Fetizon's reagent) was developed for the oxidation of bile acid methyl esters to the desired 3-keto compounds in 95% yield with minimal manipulation. These compounds were then converted to 3β-d_1 bile acids by reduction with sodium borodeuteride. Small amounts of 3α-d_1 product were easily removed by chromatography or fractional crystallization.

Procedures for the synthesis of glycine and taurine conjugates of bile acids are doubly useful; They provide the basis for synthesizing cholyl-1,2-^{13}C glycine used in the clinical breath test for bile acid deconjugation, and they provide the 3β-d_1 varieties of the bile acid conjugates required as standards in quantitative measurements. A new, highly efficient synthesis was developed for this purpose in which glycine conjugates were obtained in greater than 90% yield. Bile acids were coupled with glycine by refluxing in ethyl acetate solution in the presence of N-ethoxy carbonyl-2-ethoxy-1,2-dihydroquinone. Similarly, taurine conjugates were obtained in greater than 90% yield by conducting the coupling reaction in dimethyl formamide.

Evidence in other laboratories of the role of bile acid sulfation in cholestasis and of the need to develop new class separations that segregate and quantitate these bile acid sulfates has posed still further synthesis requirements. Until now, only a few bile acid sulfates have been synthesized, and of these only one or two have been adequately characterized to provide unambiguous identification and proof of purity. A new procedure developed in our laboratory for the sulfation of bile acids has had considerable impact on the solution of the problem of the large number of sulfated bile acids needed. The mono-hydroxy bile acid lithocholic acid and its glycine conjugate, glycolithocholic acid, are sulfated by reaction with sulfur trioxide-triethyl amine complex. 3-Mono-sulfates of cholic acid, chenodeoxycholic acid, ursodeoxycholic acid, and deoxycholic acid or of their glycine conjugates were sulfated

by using the 3-hydroxy formyl derivatives as starting materials. Use of these derivatives assures a higher yield, fewer side products, and hence greater purity and simpler isolation procedures. It has also proved to be much easier to remove the formyl protective groups by aqueous alkaline hydrolysis than to do so for the other conventionally used protective groups. Interestingly, the synthesis of taurine conjugated bile acid sulfates in high yield turned out to require that the sulfation procedure precede the conjugation with taurine. By this means, taurolithocholic acid sulfate was produced in a one pot synthesis in greater than 90% yield overall, as opposed to less than 20% by the conventional sequence.

Additional bile acid syntheses have included the preparation of several bile acids in which the hydroxyl group is in the β orientation. These bile acids appear in human biliary bile acids as the consequence of the action of intestinal flora, and the identification of such bile acids has been hampered by the lack of authentic standards. The newly prepared substances will provide gas chromatographic and mass spectral information that will assist in the identification of unknown bile acids found in clinical samples.

CLINICAL APPLICATIONS

Following the initial studies of d_5-methadone in methadone maintenance patients at The Rockefeller University by Dr. M. J. Kreek, in which we were able to show the existence of two pools ($T_{1/2}$ = 5-7 hours and $T_{1/2}$ = 30-50 hours), a study of the optical isomers of methadone was undertaken. Methadone is used clinically as the racemic mixture of two optically active forms of which the (-)-methadone is believed to be the active form. Since administration of the putatively inactive (+)-methadone would precipitate withdrawal symptoms in the patient, it is not clinically ethical to test the isomers separately. Instead, we have been able to make use of the fact that the d_5 label is introduced into the eventual methadone molecule before the synthesis of the asymmetric center. Separation of the two enantiomers before the final steps of the synthesis provides d_5(-)- and d_5(+)-methadone. A similar resolution of unlabeled methadone then enables the formulation of a pseudo-racemic mixture in which either isomer can be varied between labeled and unlabeled to obtain $d_5(-)d_0(+)$, $d_0(-)d_5(+)$, and $d_5(-)d_5(+)$ mixtures. Pharmacokinetic measurements were then carried out successively in the same patient using each of the racemic mixtures. In this manner one can obtain a valid comparison within the same patient of the biological half-life of each optical isomer. These studies, which have been carried out in five patients, show that the inactive (+) methadone is eliminated from the body almost twice as readily as the active (-) form, and the removal of the racemate closely approximated that of the average of the individual isomers. The absolute ratio of elimination rates for active/inactive isomers showed considerable variation between subjects and ranged from 1.39 to 1.95.

These measurements, which were conducted on plasma, urine, and feces, have been augmented by simultaneous collection of saliva samples at The Rockefeller University. It is well established that drugs freely diffusible in plasma readily and rapidly appear in saliva after ingestion, but it is also known that methadone is extensively (> 90%) bound to plasma proteins. Analysis of the methadone content of saliva showed the surprising result that salivary levels exceeded plasma levels present at the same time. The results

of pharmacokinetic measurements on salivary methadone are being compared with the values obtained from plasma to see if there is close agreement. If salivary measurements can be validated, several benefits are immediately apparent: first, drug kinetics can be determined noninvasively, thus permitting more frequent and larger samples to be obtained; second, the purification process required before analysis by gas chromatography-mass spectrometry can be simplified because of the absence of lipoproteins in saliva.

STABLE ISOTOPE PUBLICATION PROJECTS

The Proceedings of the Second International Conference on Stable Isotopes were completed and have been published (ERDA CONF 751025) and distributed. A new undertaking, namely the compilation of all references to biological, medical, and environmental applications of ^{2}H, ^{13}C, ^{15}N, ^{17}O, ^{18}O, and ^{32}S has been initiated. The first compilation will cover the period 1971-1976, for each isotope individually, and it is hoped that thereafter an annual bibliography for all isotopes can be compiled. Publication of these bibliographies by Biomedical Mass Spectrometry as a service to the stable isotope community has been arranged. Such publication will assure maximum diffusion of the information, and make it possible for reprint distribution of the bibliographies at low cost.

SYNTHESIS OF 11,12-$^{2}H_2$- AND 11,12-$^{3}H_2$-LABELED CHENODEOXYCHOLIC AND LITHOCHOLIC ACIDS*

A. E. Cowen,† A. F. Hofmann,† D. L. Hachey, P. J. Thomas,† D. T. E. Belobaba,† P. D. Klein, and L. Tökes‡

Deuterium- and tritium-labeled chenodeoxycholic acid and lithocholic acid were prepared by catalytic reduction of their respective Δ^{11} derivatives. Structures of the intermediates and their isotopic purity were verified by chemical ionization and electron impact mass spectrometry and by nuclear magnetic resonance spectroscopy. Experimental conditions for reductive deuteration were defined which gave complete reduction of the olefin and a product of high isotopic purity. Conditions for optimal tritiation were developed with which little exchange of protons with the solvent occurred; the product had high specific activity. To test biological stability of the label, the ^{3}H-labeled chenodeoxycholic acid was administered simultaneously with ^{14}C-labeled chenodeoxycholic acid to two healthy subjects and the $^{3}H/^{14}C$ ratio in bile was determined daily for several days. The ratio remained identical to that administered, suggesting that the 11,12-^{3}H label in chenodeoxycholic acid is stable during enterohepatic cycling and can be used for valid estimates of bile acid kinetics in man by the isotope dilution technique.

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*Abstract of a paper published in J. Lipid Res. 17, 231 (1976).

†Mayo Clinic and Mayo Foundation.

‡Institute of Organic Chemistry, Syntex Research, Palo Alto, CA.

STEREOSPECIFICITY OF THE HYDROGEN TRANSFER CATALYZED BY HUMAN PLACENTAL ALDOSE REDUCTASE*

Howard B. Feldman,† Patricia A. Szczepanik, Pamela Havre,† Roger J. M. Cornell,† Ling C. Yu,† Harvey M. Rodman,† Bryon A. Rosner,† Peter D. Klein, and Bernard R. Landau†

Placental aldose reductase (EC 1.1.1.21) was incubated with glucose in the presence of [*4A*-^{2}H]NADPH prepared in the oxidation of [2-^{2}H]isocitrate by isocitrate dehydrogenase (EC 1.1.1.42) or [*4B*-^{2}H]NADPH prepared in the oxidation of [1-^{2}H]glucose by glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The sorbitol formed from [*4A*-^{2}H]NADPH contained deuterium and from [*4B*-^{2}H]NADPH it did not. Therefore, aldose reductase is an A-type enzyme.

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*Summary of a paper published in Biochim. Biophys. Acta 480, 14 (1977).

†Case Western Reserve University School of Medicine.

QUANTITATIVE ANALYSIS OF METHADONE IN BIOLOGICAL FLUIDS USING DEUTERIUM LABELED METHADONE AND GAS CHROMATOGRAPHY-CHEMICAL IONIZATION-MASS SPECTROMETRY*

D. L. Hachey, M. J. Kreek,† and D. H. Mattson

(+)-, (-)-, and (±)-Methadone-$^2H_5$, which contained five deuterium atoms in one aromatic ring, were synthesized for use in clinical pharmacologic studies and also as internal standards. Gas chromatography-chemical ionization-mass spectrometry was used to determine plasma and urinary methadone levels by an inverse isotope dilution assay. Plasma levels of the drug could be determined to 10 pmole/ml and urine levels could be measured to 5 pmole/ml. Plasma methadone levels were examined in several patients undergoing methadone maintenance therapy. These levels generally range between 100 and 400 ng/ml (320-1300 pmole/ml) after an average oral dose of 1 mg/kg per day. Half-life of methadone was estimated at 28.8 ± 4.8 hours.

*Abstract of a paper to be published in J. Pharm. Sci. A preliminary report of this work appeared in Proceedings of the Second International Conference on Stable Isotopes, Eds. E. R. Klein and P. D. Klein. USERDA CONF-751027, pp. 518-523, 1976.

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METABOLISM OF 7α-HYDROXY-4-CHOLESTEN-3-ONE IN NORMAL SUBJECTS WITH AN INTACT ENTEROHEPATIC CIRCULATION*

Russell F. Hanson,† Patricia A. Szczepanik, Peter D. Klein, Eugene A. Johnson,† and Gale C. Williams†

The formation of bile acids in man is thought to involve a series of reactions in which the initial steps are the same for both cholic acid and chenodeoxycholic acid. The point of bifurcation of the pathway is postulated to occur after the formation of 7α-hydroxy-4-cholesten-3-one. To test the hypothesis that the entire synthesis of both bile acids proceeds through this intermediate we studied the metabolism of labeled 7α-hydroxy-4-cholesten-3-one in eight normal subjects with an intact enterohepatic circulation.

If all the production of cholic acid and chenodeoxycholic acid takes place via 7α-hydroxy-4-cholesten-3-one, the areas under the specific decay curves of cholic acid and chenodeoxycholic acid should be identical following a single injection of this labeled intermediate. However, in 6 of the 8 subjects studied the area under the cholic acid specific activity decay curve was significantly less than the area under the chenodeoxycholic acid specific activity decay curve.

*Summary of a paper published in Biochim. Biophys. Acta 431, 335 (1976).

†University of Minnesota, Minneapolis.

These results indicate that the production of cholic acid in man may not always involve the intermediate 7α-hydroxy-4-cholesten-3-one.

QUANTITATION OF PATHWAYS OF ETHANOL METABOLISM*

Pamela Havre,† Marc A. Abrams,† Ling C. Yu,† Roger J. M. Corrall,† Patricia A. Szczepanik, Howard B. Feldman,† Peter D. Klein, Ming S. Kong,† Joseph M. Margolis,† and Bernard R. Landau†

A method has been developed for the estimation of the sum of the contributions to ethanol oxidation by the microsomal ethanol oxidizing system (MEOS) and catalase in the intact liver cell. It depends upon a comparison of the fate of the R hydrogen of ethanol and the hydrogen bound to carbon 2 of sorbitol under identical conditions. The limitations of the approach are defined. From incubations of rat and monkey liver slices, it is concluded that at a concentration of ethanol of 3 mg/ml, and for the rat at 1 mg/ml, and including incubations with slices from rats chronically fed with ethanol, MEOS and catalase make at most a small (less than 20%) contribution to overall ethanol metabolism. As by-products of this study the stereospecificity of the sorbitol dehydrogenase catalyzed reaction is shown to be of the A type in the rat, and definite evidence is obtained for the irreversibility of sorbitol oxidation in the intact liver cell. A greater isotopic discrimination for the R hydrogen of ethanol is shown for its oxidation via catalase than via alcohol dehydrogenase.

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*Summary of a paper submitted for publication.

†Case Western Reserve University.

SOURCES OF VARIABILITY IN THE USE OF ^{13}C-LABELED SUBSTRATES AS "BREATH TEST" IN CLINICAL RESEARCH AND DIAGNOSIS*

P. D. Klein and D. A. Schoeller

The minimum excursion of $^{13}CO_2$ abundance with analytical significance in clinical studies involving $^{13}CO_2$-labeled compounds is, under optimum conditions, 1.4 per mil. This corresponds to the excess production of 0.14 μM $^{13}CO_2$/kg/h. Oxidation of diagnostic substrates must, in general, produce 1.4 to 5 μM $^{13}CO_2$/kg/h.

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*Conclusion of a paper published in Z. Anal. Chem. 279, 134 (1976).

EFFECT OF DEOXYCHOLIC ACID INGESTION ON BILE ACID METABOLISM AND BILIARY LIPID SECRETION IN NORMAL SUBJECTS*

Nicholas F. LaRusso,† Patricia A. Szczepanik, and Alan F. Hofmann†

The effect of deoxycholate ingestion, 750 mg per day, on bile acid kinetics, biliary bile acid composition, and biliary lipid secretion was studied in 7 healthy volunteers. Bile acid kinetics were measured by isotope dilution, and hourly outputs of bile acid, cholesterol, and phospholipid were quantitated by a duodenal perfusion technique during a 24-hr period which included three liquid meals and an overnight fast. Biliary bile acid composition was assessed by coupled gas chromatography-mass spectrometry. After deoxycholic acid ingestion, biliary bile acids became composed of predominantly deoxycholyl conjugates, and deoxycholic acid pools increased 4-fold. Both chenodeoxycholic and cholic acid pools decreased, and daily synthesis of each of the primary bile acids was inhibited by 50%. Total bile acid pools did not change in any consistent manner. Daily bile acid secretion increased slightly during deoxycholic acid ingestion, and recycling frequency varied reciprocally with the total bile acid pool both before and during deoxycholic acid treatment. Deoxycholic acid ingestion caused no change in either the daily secretion of cholesterol or lecithin, or the cholesterol saturation of fasting-state bile, which remained unsaturated throughout the study. SGOT levels increased to 4 times the upper limits of normal in 2 of 7 subjects, but these levels promptly returned to normal when deoxycholate feeding was stopped. Serum cholesterol levels decreased in every subject (average 15%) during deoxycholic acid administration. No evidence for a direct role of deoxycholate in the pathogenesis of cholesterol cholelithiasis was obtained in these studies.

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*Abstract of a paper published in Gastroenterology 72, 132 (1977).

†Mayo Clinic and Mayo Foundation.

FETAL AND NEONATAL HEPATIC FUNCTION II*

Roger Lester,† Benjamin T. Jackson,† Richard A. Smallwood,† John B. Watkins,† Peter D. Klein, and Joanna M. Little†

Marked deficiencies of both bilirubin and bile salt metabolism are evident during the prenatal period in primates. Immaturity of the apparatus for bilirubin elimination produces physiologic jaundice and may contribute to the development of kernicterus. Immaturity of the biosynthetic mechanisms for bile acid synthesis probably contributes to the development of neonatal

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*Summary of a paper published in Birth Defects: Orig. Artic. Ser., Vol. XII, No. 2, 307 (1976).

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steatorrhea and may be associated with undernutrition in premature infants and infants with other associated abnormalities. Both forms of immaturity can be altered by prenatal administration of suitable agents to the mother. This principle has been applied in the experimental therapy of disorders of neonatal bilirubin metabolism. It remains to be seen if it will be appropriate to apply it to the as yet ill-defined problems of neonatal bile salt metabolism.

SYNTHESIS OF PTEROYLGLUTAMIC ACID-3',5'-$^2H_2$ BY TRIFLUOROACETIC ACID CATALYZED EXCHANGE WITH DEUTERIUM OXIDE*

L. Palladino,† *J. A. Blair,*† *I. H. Rosenberg,*† *D. L. Hachey, and P. D. Klein*

Pteroylglutamic acid (PGA) was deuterated by trifluoroacetic acid catalyzed exchange with deuterium oxide. The product, pteroylglutamic acid-3',5'-$^2H_2$, was specifically deuterated in the aromatic protons of the p-aminobenzoyl (PABA) moiety; the protons on C_7, and C_9 and in the glutamic acid residue were not exchanged. Deuterium incorporation was measured by chemical ionization mass spectrometry (CI-MS). Pteroylglutamates were cleaved by a base-catalyzed, oxidative hydrolysis to PABA, which was converted to the methyl ester, N-trifluoroacetate for analysis by gas chromatography-chemical ionization-mass spectrometry. Products from the exchange typically contained 1% $^2H_1$ and 90% $^2H_2$ species. The procedure may be used to label specifically various analogs of PGA with deuterium in the PABA portion of the molecules.

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*Abstract of a paper published in Proceedings of the Second International Conference on Stable Isotopes, Eds. E. R. Klein and P. D. Klein. USERDA CONF-751027, pp. 579-584, 1976.

†The University of Chicago.

BREATH ANALYSIS OF $^{13}CO_2$ FOLLOWING N-DEMETHYLATION OF ^{13}C-AMINOPYRINE: A MEASURE OF LIVER MICROSOMAL FUNCTION*

J. F. Schneider,† *D. A. Schoeller, B. Nemchausky,*† *J. L. Boyer,*† *and P. D. Klein*

The hepatic microsomal mixed function oxidase enzyme activity has been measured by N-demethylation of 4-dimethyl-^{14}C-aminopyrine (DAP). Analysis of $^{14}CO_2$ in expired breath has recently been validated in the rat and man as a

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*Abstract of a paper published in Proceedings of the Second International Conference on Stable Isotopes, Eds. E. R. Klein and P. D. Klein. USERDA CONF-751027, pp. 259-264, 1976.

†The University of Chicago.

measure of this function. In the present study we examine the use of DAP labeled with the stable isotope carbon-13, in order to permit broader clinical application of this test by avoiding radiation exposure. Two mg/kg of 86% enriched ^{13}C-DAP was given orally to 4 normal subjects and 5 patients with cholestatic liver disease. All subjects were fasted overnight and studied at rest. Breath samples were collected at 1/2 hour intervals for 3 hours. In all samples the excess of $^{13}CO_2$ was significantly greater than the variation in baseline after ingestion of unlabeled DAP. In normal subjects the peak production of $^{13}CO_2$ occurred in the first 1/2 hour sample. Unlabeled DAP (8 mg/kg) clearance from serum correlated with excess $^{13}CO_2$ production measured in exhaled breath confirming the $^{14}CO_2$ results. Although there was considerable variation among subjects in excess $^{13}CO_2$ production, values were reproducible in the same individual. When phenobarbital (180 mg/day) was administered, an increase in exhaled $^{13}CO_2$ was observed. Measurement of $^{13}CO_2$ in breath following DAP provides a reproducible clinical measure of microsomal function and drug induction. The use of stable carbon-13 labeled DAP permits measurement of liver microsomal function in patients who cannot receive radioactive labeled DAP.

A REVIEW OF THE STATISTICAL CONSIDERATIONS INVOLVED IN THE TREATMENT OF ISOTOPE DILUTION CALIBRATION DATA*

D. A. Schoeller

The use of linear regression analysis for the reduction of isotope dilution data is reviewed. The calculation of linear regression statistics is based upon four assumptions: zero variance in the independent variable, equal variance for all values of the dependent variable, linearity and continuity. Unfortunately, isotope dilution data often violate one or more of these assumptions, which results in the calculation of an inaccurate calibration line. The inaccuracies can be avoided through careful inspection of the data, including analyses of variance and linearity. Large differences in the variances of the dependent variable require the use of a weighted linear regression. Nonlinearity necessitates either discarding data in the nonlinear portion of the calibration or the calculation and use of atom % excess and dilution instead of the simple isotope ratios.

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*Abstract of a paper published in Biomed. Mass Spectrom. 3, 265 (1976). A preliminary report of this work appeared in Proceedings of the Second International Conference on Stable Isotopes, Eds. E. R. Klein and P. D. Klein. USERDA CONF-751027, pp. 421-426, 1976.

CLINICAL DIAGNOSIS USING THE STABLE ISOTOPE ^{13}C IN CO_2 BREATH TESTS: METHODOLOGY AND FUNDAMENTAL CONSIDERATIONS*

D. A. Schoeller, J. F. Schneider,† N. W. Solomons,† J. B. Watkins,‡ and P. D. Klein

The methodology for measuring *in vivo* oxidation of substrates labeled with the nonradioactive carbon isotope ^{13}C has been developed using isotope ratio mass spectrometry. The use of ^{13}C offers the possibility of utilizing CO_2 breath tests in infants, children, pregnant women and all subjects in whom $^{14}CO_2$ breath tests cannot be used. The excretion of 140 nanomols/(kg-hr) of $^{13}CO_2$ produced from the oxidation of the labeled substrate could be detected with 95% confidence during a total CO_2 excretion of 9 mM/(kg-hr). The precision of CO_2 breath tests using ^{13}C is limited by the natural fluctuations of the ratio of $^{13}C/^{12}C$ in expired CO_2, which occur with a standard deviation of 0.72°/₀₀, or approximately 7 parts $^{13}CO_2$ per 10^6 parts expired CO_2. Larger excursions in the ratio were observed if the subject ate shortly before or during the breath test. Clinically significant diagnostic tests can reasonably be expected to require the excretion of 2 to 20 times as much labeled CO_2, or 0.28 to 1.4 μM/(kg-hr).

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*Abstract of a paper to be published in J. Lab. Clin. Med. A preliminary report of this work appeared in Proceedings of the Second International Conference on Stable Isotopes, Eds. E. R. Klein and P. D. Klein. USERDA CONF-751027, pp. 246-251, 1976.

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DIAGNOSIS OF BACTERIAL OVERGROWTH AND ILEAL DYSFUNCTION BY RESPIRATORY CO_2 ISOTOPIC MEASUREMENTS OF CARBON 13*

N. W. Solomons,† D. A. Schoeller, J. B. Wagonfeld,† D. G. Ott,‡ I. H. Rosenberg,† and P. D. Klein

The interval sampling of expired air offers a non-invasive methodology for the study of intestinal physiology. In the present study, stable isotope chemistry and mass spectroscopic analysis have been applied to the development of a glycocholate deconjugation breath test using carbon-13 as the isotopic marker. In 13 adult subjects with a wide range of conditions associated with upper intestinal bacterial overgrowth or ileal dysfunction, the intraluminal

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*Abstract of a paper submitted for publication.

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deconjugation of standard cholyl-1-^{14}C-glycine was compared to that of simultaneously administered cholyl-1-^{13}C-glycine or cholyl-1,2-^{13}C-glycine. The correlation of net deconjugation as measured by the radioactive isotope and stable isotope labeled bile salt was excellent (correlation coefficient 0.952). Exogenous bile salts did not affect the reproducibility of the breath test. There was statistical identity between the results of tests using cholyl-1-^{13}C-glycine or cholyl-1,2-^{13}C-glycine.

The calculated detection limits employing a dose of 7.5 mg/kg body weight of cholyl-1,2-^{13}C-glycine are 4.7% and 6.2% at the 95 and 99% confidence levels, respectively. This test offers potential advantages of safety and acceptability for infants, children, pregnant women, and women at risk of pregnancy in whom the use of carbon 14 might be considered hazardous.

CHARACTERIZATION OF BILE ACID METHYL ESTER ACETATE DERIVATIVES USING GAS-LIQUID CHROMATOGRAPHY, ELECTRON IMPACT, AND CHEMICAL IONIZATION MASS SPECTROMETRY*

Patricia A. Szczepanik, David L. Hachey, and Peter D. Klein

The gas-liquid chromatographic retention times on 0.5% SP-525 for 48 bile acids and related compounds as their methyl ester acetate derivatives are given. Ion tables for electron impact spectra have been compiled that permit direct access to ion structures for any given ion mass. Chemical ionization yields highly simplified mass spectra with two or three ions predominating for each compound. When the relative retention times of bile acids as their methyl ester acetates are combined with selective ion monitoring techniques in chemical ionization mass spectrometry, the retention time and ion mass number form a coordinate system which can be a powerful tool in the characterization of bile acid mixtures.

*Abstract of a paper published in J. Lipid Res. 17, 314 (1976).

EVALUATION OF POLY S-179 AS A STATIONARY PHASE FOR THE GAS CHROMATOGRAPHY/MASS SPECTROMETRY OF BILE ACID METHYL ESTER ACETATES*

Patricia A. Szczepanik, David L. Hachey, and Peter D. Klein

The stationary phase Poly S-179 has been found to offer distinct advantages over the previously reported SP-525 for the gas chromatographic separation of bile acid methyl ester acetates. Relative retention times of these bile acid derivatives are compared on the two phases.

*Summary of a paper submitted for publication.

AN IMPROVED PROCEDURE FOR THE SYNTHESIS OF GLYCINE AND TAURINE CONJUGATES OF BILE ACIDS*

K. Y. Tserng, D. L. Hachey, and P. D. Klein

Glycine and taurine conjugates of 5β-cholanic acids have been synthesized using improved procedures based on the peptide coupling reagent, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. The conjugates are obtained in chromatographically pure form in yields higher than 90%. The use of this procedure in the large scale preparation of cholyl[1,2-$^{13}C_2$]-glycine is described.

*Summary of a paper to be published in J. Lipid Res.

AN IMPROVED SYNTHESIS OF 24-^{13}C-LABELED BILE ACIDS USING FORMYL ESTERS AND A MODIFIED LEAD TETRAACETATE PROCEDURE*

K. Y. Tserng and P. D. Klein

An improved synthesis of [24-^{13}C]-labeled bile acids has been achieved using formyl derivatives of bile acids and a modified lead tetraacetate procedure. The formylated bile acids were degraded by lead tetraacetate and lithium chloride to formylated 23-chloronorcholanes in 72-83% yield. Formylated 23-chloronorcholanes were converted to nitriles in dimethylformamide, which were then hydrolyzed to obtain C-24 labeled bile acids in yield of 80-90% of labeled sodium cyanide used. This method results in a higher yield and a purer product with less manipulation than previously reported procedures for synthesis of labeled bile acids.

*Summary of a paper to be published in J. Lipid Res.

FORMYLATED BILE ACIDS: IMPROVED SYNTHESIS, PROPERTIES, AND PARTIAL DEFORMYLATION*

Kou-Yi Tserng and Peter D. Klein

Pure performylated bile acids are obtained in quantitative yield by a new formylation procedure. The procedure involves heating the bile acids in 90% formic acid containing catalytic amount of perchloric acid and then

*Abstract of a paper to be published in Steroids.

adding acetic anhydride slowly until effervescence occurs. Pure performylated bile acids are then isolated simply by diluting the reaction mixture with water. Contrary to what was believed by past investigations, the formyl groups on these compounds are quite stable to various reaction conditions. The stability and ready availability of these compounds make them more suitable candidates than their counterpart--bile acid acetates--for use as starting material in various synthetic schemes, such as C-24 labeled bile acids, etc. The partial deformylation of these formates can be effected by using methanolic ammonia, sodium methoxide in methanol, or sodium hydroxide in aqueous acetone. The resulting 3-hydroxy formyl bile acids are obtained in high yield and are the best starting materials for the synthesis of bile acids with specific modification at 3-hydroxyl group, such as the synthesis of bile acid 3-monosulfates and 3-monoglucuronides.

SYNTHESIS OF SULFATE ESTERS OF LITHOCHOLIC ACID, GLYCOLITHOCHOLIC ACID, AND TAUROLITHOCHOLIC ACID WITH SULFUR TRIOXIDE-TRIETHYLAMINE*

K. Y. Tserng and P. D. Klein

The facile synthesis of lithocholic acid sulfates by a procedure which produced the desired products in over 90% yield is described. Lithocholic acid sulfate and glycolithocholic acid sulfate were synthesized by reacting lithocholic acid or glycolithocholic acid with sulfur trioxide-triethylamine complex in dimethylformamide for 0.5-1 hour. Taurolithocholic acid sulfate was obtained by conjugating lithocholic acid sulfate with taurine in dimethylformamide at 90°C for 0.5 hour. The one-pot synthesis of taurolithocholic acid sulfate starting from lithocholic acid is also described. This procedure, which generated lithocholic acid sulfate, *in situ*, produced taurolithocholic acid sulfate in 98% yield, compared to an overall yield of less than 10% obtained by previously published procedures.

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*Abstract of a paper submitted for publication.

^{13}C-TRIOCTANOIN: A NONRADIOACTIVE BREATH TEST TO DETECT FAT MALABSORPTION*

*J. B. Watkins,† D. A. Schoeller, P. D. Klein, D. G. Ott,‡ A. D. Newcomer,** and A. F. Hofmann***

Fat malabsorption may be accurately detected in adults by measuring the excretion of $^{14}CO_2$ in breath following oral administration of a tracer dose of ^{14}C-labeled triglyceride. In order to detect fat malabsorption in children and in women of child-bearing age without radiation hazard, the use of trioctanoin labeled with the stable, nonradioactive isotope ^{13}C has been inaugurated and validated for use in this breath test. The validation tests with both ^{14}C- and ^{13}C-trioctanoin were conducted in 14 adult patients with varying degrees of fat malabsorption and demonstrated that the labels were excreted at nearly identical rates ($r = 0.97$). After establishment of dose requirements and measurement of endogenous $^{13}CO_2$ production rates, 9 children aged 3 months to 5 years were evaluated for fat malabsorption. The results obtained using the ^{13}C-trioctanoin breath test were compared to those obtained by a quantitative 72-hour fat balance study. The cumulative excretion of $^{13}CO_2$ by 2 hours was 25 ± 2.5% (Ave ± S.D.) of the dose in patients with normal fat absorption and provided a clear differentiation ($p < .001$) from the 3.5 ± 2.5% of the dose excreted by those with steatorrhea due to untreated pancreatic insufficiency resulting from cystic fibrosis. Peak $^{13}CO_2$ levels occurred at 1.5 hours in both groups with some overlap. Addition of exogenous pancreatic enzymes improved fat absorption and increased $^{13}CO_2$ excretion four-fold. The correlation between the percent of fat intake excreted and the cumulative $^{13}CO_2$/mmol CO_2 excreted by 3 hours was very good ($r = -0.88$) in all patients. These data indicate that the ^{13}C-trioctanoin breath test provides accurate detection of fat malabsorption in children with pancreatic insufficiency. This noninvasive technique is more convenient than 72-hour stool collection and permits safe and sensitive metabolic studies in children without exposure to radiation.

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*Abstract of a paper to be published in J. Lab. Clin. Med. A preliminary report of this work appeared in Proceedings of the Second International Conference on Stable Isotopes, Eds. E. R. Klein and P. D. Klein. USERDA CONF-751027, pp. 274-281, 1976.

†Harvard Medical School.

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11. BIOCHEMISTRY

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ANL 61100
NASA-P-7142-C

GROUP LEADER'S INTRODUCTION

John F. Thomson, Group Leader

Since the last report, two changes have taken place in the Biochemistry Group: (1) Dr. Y. E. Rahman's project on liposome-encapsulated drugs was transferred to the Medical Development Section of the Division of Biological and Medical Research. (2) Drs. R. N. Feinstein and D. A. Haugen were transferred from the Carcinogenesis program to the Biochemistry Group. However, since their budget support still comes from Carcinogenesis, and their research interests are still intimately allied with that program, their submissions to this report are incorporated within Section 5, dealing with the Carcinogenesis Group.

The contributions of this group consist of three reports. The first is concerned with recent developments in the isolation and characterization of subcellular components of mammalian cells: (1) stabilization of mitochondrial membranes by the antidepressant drug imipramine, (2) activation of peroxisomal enzymes by digitonin, (3) kinetic studies of urate oxidase, (4) separation of rat liver nuclei according to ploidy by zonal centrifugation, and (5) separation and characterization of enzymes and other proteins of specific organelles.

The other two reports are in the field of plant physiology. One deals with the effects of simulated acid rainwater on the growth and heavy metal uptake of soybean plants, and the other with the search for the gravity-sensing mechanism in plants. It may be noted that both of these projects, the former supported by ERDA and the latter by NASA, will be discontinued.

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‡Transferred to the Liposomes as Biological Carriers Group; terminated during 1976.

ERDA RT-02-01
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ISOLATION OF CELLS AND SUBCELLULAR COMPONENTS BY CENTRIFUGATION TECHNIQUES

*John F. Thomson, Sharron L. Nance, Sandra L. Tollaksen, and Bradford B. Smith**

Isolation of subcellular fractions is an important analytical tool for the study of cellular physiology and biochemistry, particularly when supplemented by correlative morphologic examination. This study is directed in part toward the development and application of methods of density-gradient centrifugation that relate the concentration of cellular components to the size of the particulates with which they are associated, rather than to morphologic labels of doubtful meaning. Enzymes or other cellular constituents known to be specifically associated with given organelles are used as biochemical markers.

The procedures developed in this laboratory have been valuable in the comparison of tissues from normal animals and those treated in various ways. They have also been successfully adapted as preparative methods. The basic technique has been to layer a preparation (e.g., a tissue homogenate or a suspension of cells) over a density gradient; after centrifugation, successive fractions are collected and analyzed by appropriate methods: enzyme assays, microscopic examination, cell counts, chemical compositions, etc. Since the average particle size in each fraction can be estimated from a sedimentation equation derived from Stokes' law, the relationship between concentration and particle size can be estimated; we have written computer programs to facilitate the mathematical and statistical analyses of such data. Currently we are carrying out tissue fractionations in zonal centrifuges, which provide much finer resolution than can be obtained by other procedures that involve centrifugation.

We have continued our studies on the protective effect of the antidepressant drug imipramine against lysis by digitonin of mitochondrial membranes. Sufficient data are now available to permit an analysis of the rather complex stoichiometry of the system.

Swelling of mitochondria produced by triiodothyronine (T-3) is not prevented by imipramine; further, imipramine inhibits the reversal of T-3-induced swelling effected by addition of ATP, magnesium ions, and serum albumin.

Imipramine has no effect on lysis by digitonin of peroxisomal membranes. However, in the course of this work we observed an unexpected activation of the peroxisomal enzymes urate oxidase and catalase. In the case of the former, the activation increased linearly with respect to the surface area of the peroxisome, as estimated from zonal centrifugation studies, up to a certain area, somewhat larger than the square of the diameter of the midpoint of the distribution pattern. We have systematically ruled out possible artifacts such as pH effects, ionic strength, inhibition by sucrose, etc., and have concluded

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*Thesis Parts Student, San Diego State University.

that the problem is one of localization of urate oxidase within the organelle. The enzyme is believed to be associated with the insoluble peroxisomal "core"; digitonin destroys the peroxisomal membrane, but does not affect the core. The larger the peroxisome, the larger the core, up to the point described above, at which activation decreases. At this point, evidence from centrifugation studies suggests that large peroxisomes contain either multiple small cores, or some soluble urate oxidase. The system is an interesting problem in heterogeneous enzyme catalysis, involving a soluble substrate and an insoluble enzyme.

Our collaborative work with Y. E. Rahman of the Liposomes as Biological Carriers Group came to a halt when her program on cancer chemotherapeutic agents was terminated.

We have begun a collaborative program with C. Peraino, Carcinogenesis Group, on rat liver nuclei. For this work we adapted a published method for separation of nuclei by zonal centrifugation. The procedure not only provides a clean separation between diploid and tetraploid nuclei, but also permits detection of S-phase diploids from the diploid peak.

With the assistance of B. B. Smith, a graduate student from San Diego State University, we carried out additional studies on peroxisomal enzymes. The principal accomplishment was to establish unequivocally that glyoxalate reductase and lactate dehydrogenase were not peroxisomal enzymes, as had been previously reported from another laboratory.

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ACID RAIN ON PLANT GROWTH AND HEAVY METAL UPTAKE*

*Jane Shen-Miller, Mason B. Hunter,† Jannette L. Carey,‡ and Leo D. Savare, Jr.***

This study was initiated to investigate the effect of atmospheric pollutants generated from the burning of fossil fuels on plant growth; in particular, we studied the long-term effect of acid rain on growth and cadmium uptake in soybean plants.

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*We gratefully acknowledge the assistance of Joseph E. Miller, Radiological and Environmental Research Division, and Richard Olsen, Environmental Impact Studies Division, in atomic absorption analyses.

†Fall 1975 participant in the Undergraduate Honors Participation Program, Carroll College.

‡Spring 1976 participant in the Undergraduate Honors Participation Program, Central Connecticut State College.

**Summer 1976 participant in the Undergraduate Honors Participation Program, LeMoyne-Owen College, Memphis, Tennessee.

Acid rain is increasingly being recognized as a serious regional environmental problem. Levels of acidity as high as 10^{-2} M H^+ have been recorded in southern Scandinavia and the northeastern United States. Summer rainfalls are more acid than those in winter months, and summer is the season in the temperate zone when crops attain rapid growth and maturity. The increased acidity in rain water is likely related to the increased concentration of atmospheric pollutants such as sulfur dioxide and nitrogen oxides (Galloway, J. N., et al., Science 194, 722, 1976) generated from the burning of fossil fuels. Sulfur dioxide can be transported in the air for more than 1500 km from the site of origin. The depletion of fish stocks and the reduction of forest productivity have been observed in Scandinavian countries. Studies of rainfall chemistry just being initiated in the United States found H_2SO_4 and HNO_3 to be the two major components in the rain. The effects of long-term acid rain on crop performances are not known. The consequences of this environmental addition could have a great impact on food and fiber production.

Heavy metals are another set of pollutants generated from the burning of fossil fuels. Cadmium, the metal examined in this study, showed strong binding to mitochondrial ligands, and diverted energy from the electron transport chain. It further interfered with ATP synthesis and maintenance of ionic concentration gradients across membranes (Bittell, J. E., et al., Plant Physiol. 30, 326, 1974).

As a beginning, a single strong acid was investigated. Sulfuric acid was introduced to the spray at acidity levels ranging from pH 2 to pH 5 (determined by titration). Soybean plants (Amsoy 71) were sprayed with acid rain from 8 to 9 days of planting. Plants were treated with $CdCl_2$ (2-8 ppm) at about 52 days of age. Plant tissues were measured for Cd^{++} uptake by atomic absorption analysis, and for leakiness and for leghemoglobin content in the root nodules by spectrophotometry.

Statistically significant decreased growth rate and dry weight, severe foliage damage, lowered bean pod numbers, greater cadmium injury and uptake, and increased leakiness from foliage were observed in soybean plants sprayed with acid rain of pH 2. Plants receiving rains of lower acidities showed no difference in growth as compared to the control (no H_2SO_4). In these experiments, the soil surface was protected from the acid runoff. When the soil was not shielded, a significant increase in the number of root nodules and a decrease in nodule diameter were seen in the pH 2 treatment. An indication of a lowering in leghemoglobin content (ferric form) in the root nodules was noted in soybean plants sprayed with pH 2 and pH 3 solution. Leghemoglobin is essential for nitrogen fixation by bacteroids in the nodules.

These studies indicate that highly acid rainfall is harmful to crop yield (1) by reducing photosynthetic surfaces, and (2) by reducing nitrogen fixation capability by rendering plant membranes more susceptible to the injury and uptake of other pollutants in the atmosphere, and by allowing excessive exit of nutrients and proteins from plants. The addition of H_2SO_4 at lower acidities could be beneficial, particularly to legumes for the increased synthesis of sulfur proteins containing methionine and cysteine.

NASA-P-7142-C

GROWTH AND DEVELOPMENT OF PLANTS IN COMPENSATED AND NORMAL EARTH FIELDS: INTERACTION OF LIGHT AND GRAVITY ON THE DIFFERENTIAL MITOSIS AND ROOT MERISTEM MORPHOLOGY IN CORN ROOTS

Jane Shen-Miller

The broad objective of this project is the identification of the gravity-sensing mechanism in plants. This study was initiated to localize the precise region where geotropic response occurs in roots, so as to form a basis for harvest of specific tissues for electron microscopic examination of the involvement of cellular organelles in geotropism.

The corn root (Wisconsin hybrid 64A x 22R) used in this study requires an exposure to light before it becomes reactive to gravity. Red light (660 nm) is the most effective wavelength for the initiation of the response (Shen-Miller, J., Photochem. Photobiol., submitted for publication); and the curvature response occurs most prominently in the region 2 to 3 mm from the root tip (Shen-Miller, J., et al., ANL-76-99, 1975, p. 132). In addition to the localization of differential cell expansion, where the cells in the upper cortical tissue significantly increase in length, we observed a differential cell division in upper and lower tissues in geotropically stimulated roots, and a change in root meristem morphology in these roots.

Primary roots of corn were irradiated with 660-nm light for 60 seconds at 1 Jm^{-2}. At 15-minute intervals following irradiation and gravity stimulation, the roots were harvested for light microscopy examination. Cell mitoses were scored beginning from the root meristem and extending basally for 1.5 mm. The root meristem is separated from the root cap by a densely stained layer, designated the "cuticle." The cuticle is thinner at the tip of the root meristem and becomes thicker toward the periphery. The diameter of the thin region was measured at different times following irradiation and gravity stimulation.

After red light irradiation and gravity stimulation, a greater amount of cell division occurred in the lower tissues, in contrast to the situation in cell expansion. The divisions in the cortex and stele are asynchronous, with the peak of cortical division preceding that of the stele, and both peaks occurring before the peak of geotropism. The thin region of the cuticle layer covering the root meristem increased in diameter and peaked at 180 minutes following red irradiation and gravity stimulation, which was coincidental with the peak of geotropic response.

The pattern of growth and mitosis observed can provide a possible clue to the distribution of hormones in corn root apices. Indoleacetic acid, gibberellic acid, cytokinin, and abscisic acid all have been found in roots. Their synthesis, transport, differential distribution, and interaction could initiate the cellular responses we found here. The lessening of a cuticle barrier between the root cap and the root proper by red irradiation and gravity stimulation would facilitate the transport of messages. The root cap is the site of perception for the gravity stimulus (Juniper, B. E., et al., Nature 209, 93, 1966) which is transported to the root proper for the expression of geotropism.

SIMILARITY IN DOSE RESPONSES, ACTION SPECTRA AND RED LIGHT RESPONSES BETWEEN PHOTOTROPISM AND PHOTOINHIBITION OF GROWTH*

W. M. Elliott and J. Shen-Miller

First positive phototropism and photoinhibition of growth of oat coleoptiles share similar dose response curves and action spectra. Both responses increase with increasing dosage of blue light (440 nm) up to 10^{13} photons·cm^{-2}, then both decrease with increasing dosage. Action spectra for both responses have peaks at 360, 440, and 470 nm. When red light (660 nm) was given beforehand, the sensitivity of each response to blue light was lessened. These data indicate a close correlation between phototropism and photoinhibition of growth. Both phenomena can be explained as a result of photoinhibition of basipetal transport of auxin.

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*Abstract of a paper published in Photochem. Photobiol. 23, 195 (1976).

THE ACTIVITY OF ADENOSYL-D-METHIONINE AND ADENOSYL-2-METHYLMETHIONINE IN TRANSMETHYLATIONS*

K. D. Nakamura and F. Schlenk

The D-methionine- and 2-methyl-DL-methionine analogs of the enzymatic methyl donor, (-)*S*-adenosyl-L-methionine, were synthesized by methylation of *S*-adenosyl-D-homocysteine and *S*-adenosyl-2-methyl-DL-homocysteine with methyl iodide. By chromatographic purification, *S*-adenosyl-D-methionine and *S*-adenosyl-2-methyl-DL-methionine were obtained. The structure of the latter was ascertained by hydrolysis to 2-methylmethionine in strong acid, and to 5'-methylthioadenosine and 2-methylhomoserine at pH 4. Reference material of the latter compound was obtained by alkaline hydrolysis of 2-methylmethionine methylsulfonium iodide. The sulfonium compounds were tested as methyl donors with *N*-acetylserotonin *O*-methyltransferase, L-homocysteine *S*-methyltransferase, histamine *N*-methyltransferase, and guanidinoacetate *N*-methyltransferase. In most instances, methyl donor activity was observed.

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*Abstract of a paper published in Arch. Biochem. Biophys. 177, 170 (1976).

HARVESTING THE SUN: A BIOLOGICAL APPROACH*

J. Shen-Miller

The exploitation of natural reserves by man, and the squandering of energy in our daily living will soon leave an earth barren of riches. On the day when we use up our last drop of oil, what will the landscape be? Will it be fields of luscious green trees and plants, or will it be a bleak scene of nuclear plants? A happy medium would be a combination, and now is the time for an interdisciplinary collaboration between biologists and engineers to seek and apply other resources, resources such as the energy of the sun in fuel production. The annual radiation that reaches the earth's surface is 3×10^{21} kJ, which is four orders of magnitude greater than the total global energy consumption of 1970. The United States seems to see no great urgency in developing this renewable resource, but I hope this view will change. It cannot be emphasized enough that this is the time to give this matter a high priority and substantial funding.

According to one estimate, the times for average depletion rate of global coal, oil, and gas reserves are 150, 50, and 49 yr, respectively. Judging from the demand for energy, with the assumption of an increasing consumption per capita, the fossil fuel reserves of the United States have a depletion time of 500 yr. Nuclear power is most likely the major practical hope, but the development of reactors and other energy sources (geothermal, wind, etc.) will take time, and alternate approaches are just as essential for supplementing energy needs. (Even the diehard nuclear enthusiasts do not wish to put all our eggs in the nuclear basket.) Tapping of solar energy for fuel is just such an alternative. This article will cover only the biological harvest from the sun; photoelectric and photochemical approaches are other means of harvest.

Green plants, converting energy from the sun, synthesize cellulose, which is the most abundant organic compound on earth; about 10^{11} tonnes (equivalent to 2204 lb, or 1.102 ton) are produced annually. Cellulose is a clean fuel which does not add accumulated CO_2, sulfur and radionuclides into the atmosphere, and it is a renewable resource. The major sources of cellulose come from agricultural, forest, and aquatic production, most of which are disseminated into agricultural, industrial, and municipal wastes. The contribution of individual sources of cellulosic waste is seemingly minute, a fraction of a percent of the total energy demand; however, adding them together could amount to 8% of the total estimated U.S. energy demand for 2000, and a monetary value of 48.3 billion dollars (assuming an average retail cost for 2000 to be $\$2.84/10^6$ kJ). The above estimate is based on the cellulosic waste alone. If large scale "energy farming" is employed, and research (basic and applied) in this area receives greater support, the increase in the supply of cellulose will be manyfold greater. Although nuclear power plants play a dominant role in fuel development, presently they contribute only 3% of the total energy supply in the U.S. In this article, I will discuss the various cellulosic sources, gross fuel production from each source, some of the fuel production processes and problems, and the necessary areas of biological research.

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*From the Introduction of a paper submitted for publication.

LIGHT AND CORN-ROOT GEOTROPISM: THE INVOLVEMENT OF PHYTOCHROME*

J. Shen-Miller

The primary roots of corn (Wisconsin hybrid 64A x 22R) show positive geotropism following exposure to light. This confirms the works of other investigators. The curvature response begins at about 1 hr following irradiation and reaches a plateau at 5 hr. A study of wavelengths 350-760 nm, using energies of 2.24×10^{14} photon·cm^{-2} and exposure times 60 sec, shows that the most effective light is at 660 nm with lesser effectiveness at 460 and 560 nm. The responses at 660 and 460 nm are reversible by a far-red (730 nm) exposure, indicative of the participation of phytochrome. Analyses of fresh tips of corn roots with a dual-wavelength difference photometer show the phytochrome content in the root to be about 0.16 $\Delta(\Delta OD)$ per gram fresh weight. The requirement of light for the geotropic growth response of corn roots might be an adaptive phenomenon.

It has been shown by others that some intermediates of phytochrome absorb in the green wavelengths. If the 560 nm peak we observed represents an intermediate, then this intermediate is active in the regulation of corn root geotropism. That the 560-nm response showed no reversibility by the 730 nm radiation could be due to an insufficient level of energy used in the far-red. The occurrence of photomorphogenic activity in the green should be of concern to those who use green as the "safe" light in "dark" experiments.

*Abstract of a paper submitted for publication.

PARTICIPATION OF CELLULAR ORGANELLES IN GROWTH AND GEOTROPISM IN OAT COLEOPTILES*

J. Shen-Miller

For three quarters of a century, botanists have been concerned with the gravity sensing mechanism in plants. Geotropism, the plant response to gravity, is the curvature of roots and shoots when their positions are deviated from the normal position relative to gravity. Although plants have no special organs for sensing gravity, such as the statocyst of the crayfish and semicircular canals of the inner ear of vertebrates, plants can detect and respond to centrifugal forces less than 0.001 *g*. In 1900, Nemec and Harberlandt independently showed that plastid amyloplasts were the cellular inclusions that quickly showed an asymmetrical distribution when the plant was reoriented. These starch-filled plastids fell rapidly to the bottom wall of the cells upon reorientation, thus establishing a foundation for the statolith theory of geotropism in plants. Studies over the years have been

*From the Introduction of a paper submitted for publication.

overwhelmingly in support of the theory that the amyloplast is the statolith: sedimentation of this organelle imparts a pressure on the cell membrane, thereby initiating a growth response. However, as yet, there is no unequivocal evidence that the correlative phenomena have a causal relation to gravity perception involving the amyloplasts. Further, no correlation has been found between the quantity and sedimentation of amyloplasts and the initial tropistic curvature.

Our interest in the participation of cellular organelles in geotropism began with the dictyosomes, which collectively form the Golgi apparatus. The geotropic curvature is a differential growth response; the plant hormone indoleacetic acid (IAA) participates in this growth. The dictyosomes and their vesicles take part in cell expansion and growth where new cell wall and membrane originate. In actively growing cells, the rate of dictyosome production and the movement of dictyosomal vesicles to the cell periphery are rapid; the timing is in keeping with the first appearance of the curvature response (25-30 min).

EFFECTS OF INDOLEACETIC ACID ON THE QUANTITY OF MITOCHONDRIA, MICROBODIES, AND PLASTIDS IN THE APICAL AND EXPANDING CELLS OF DARK-GROWN OAT COLEOPTILES*

J. Shen-Miller and S. R. Gawlik

We determined the number of mitochondria, microbodies, and plastids in dark-grown oat coleoptiles following incubation in indoleacetic acid (IAA) for a period of 60 min at 6-min intervals. In the apical outer epidermis of coleoptiles, the mitochondria increased from 31.4 to 35.0 per cell section within 6-min incubation in IAA, and this persisted over the 60-min incubation. Neither the microbodies, plastids, nor the dictyosomes responded to the hormone. The apical parenchyma showed no change in quantity of any of the organelles including the dictyosomes during IAA incubation. The quick response of mitochondria in the coleoptile tip could be interpreted as an association of this organelle with hormone transport, growth, or perhaps with gravity perception. In the subapical expansion region, IAA caused significant reduction of mitochondria, microbodies, and dictyosomes in the outer epidermis compared to the control, the timing of which preceded the IAA-induced elongation and geotropism. The fast response of organelles in the various cells is most likely a change in organelle volume rather than number. That microbodies show a response to the plant hormone in the permanently achlorophyllous epidermis indicates that these organelles, in addition to their peroxisomal functions in green leaves, also have a growth regulation function. IAA treatment was without effect on the quantity of the various types of plastids (including the amyloplasts) in the different oat coleoptile cells. This finding sheds no light on the role of amyloplasts as sensors in geotropism.

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*Abstract of a paper submitted for publication.

REGIONS OF DIFFERENTIAL CELL ELONGATION AND MITOSIS, AND ROOT MERISTEM MORPHOLOGY IN DIFFERENT TISSUES OF GEOTROPICALLY STIMULATED CORN ROOT APICES*

J. Shen-Miller, Rand E. McNitt, and Marty Wojciechowski

We examined cell length, mitosis, and root meristem "cuticle" in different tissues of geostimulated, red-light exposed primary roots of corn (Wisconsin hybrid 64A x 22R). The examination was done at 15-min intervals for a period of 240 min. The differential cell elongation was most prominent between 1.5 and 2.5 mm from the root meristem; the outer cortex has the greatest elongation growth, and the upper cells showed significant increase in length compared to the lower. A differential mitosis was also found, the lower tissues having more cells in division. Cell divisions in the cortex and stele were asynchronous; the peak of cortical division preceded that of the stele. Both peaks occurred before the peak of geotropism. A densely stained layer, which we call the cuticle, separates the cap from the root meristem. This layer is thinner at the apex of the root meristem. The diameter of the thin region increased with time and peaked at 180 min after geostimulation, which was coincidental with the peak of the geotropic response. We discuss these cellular responses in relation to the distribution and activity of plant hormones.

*Abstract of a paper submitted for publication.

INHIBITION OF 5'-NUCLEOTIDASE BY CONCANAVALIN A: EVIDENCE FOR LOCALIZATION ON THE OUTER SURFACE OF THE PLASMA MEMBRANE*

Francis A. Williamson, D. James Morré,† and J. Shen-Miller

5'-Nucleotidase activity of rat liver plasma membrane is markedly inhibited by concanavalin A. Taken together with a unilateral pattern of labelling of concanavalin A binding sites with hemocyanin, the results indicate that an allosteric site of the enzyme is at the outer surface of the membrane.

*Summary of a paper published in Cell Tiss. Res. 170, 477 (1976).

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12. MOLECULAR STUDIES

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GROUP LEADER'S INTRODUCTION

Steven S. Danyluk, Group Leader

In the Divisional reorganization, the original Biophysics Group, which had four programs, was reconstituted into the Molecular Studies Group, including molecular studies and research on circadian rhythms, and separate groups in X-ray crystallography and clinical applications of stable isotopes (see Sections 10 and 13 of this report).

Currently the research activities in the Molecular Studies Group are comprised of two major projects; one deals with the structure and function of biological molecules in solution, while the second focuses on mechanisms of circadian regulation in simple eukaryotes and mammals. Both projects are concerned with the definition of organismic, cellular, and subcellular processes in terms of molecular changes and events. Such knowledge is especially vital for elucidation of cytotoxic, mutagenic, and carcinogenic effects of toxicants generated in nuclear and nonnuclear energy production.

Among specific objectives of the first project (S. S. Danyluk and M. MacCoss) are accurate determination of secondary and tertiary structures of key biological molecules in solution, analysis of conformational dynamics for these molecules and the effects of environmental agents thereon, and correlation of structural-conformational data with modes of biological function. To achieve these objectives, a multifaceted program encompassing spectroscopic measurements (principally magnetic resonance methods), synthesis of selectively labeled (^{2}H, ^{13}C) biomolecular analogues, and theoretical calculations has been developed and applied to nucleic acids and other selected biomolecules.

Results achieved to date for nucleic acids include complete conformational descriptions for all commonly occurring ribo- and deoxyribonucleotides and for fifteen diribonucleoside monophosphates in aqueous solution at neutral pH. The dimers cover nearly all of the pair-wise base-base interactions present in tRNA and mRNA. Specific findings of significance for ribonucleic acid structures include evidence for existence of two compact folded structures for dimers, a base-stacked right helical form characterized as *anti*, C3'-*endo*, g^{-}, ω,ω' (300°, 300°), $g'g'$, *gg*, C3'-*endo*, *anti*, and a more loosely stacked loop

form with *anti*, C3'-*endo*, g^-, ω,ω' (80°, 80°), $g'g'$, gg, C3'-*endo*, *anti* orientations. Both forms are in dynamic equilibrium with one or more extended forms, with the extent of stacking increasing in order pyr-pyr < pyr-pur < pur-pur $\lesssim$ pur-pyr, and the ratio of right helical/loop forms determined by base sequence. Interchange between different stacked and extended forms occurs by rotation about P-O3' and P-O5' bonds; the latter are thus pivotal points for the overall dimer conformation. These results reveal an extraordinary balance of conformational forces so linked that a perturbation in one region of the dimer leads to a series of coupled conformational changes throughout the entire molecule. Whether such conformational interrelationships extend to higher subunits is an important question to be answered in studies now underway of key functional segments of tRNA molecules, i.e., the anti-codon loop, dihydrouridine loop, and CCA aminoacyl charging site. Further complementing this work are parallel investigations of chemical and structural properties of potential nucleoside/tide antitumor drugs, drug-nuclei acid complexes, and carcinogen-nucleotide adducts.

The central aim of the second project (C. F. Ehret) is to define in molecular terms the nature and commonality of circadian regulatory processes occurring across a wide range of organisms. The approaches adopted employ a variety of methods including those of comparative molecular biology and biochemistry coupled with novel circadian protocols for entrainment such as light, food, and temperature. A great deal of progress has evolved in recent years, and studies by the circadian regulation group have shown that the circadian clock is a general property of free-living cells; that the circadian oscillation occurs only during infradian growth mode; that UV plays a dominant role in the action spectrum for resetting; that UV resetting is photoreactiveable; and that temporally characteristic (chronotypic) nucleic acid synthesis occurs in the circadian cell cycle.

A particularly interesting series of results obtained in the past year deals with the circadian regulation of cellular energy reserves. Thus a close synchrony is seen among key cellular physiological indices, regulatory enzyme concentrations, and circadian phase in protozoans. This synchrony appears to have analogues in mammalian systems, and if studies currently underway confirm this possibility, a major step forward will have been achieved in defining the basic nature of circadian rhythms in living systems.

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STRUCTURE AND FUNCTION OF BIOLOGICAL MOLECULES IN SOLUTION

Clinton F. Ainsworth, Daniel Cameron, Steven S. Danyluk, Fouad S. Ezra, Steven H. Gray,† Malcolm MacCoss, George Svihla, Ravindra Tewari, and Alice M. Wyrwicz*

This program is primarily concerned with quantitative evaluation of structural/conformational properties of biological molecules in solution, and the use of such information for detailed mechanistic explanations of biological function at the molecular level. Although the structural characteristics of nucleic acids (tRNA, mRNA, DNA) are currently the main focal point, related areas of interest include DNA-binding drugs, immunoglobulins, modified nucleosides/tides, and cell membrane constituents. Of particular interest are conformational changes produced by chemical and/or physical interactions between biological molecules and toxicants released in energy generation.

Over the past several years, the program has evolved along three related directions. A central theme continues to be the determination of accurate conformational data for nucleic acid fragments and other biological molecules in aqueous solution by high-resolution nuclear magnetic resonance spectroscopy. This facet is paralleled by synthesis of novel biomolecular derivatives and precursors in protio and, as needed, in selectively deuterated analogues. Synthesis of the latter compounds led to a breakthrough in NMR spectroscopic analysis of ribonucleic acid dimers (Kondo, N. S., and S. S. Danyluk, J. Amer. Chem. Soc. 94, 5121, 1972). A third component utilizes theoretical quantum mechanical methods to calculate structural/conformational properties, particularly in those instances where spectroscopic measurements are not feasible, i.e., because of compound instability or unavailability. This integrated approach was followed in the successful definition of conformational properties for all of the commonly occurring constituents of RNA and DNA (Davies, D. B., and S. S. Danyluk, Biochemistry 13, 4417, 1974; Davies, D. B., and S. S. Danyluk, Biochemistry 14, 543, 1975) and, in work recently completed (Kondo, N. S., and S. S. Danyluk, Biochemistry 15, 756, 1976; Lee, C-H., et al., Biochemistry 15, 3627, 1976; Ezra, F. S., et al., Biochemistry 16, 1977, 1977), has yielded a complete conformational profile for all possible pairs of diribonucleoside monophosphates. These compounds are the simplest repeating structural units of ribonucleic acids, and the detailed NMR studies have revealed for the first time the pivotal nature of P-03' and P-05' phosphodiester bonds, the relative flexibilities of conformational bonds and sequence effects thereon, and the interconnectedness of conformational perturbations across the entire dimer molecule. From such findings it will ultimately be

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*Fall 1976 participant in the Undergraduate Honors Research Participation Program, University of Minnesota.

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possible to answer fundamental questions concerning the origins of unique tertiary structures in tRNA molecules, the dynamical nature of such structures, and the influence of environmental factors on key structural features.

Further progress was made in the past year in defining conformational properties of nucleic acids, and in identifying the influence of chemical and configurational modifications on such properties. Results from representative projects currently being pursued in spectroscopic, theoretical, and synthesis areas are outlined below.

QUANTITATION OF GLYCOSYL TORSION ANGLES

One of the most important conformational bonds in DNA and RNA molecules links base and sugar rings in nucleotidyl residues. The orientation about the glycosidic bond, χ_{CN}, is critical for complementary hydrogen-bond formation, and is a primary determinant of helix dimensions. Although much research has been done in attempts to fix χ_{CN} values in solution, the results only give a qualitative indication at best of the existence of *syn* and *anti* domains. Over the past year, a novel approach which promises to permit accurate quantitation of χ_{CN} values for pyrimidines was developed. Essentially it involves the accurate measurement of vicinal ^{13}C-proton spin coupling constants along C2-N-C1'-H1' and C6-N-C1'-H1' coupling paths in molecules where rotation about the glycosidic C1-N bonds is restricted completely. By measuring the relevant couplings for a carefully selected series of compounds, an empirical correlation can be established relating χ_{CN} and $J(^{13}C\text{-}H)$. The way is then open for calculation of χ_{CN} from experimentally determined coupling constants. A series of uridine cyclonucleosides, in which the uracil ring is rigidly coupled to the sugar ring thereby fixing χ_{CN} over a range of magnitudes encompassing *syn* and *anti* conformers, was therefore synthesized and the ^{13}C spectra measured at 25 MHz. Analysis of the spectra showed that both couplings, J(C2-H1') and J(C6-H1'), indeed vary with χ_{CN} in a highly specific manner. The data further suggest that when the analytical form of the J versus χ_{CN} relationship is established, the way will be open for quantitative determinations of χ_{CN} in all pyrimidine derivatives.

THEORETICAL CALCULATIONS OF CONFIGURATIONAL EFFECTS IN NUCLEIC ACIDS

Studies from our laboratory and elsewhere have delineated in considerable detail the conformational properties and the nature of intra- and intermolecular forces determining these properties for naturally occurring nucleic acid segments. Thus far only cursory attention has been directed toward the influence of configurational effects on such properties, largely because nucleotides occur in nature predominantly in the β-anomeric form. However, recent work indicates that α-anomers may play a more important role in biological processes than is commonly accepted. A theoretical study was therefore initiated of configurational effects on conformational properties of α- and β-anomers of 3',5'-cyclic purine and pyrmidine nucleotides and 2'-arabino epimers. Using the PCILO (Perturbed Configuration Interaction using Localized Orbitals) method, theoretical calculations were made of the effect of base and 2'-hydroxyl group orientations and ribose ring pucker on overall energy. The results show that steric repulsions and stabilizing effects of intramolecular hydrogen

bonding between the base and 2'-OH group are of major importance in determining conformations of α-anomers and 2'-arabino-β-epimers. For example, hydrogen bonding between 2'OH and polar centers on the base ring is clearly implicated as a determinant of *syn-anti* preferences, i.e., χ_{CN} value in all anomers. Moreover, barrier heights for interconversion between the latter conformers are sensitive to ribose pucker and 2'OH orientations, e.g., when base-ribose interactions are minimal the barrier is small and the *syn/anti* ratio approaches unity. The calculations also show that ribose ring pucker plays an essential role in relieving repulsive interactions between the base and 2'-hydroxyl group. Thus a C3'-*endo*-C2'-*exo* ($^3_2$T) pucker is favored for α-anomers in contrast with the C4'-*exo*-C3'-*endo* ($_4$T^3) form found in β-compounds. Although NMR data are not yet available for all of the compounds, where comparisons are possible there is a gratifying concordance between experimentally and theoretically deduced conformations.

SYNTHESIS OF NOVEL NUCLEIC ACID DERIVATIVES

Synthetic preparations of new and novel nucleosides/tides, and selectively labeled oligonucleotides are a vital component of the overall structural program. Recognition of the importance of this activity was formalized with addition of a new staff member (Dr. Malcolm MacCoss) having extensive experience in synthetic nucleic acid chemistry. Among some of the more significant achievements were: (1) Synthesis of all uridine cyclonucleosides (O^2,3'-cycloU, O^2,5'-cycloU, 2',3'-isopropylidine-O^2,5'-cycloU, and $O^{2'}$,6-cycloU) needed to provide model compounds with fixed glycosyl torsion angles. These have been used to produce a semiempirical method of determining χ_{CN} values using ^{13}C NMR spectroscopy (*loc cit*). (2) Development of a new detritylation procedure (trifluoracetic acid in n-butanol) which permits the mildest known acidic deblocking conditions for trityl groups and allows synthetic transformations not previously possible with this blocking group. This procedure promises to be of great utility in synthesis of modified nucleosides/tides. (3) The synthesis of nucleosides and nucleotides selectively deuterated at known positions continues as an important adjunct of the NMR measurements. During the past year syntheses were undertaken of nucleosides/tides deuterated at specific positions in the sugar ring. This work was initiated in order to determine spectroscopic and conformational parameters in various nucleic acid derivatives, e.g., NAD, various dimers, and the assignment of the H5', H5" resonances in nucleosides and nucleotides.

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CIRCADIAN CYBERNETICS AND CHRONOTYPIC ORGANISMIC SENSITIVITY TO ENVIRONMENTAL FACTORS

Charles F. Ehret, Kenneth Groh, John C. Meinert, and George Svihla

Progress in understanding the mechanisms underlying the circadian biological clock at cellular and organismic levels has advanced considerably in recent years, and the circadian regulation group at Argonne has pioneered in

many of the basic advances. It was first shown here that the circadian clock is a general property of free-living cells and occurs independently of photosynthesis; that the circadian oscillation occurs only during the infradian growth mode; that UV plays a dominant role in the action spectrum for resetting; that resetting by UV is photoreactiveable; and that temporally characteristic (chronotypic) nucleic acid synthesis occurs in the circadian cell cycle. Out of these studies, a general theory for the control of the circadian biological clock by gene action (the Chronon Theory) emerged, and this has remained the single tenable and dominant heuristic molecular theory for nearly 10 years.

During the past year fundamental studies at the cellular level have focused upon what appears to be in intermediary metabolism another very general yet critical component of the eukaryotic circadian clock: the circadian storage and depletion of energy reserves. During the infradian growth mode, as a function of circadian phase, key regulatory enzymes reach their peaks and troughs at predictable phases of the circadian cycle. Such chronotypic enzymes (Figure 12.1) show up on precise schedule with the respiratory and molecular expectations and contingencies of programmed glycogenolysis. Thus a strong correlation is seen to exist between the regulation of glycogen metabolism (circadian chronotypic glycogen depletion) and other known physiological manifestations of the circadian clock (respiration, cell-division probability). Comparison of these findings in the protistan *Tetrahymena* with comparable measures of the chronotype of the vertebrate hepatic system (rat) suggests that the circadian regulation of energy reserves is very similar in widely disparate species, and is a general eukaryotic feature, summarized in Figure 12.2 as a general model for the circadian clock. Elucidation of these fundamental molecular properties of the circadian biological clock provides not only the best clues toward an ultimate satisfactory explanation of the basic nature of the clock, but also provides a handle on environmental and pharmacological control options in the applied domain of circadian bioengineering. In the latter area, the chronobiotic action of theophylline and of pentobarbital had already been shown by the Argonne group in the rat some years ago, and is now being applied, along with programmed feeding protocols which stimulate either the tyrosine → catecholamine pathway (a high protein meal) or the tryptophan → serotonin pathway (a high carbohydrate meal), in order to learn more about rapidly resetting the circadian clock in higher organisms. That resetting cannot occur without some cost in the form of dyschronism and increased risk of cancer is already suggested by the cellular theory and the mammalian data: chronobiotics and carcinogens may thus strongly overlap each other categorically.

Further details of the work described above will be found in the following abstracts.

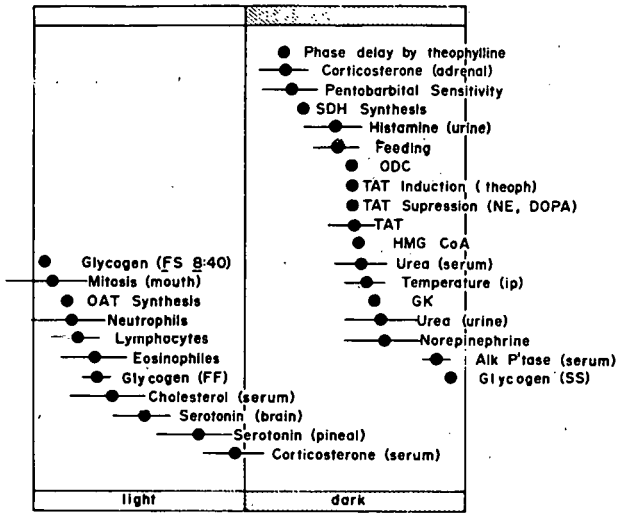

Fig. 12.1. Circadian chronotype of the rat. Temporally characteristic molecular and physiological properties of the rat taken from experiments by various investigators. The horizontal bars represent 95% confidence intervals around the mean for the acrophase as calculated by the cosinor method of F. Halberg et al. (Physiol. Teacher 1, 1, 1972).

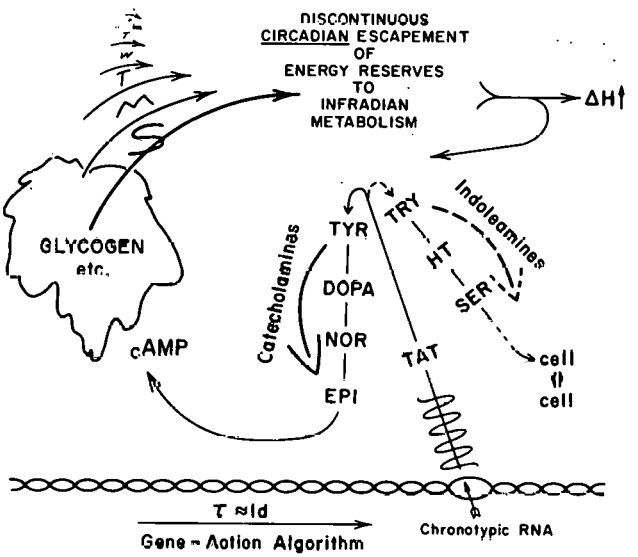

Fig. 12.2. An energy-reserve escapement with alternate path options (the one choice being if catecholamines, then "release"; vs. if indoleamines, then "hold") is coupled to a gene-action circadian oscillator that generates chronotypic enzymes, such as tyrosine aminotransferase (TAT) that control the path of choice. This scheme results in long-term conservation of energy reserves through circadian metering during infradian growth. During diurnal entrainment, glycogen depletion occurs daily, represented by the arrows and days-of-the-week (upper left).

THE INFRADIAN EUKARYOTIC CELL: A CIRCADIAN ENERGY-RESERVE ESCAPEMENT*

Charles F. Ehret and Kenneth W. Dobra

Eukaryotic cells in the infradian growth mode possess the extraordinary capacity to continue their cell cycle (of syntheses of chronotypic macromolecules) with a "constant" (circadian) periodicity, even when external environmental pressures have caused a virtual cessation of the cell division cycle, and independently of the generation time. In evolutionary perspective this circadian-infradian option appears as a novel strategy of primitive eukaryotes confronted by a major problem of survival: how to endure the famine that follows a feast? The solution arrived at by the fittest, and since then exploited in other ways in all of the eukaryotic phyla of higher plants and animals, was to enter the infradian mode well endowed with a store of energy-reserve polymer, and then to dole, parcel, budget, aliquot, and meter-out in discontinuous daily rations these reserves by means of a circadian energy-reserve escapement, common in each of its most general properties to all eukaryotes.

*Abstract of a paper to be published in Chronobiologia.

CIRCADIAN REGULATION OF GLYCOGEN, TYROSINE AMINOTRANSFERASE, AND SEVERAL RESPIRATORY PARAMETERS IN SOLID AGAR CULTURES OF *Tetrahymena pyriformis**

Kenneth W. Dobra and Charles F. Ehret

At the cellular level, in *Tetrahymena* cells entrained by light-dark cycles to circadian synchrony, a characteristic chronology of molecular activity is seen: tyrosine aminotransferase (TAT) reaches its peak in the middle of the dark phase, and is followed by cyclic AMP, which peaks several hours later; the rise of cAMP immediately precedes a precipitous decline in glycogen which continues into early light phase; ATP levels reach a peak during or shortly after the highest rates of glycogen depletion, and are about 180° (12 h) "out of phase" with the peak for TAT. Comparison of these findings with earlier measures of the chronotype of the rat show that the circadian chronology of glycogen storage and depletion is remarkably similar in the two species. The findings that single celled protozoa have the same biochemical components, that both glycogen and TAT oscillate with a circadian period, and that catecholamines can alter the activity of adenyl cyclase in the protozoan as well as in the rat, suggest the involvement of chronotypically homologous regulatory pathways for the circadian metabolism of glycogen in very diverse phyla.

*Abstract of a paper to be published in Chronobiologia.

CIRCADIAN REGULATION: GROWTH KINETICS OF THE INFRADIAN CELL*

Charles F. Ehret, John C. Meinert, Kenneth R. Groh, Kenneth W. Dobra, and Gregory A. Antipa

There are two ways in which most eukaryotic cells live--one is in the fast exponential or ultradian mode of growth, in which cell generation times (GT's) are considerably shorter than a day, the other is in the slow exponential or infradian mode of growth in which average cell generation times ($\overline{GT}$'s) are somewhat (or considerably) longer than a day. Although the preponderance of eukaryotic cells in the plant and animal life of the biosphere at any given moment (including the cells present in a mature metazoan such as man) are in the infradian mode of growth, the preponderance of research on the regulation of cell division has been performed to date on cells in the ultradian mode. Circadian oscillations occur during the infradian growth mode. As cells enter the infradian mode of growth, most often induced by a precipitous decline in dissolved oxygen as the respiratory rate of the cell population exceeds the oxygen capacity of the medium and diffusion rate of oxygen into the medium, the ability to synthesize energy storage compounds (e.g., glycogen for *Tetrahymena*) increases by a factor as great as 20 times that of the well-oxygenated ultradian mode. This increase in glycogen storage, manifested at the cellular level as an enormous increase in the amount of glycogen granules in the cytoplasm of *Tetrahymena*, prepares the cells for the relatively anaerobic infradian mode where the energy available through aerobic respiration is about 5% that available during the ultradian mode. Thus, control of culture conditions, particularly the temperature and oxygen levels, is important in determining not only the maximum levels of the compounds of interest but also rapid changes that might be observed in the amount of activity of enzymes with small perturbations in the physiological environment. Infradian, essentially anaerobic cultures of *Tetrahymena* can be induced to begin aerobic respiration leading to division by increasing the amount of available oxygen (Pasteur effect), and this inducibility is in turn dependent upon the circadian phase of the infradian culture. The fact that the Pasteur effect occurs with a circadian rhythm causes lag itself (i.e., the infradian-ultradian transition interval) to behave as a circadian function during induction of an infradian population, provided that the infradian cells are in circadian synchrony. If the infradian population is asynchronous at the time of induction (as is commonly the case), then lag becomes an integral function of the circadian phase angles of all participating cells.

A fundamental mark of circadian regulation is the capacity of infradian eukaryotes to uncouple generation time from cell cycle time, yielding periods that are nearly constant and usually circadian. The remarkably similar oscillations of depletion and synthesis of energy reserves in protistans and in mammalian organs during infradian growth lend support to a unifying view that chronotypic gene action and chronotypic regulatory enzymes (such as tyrosine aminotransferase) and overt behavior are linked by the causally interconnected oscillations of glycogenolysis and of biogenic amine metabolism.

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One may thus discern, throughout the eukaryotes in general, parallels in cellular and molecular regulatory mechanisms that transcend three previously disparate fields of biological study: the ultradian cell division cycle, the protistan and cellular circadian cycle, and the circadian cycle of chronotypic enzymes, hormones, and behavioral patterns in higher organisms. The simplest theory consistent with the data is that the gene-action clock that programs the ultradian cell division cycle to iterate the genomic algorithm as rapidly as possible (even at the cost of temperature dependence) in well-nourished cells continues to program the circadian cycle of diffusion-limited and temperature-independent iteration of energy-reserve depletion in infradian cells. The iteration period (CT) may be very short (in feast) but in the course of $\sim 10^9$ years of natural selection is, in wild populations, genetically restricted (even in famine) to be no longer than $\tilde{\sim}$ 24 hours.

THE ONCOGENIC IMPLICATIONS OF CHRONOBIOTICS IN THE SYNCHRONIZATION OF MAMMALIAN CIRCADIAN RHYTHMS: BARBITURATES AND METHYLATED XANTHINES*

Charles F. Ehret and Kenneth W. Dobra

Methylated xanthines and barbiturates are not only chronobiotics, but are also oncogens or cocarcinogens. Low levels of phenobarbital in the diet induce circadian dyschronism in rats: conversely, *ad libitum* dietary regimens that tend to favor dyschronism in old rats also correlate with high tumor incidence. Epidemiological evidence shows that persons at high risk of cancer include those who have had genetic deficiency diseases or who have taken drugs that are known to interfere with the putative molecular-genetic pathways for circadian regulation: those interfering especially with the storage and regulation of energy reserves, and the regulation of the catecholamine and indoleamine synthetic pathways. It is suggested that each time that the circadian biological oscillator in a cell is reset by a chronobiotic, or by any other circadian zeitgeber, there is increased risk of oncogenic damage to the gene-action machinery of the cell; for this reason all chronobiotics may be oncogenic when taken at a vulnerable phase of the circadian cycle.

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*Abstract of a paper to be published in the Proceedings of the Third International Symposium on the Detection and Prevention of Cancer, Marcel Dekker, New York.

CIRCADIAN CYBERNETIC STUDIES OF THE MAMMALIAN CLOCK: A NEW SYSTEM TO STUDY THE ROLES OF PROGRAMMED FEEDING AND OF CHRONOBIOTICS IN RAPIDLY RESETTING THE PHASE OF THE DEEP-BODY TEMPERATURE RHYTHM*

Charles F. Ehret, John C. Meinert, Jay Schlabach,† Lawrence Lauder,‡ and John Waters‡

Since we had shown earlier that the clock-resetting capacity of a chronobiotic such as theophylline can be detected even in the presence of two other powerful zeitgebers such as food and light, it appeared essential to construct a facility equipped to handle multiple zeitgebers and temperature telemetry data acquisition automatically for 40 rats. Because high protein meals tend to favor synthesis of catecholamines, and high carbohydrate meals tend to favor serotonin, and because these biogenic amines are chronotypically high or low (norepinephrine and epinephrine high during the active phase, serotonin high during sleep), it appeared desirable to split the FS (feed-starve) program into two new phases for F: one early (like "breakfast"), the other late (like "supper"). This separation has been accomplished in the new system by means of clock-controlled, motor-driven food hoppers, two to a cage, that enter or leave the cage on program. The system is supplemented by programmed watering, which permits programmed administration of chronobiotics and of water intake through a system of clock-controlled solenoid valves. The data acquisition system, recording deep-body temperature from radio-telemeters implanted in the peritoneal cavity, can interrogate 40 rats/minute for extended periods of time for the purpose of recording data on ultradian episodic oscillations. The normal interrogation frequencies are 4 times/hour for circadian data collection.

*Abstract of a paper in preparation.

†Spring 1976 participant in the Undergraduate Honors Research Participation Program, Goshen College.

‡Members of Argonne National Laboratory's Explorer Post 609, L. Lauder from Downers Grove South High School and J. Waters from Hinsdale Central High School.

THE EFFECT OF (2'-5') AND (3'-5') PHOSPHODIESTER LINKAGES ON CONFORMATIONAL AND STACKING PROPERTIES OF CYTIDYLYL-CYTIDINE IN AQUEOUS SOLUTION*

F. S. Ezra, N. S. Kondo, C. F. Ainsworth, and S. S. Danyluk

Conformational properties of (2'-5') and (3'-5') CpC have been determined by proton magnetic resonance spectroscopy at 220 MHz. The ribose ring structures are predominantly ^3E with the exception of the ring from the

*Abstract of a paper published in Nucleic Acids Research 3, 2549 (1976).

2'-phosphate fragment of C(2'-5')pC which exhibits an ^2E pucker. Bases are oriented *anti* with respect to the ribose and the conformations about C4'-C5', C5'-O5', C3'-O3' (C2'-O2') are *gg*, *g'g'*, and $g^+ \rightleftharpoons g^-$, respectively. The dimers exist as mixtures of stacked (g^+g^+ and g^-g^- about the P-O(C) bonds) and unstacked species at 20°C. Stacking is estimated to be 35% in both dimers.

CONFORMATIONAL PROPERTIES OF PURINE-PYRIMIDINE AND PYRIMIDINE-PURINE DINUCLEOSIDE MONOPHOSPHATES*

Fouad S. Ezra, Che-Hung Lee,† Norman S. Kondo, Steven S. Danyluk, and Ramaswamy H. Sarma‡

The detailed conformational features and dynamics of hetero-dinucleoside monophosphates ApU, ApC, GpU, GpC, UpA, CpA, UpG, and CpG have been studied in aqueous solution by high field NMR spectroscopy. Analysis of the resultant NMR parameters leads to a number of discernable trends throughout the series. Thus the ribose rings of the dimers exist as equilibrium mixtures of C2'-*endo*(^2E) $\rightleftharpoons$ C3'-*endo*(^3E) conformers with a proclivity for the ^3E pucker in most cases; the C4'-C5' bonds of both nucleotidyl units show significant preference (74-96%) for a *gg* conformation; and the dominant conformer (85-89%) about C5'-O5' is *g'g'*. Orientation about the C3'-O3' bond is coupled to the ribose conformational equilibrium and the system exists with a bias for the $^3Eg^-$ coupled conformation in which the H3'-C3'-P dihedral angle occupies the narrow range of 33-35°.

Dimerization, on the average, causes about 10% increase in *gg* and *g'g'* populations and the g^- domain becomes increasingly populated about the C3'-O3' bond. The ribose equilibrium $^2E \rightleftharpoons {}^3E$ shifts in favor of ^3E upon dimerization, the effect being very conspicuous for the pu-py series ($\sim$ 40 → 60%) and less noticeable for the py-pu systems ($\sim$ 47 → 58%), clearly suggesting a correlation between sequence and ribose conformational equilibrium. The temperature and dimerization data for the hetero-dinucleoside monophosphates show that the transition $^2E \rightarrow {}^3E$ is directly related to χ_{CN} changes induced by dimerization and stacking. Analysis of the ribose coupling data shows that the percentage populations of stacked species vary from dimer to dimer with GpC displaying a maximum of 45% stacked population and UpG about 10%. However, in general, the pu-py dimers show a higher preference (27-45%) for stacked conformations than py-pu dimers (10-25%).

It is proposed that the pronounced deshielding of H5' of the 5'-nucleotidyl units upon dimerization is associated with the presence of right handed stacks (g^-g^-) whereas the chemical shift trends of H5' and H5" of

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3'-nucleotidyl units are due to the presence of left handed stacks (g^+g^+) in all the dimers. In pu-py dimers the population of the g^-g^- species is found to be greater than that of g^+g^+. Also the population of g^-g^- stacks in pu-py dimers is generally greater than in their corresponding matched py-pu dimers. Thus the base sequence has not only an explicit effect on the overall populations of the stacked species, but also on the handedness of the stacks. The present results further confirm the interdependence of conformational bonds throughout the nucleotidyl framework.

PROTON MAGNETIC RESONANCE STUDY OF THE DYSPROSIUM(III) AQUEOUS COMPLEX*

Joseph Granot† *and Daniel Fiat*

Proton field shifts in dysprosium perchlorate aqueous solutions were measured as function of temperature and dysprosium concentration. The contact and psuedo-contact shifts were separated by means of a least-squares method based on their different temperature variation (as T^{-1} and T^{-2}, respectively). The hyperfine coupling constant between the dysprosium unpaired electrons and the proton nuclei, and the spin density at the proton nuclei were calculated. The results were compared with those obtained for other lanthanides and interpreted in terms of structural changes. Linewidth measurements yielded an estimation for the water exchange rate. The electronic relaxation times were calculated and the activation energies for the relaxation processes present in the solution were obtained.

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*Abstract of a paper published in J. Magn. Reson. 19, 372 (1975).

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STRUCTURE OF THE CONCANAVALIN A-METHYL α-D-MANNOPYRANOSIDE COMPLEX AT 6-Å RESOLUTION*

Karl D. Hardman and Clinton F. Ainsworth

The carbohydrate binding site of concanavalin A has been identified in crystals of the concanavalin A-methyl α-D-mannopyranoside complex and is 35 Å from the iodophenol binding site (K. D. Hardman and C. F. Ainsworth (1973), Biochemistry 12, 4442), which has been postulated to be adjacent to the carbohydrate-specific binding site (Edelman et al. (1972), Proc. Natl. Acad. Sci. USA 69, 2580). The crystals are orthorhombic in space group $C222_1$ and crystal density measurements indicate a protein mass of four monomers (molecular weight of 104,000) per asymmetric unit. However, the electron

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*Abstract of a paper published in Biochemistry 15, 1120 (1976).

density map contains eight monomers/asymmetric unit, revealing lattice disorder. The electron density map with a nominal resolution of 6 Å has been solved using three heavy-atom derivatives and the position and orientation of each monomer established. Atomic coordinates of the native protein which has previously been determined (K. D. Hardman (1973), Adv. Exp. Med. Biol. 40, 103) were transposed into this new space group and the gross conformations of the monomers, dimers, and tetramers were found to be very similar to the previous structure. However, some minor differences were apparent even at this resolution. After crystal growth, the methyl α-D-mannopyranoside was replaced by *o*-iodophenyl β-D-glucopyranoside or methyl 2-iodoacetimido-2-deoxy-α-D-glucopyranoside in separate experiments, and difference electron density maps were calculated. The highest peaks for both iodinated sugar derivatives associated with each monomer agreed within a few angstroms of each other and were found near side chains Tyr-12 and -100 and Asp-16 and -208. This region is 10-14 Å from the manganese, in good agreement with nuclear magnetic resonance (NMR) studies in solution (C. F. Brewer et al. (1973), Biochemistry 12, 4448) and with the site predicted from cross-linked I222 crystal studies (K. D. Hardman (1973), Adv. Exp. Med. Biol. 40, 103).

CONFORMATIONAL PROPERTIES OF ADENYLYL-3'→5'-ADENOSINE IN AQUEOUS SOLUTION*

Norman S. Kondo and Steven S. Danyluk

A detailed 220-MHz NMR study has been made of the conformational properties for the homodinucleotide adenylyl-3'→5'-adenosine, ApA, in D_2O. Unambiguous signal assignments of all proton signals were made with the aid of selectively deuterated nucleotidyl units, *ApA, Ap*A, and D-8ApA, and complete, accurate sets of NMR parameters were derived by simulation-iteration methods. Sets of limiting chemical shifts and coupling values were also obtained for ApA and constituent monomers 3'-AMP and 5'-AMP at infinite dilution and at identical ionization states for assessment of dimerization effects. Conformational properties were evaluated quantitatively for most of the conformational bonds of ApA and these are consistent with two compact folded dynamically averaged structures, a base-stacked right helical structure, I, characterized as anti, C3'-endo, g^-, ω,ω' (320,330°), $g'g'$, gg, C3'-endo, anti, and a more loosely base-stacked loop structure, II, with anti, C3'-endo, g^-, ω,ω' (80°,50°), $g'g'$, gg, C3'-endo, anti orientations. Dimerization produces a number of nucleotidyl conformational changes including a shift in ribose equilibrium C2'-endo (*S*) $\rightleftarrows$ C3'-endo (*N*) in favor of C3'-endo in both Ap- and -pA (60:40 vs. 35:65 in monomers), a change in glycosidic torsion angle χ_{CN} toward 0°, and a greater locking-in of rotamers along bonds involved in the phosphodiester backbone. Moreover, there is clear evidence that the transitions from $S \rightarrow N$ forms and $\chi_{CN} \rightarrow 0°$ are directly related to base stacking in ApA. Finally, ApA exists in solution as an equilibrium between I, II and an unstacked form(s) with as yet undetermined conformational features.

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*Abstract of a paper published in Biochemistry 15, 756 (1976).

Since C4'-C5', C5'-O5', and C3'-O3' bonds possess exceptional conformational stabilities, it is proposed that destacking occurs primarily by rotation about P-O5' and/or O3'-P. Predominant factors influencing the overall ApA conformation are thus base-base interaction and flexibility about P-O5' and O3'-P, with change of ribose conformation occurring in consequence of an alteration of χ_{CN}, the latter in turn being governed by the need for maximum π overlap of stacked adenine rings.

CONFORMATIONAL PROPERTIES OF DINUCLEOSIDE MONOPHOSPHATES IN SOLUTION: DIPURINES AND DIPYRIMIDINES*

Che-Hung Lee,† *Fouad S. Ezra, Norman S. Kondo, Ramaswamy H. Sarma,*‡ *and Steven S. Danyluk*

In order to obtain information about the conformational features in a polyribonucleotide at the nearest neighbor level, detailed nuclear magnetic resonance studies of the dinucleoside monophosphates ApA, ApG, GpA, UpU, CpC, UpC, and CpU were undertaken. Proton spectra were recorded at 100, 220, 270, or 300 MHz for D_2O solutions, 0.01-0.03 M, pD 7.4 at 20 ± 2°C. Spectra of ApA, ApG, UpU, and UpC were also recorded in the temperature range of 70-90°C. Unambiguous signal assignments of all proton resonances were made with the aid of selectively deuterated dimers. Complete, accurate sets of nuclear magnetic resonance (NMR) parameters were derived for each nucleotidyl unit by simulation-iteration methods. A complete set of chemical shift and coupling constant data was also obtained for all the constituent monomeric units at a concentration and ionization state comparable to that of the dimers. Conformational properties were evaluated quantitatively for most of the bonds in the dinucleoside monophosphates using procedures developed in earlier studies. All of the dimers have a flexible conformational framework in aqueous solution. While flexibility is allowed and alternate conformations are accessible, these molecules nevertheless attempt to achieve conformational identity by showing preferences--sometimes overwhelming preferences--for certain orientations. Thus the ribose rings exist as equilibrium mixtures of C2'-endo $\rightleftarrows$ C3'-endo conformers with a bias for the C3'-endo pucker in most cases. The C4'-C5' bonds of both nucleotidyl units show significant preference (70-85%) for a gg conformation. Similarly, the dominant conformer (80-90%) about C5'-O5' is g'g'. Even though an unambiguous determination of the orientation about C3'-O3' cannot be made, there is suggestive evidence that the orientation of the 3' phosphate group is coupled to the ribose conformational equilibrium and it is likely that a $^3Eg^- \rightleftarrows {}^2Eg^+$ equilibrium exists with a bias for the $^3Eg^-$ coupled conformation in which the H3'-C3'-O3'-P dihedral angle is about 34-38°. The individual nucleotidyl units in the dimers differ in several key ways from corresponding monomer conformations. Specifically, the ribose equilibrium C2'-endo $\rightleftarrows$ C3'-endo shifts in

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favor of C3'-endo upon dimerization, the only exception being UpU. The C4'-C5' and C5'-O5' bonding network in the dimer forms a stable conformational unit and no correlation exists in the dimers between the conformational preference of this fragment and ribose conformer population. The temperature data for the dimers and dimerization data clearly indicate that the transition C2'-endo → C3'-endo is directly related to χ_{CN} changes brought about by dimerization and stacking. Such coupling of ribose conformation with stacking enabled a quantitation of the ratio of stacked and unstacked populations for a given dimer from ribose couplings. The percentage populations of the stacked species vary from dimer to dimer with ApA and CpC displaying a maximum of 35-40% stacked population and UpU less than 10%. It is further shown that the stacking process can also be monitored by the shift non-equivalence for H5',H5" of the 5'-nucleotidyl unit of the dimer. The data are in accord with the existence of extended conformations in equilibrium with two folded structures, a compact base-stacked right helical structure and a loosely base-stacked loop structure characteristic of a left helical form. It is suggested that the O3'-P-O5' frame is highly flexible and acts as the swivel enabling the 3'- and 5'-nucleotidyl units to stack-destack depending on the nature of the π-ring system in the bases and solvent and temperature conditions. Stacking will cause a modest decrease in χ_{CN} with an accompanying increase in C3'-endo populations, the latter in turn shifting the 3' phosphate group from g^+ to g^- domains. In short we envision a coupled series of conformational events at the onset of stacking made feasible by the swivel nature of the O3'-P-O5' bridge.

A NEW ASPECT OF THE USE OF RIBONUCLEOSIDE 2',3'-O-ISOPROPYLIDENE DERIVATIVES FOR INVESTIGATION OF ANOMERIC CONFIGURATION*

Malcolm MacCoss, Morris J. Robins,† Bernard Rayner,‡ and Jean-Louis Imbach‡

A new method of determining the anomeric configuration of ribonucleosides using their 2',3'-O-isopropylidene derivatives is described. This procedure utilizes the multiplicity of the H4' resonance in the NMR spectra of these derivatives. Due to steric interactions between the base and the isopropylidene group in the α-anomers, the sugar takes up a conformation such that the dihedral angle between H3' and H4' approaches 90°. This gives rise to a triplet for the H4' resonance ($J_{4'5'} \simeq J_{4'5''}$). In the β-anomers, H4' gives rise to a multiplet (higher than triplet) due to coupling of H4' to H3' as well as H5' and H5". This procedure works well for derivatives which are not amenable to other spectroscopic methods for determining anomeric configuration.

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*Summary of a paper to be published in Carbohydrate Research.

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13. CRYSTALLOGRAPHY

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GROUP LEADER'S INTRODUCTION

Marianne Schiffer, Acting Group Leader

The Crystallography Group, formerly part of the Biophysics Group, was instituted as a separate group in January, 1976, under the direction of A.B. Edmundson. In the past several years, the program has made significant contributions to the understanding of immunoglobulin function by elucidating the Mcg Bence-Jones protein structure at high resolution. During 1976, A. B. Edmundson, K. R. Ely, and E. E. Abola left the Division for the University of Utah. The work on immunoglobulins will continue both at Argonne and at Utah, with some of the projects involving the Mcg Bence-Jones and myeloma proteins continued in collaboration. The study of the Mcg myeloma protein will continue mainly at Utah. This annual report primarily covers the work of the Crystallography Group as it is now constituted (see staff listing, below).

Immunoglobulins are part of the body's defense mechanisms against invading foreign agents such as pollutants and pathogens. Their function is to recognize foreign agents and then trigger the body's immune response. To understand how the immunoglobulins function, and what accounts for their high degree of specificity, X-ray diffraction studies of single crystals of Bence-Jones and myeloma proteins have been carried out. This is the only technique by which the positions of all the atoms within the molecule can be determined.

CRYSTALLOGRAPHY STAFF

REGULAR STAFF

*Edmundson, Allen B. (Senior Biochemist)
*Ely, Kathryn R. (Scientific Associate)
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TEMPORARY STAFF DURING 1976

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*Firca, Joseph R. (Postdoctoral Appointee)
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*Terminated during 1976.

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X-RAY CRYSTALLOGRAPHIC STUDIES OF IMMUNOGLOBULINS

Enrique E. Abola, Allen B. Edmundson, Kathryn R. Ely, Joseph R. Firca, Nicolas C. Panagiotopoulos, Marianne Schiffer, and Florence A. Westholm

Mcg BENCE-JONES PROTEIN AND IgG MYELOMA PROTEIN

The structure of the Mcg Bence-Jones dimer (λ-type light chain) has been determined (Schiffer, M., et al., Biochemistry 12, 4620, 1973), and its atomic positions are currently being refined by crystallographic procedures (Deisenhofer, J., and W. Steigemann, Acta Crystallogr. B31, 238, 1975). The atomic model was based on 2.3-Å resolution data. The Bence-Jones dimer closely resembles an antigen binding (Fab) fragment of a functional antibody molecule, both in its structure and function. It can bind hapten-like molecules (Edmundson, A. B., et al., Biochemistry 13, 3816, 1974), such as dinitrophenyl compounds, in its binding cavity which is formed by the variable domains of both monomers. One monomer is similar in conformation to the heavy chain component, the other to the light chain component of the Fab fragment. The structures of the like domains are the same, but the conformation of the switch peptides connecting the variable and constant domains differs in the two monomers. The variable and constant domains also have a similar fold, but during evolution their sequence changed to be appropriate to their new function (Edmundson, A. B., et al., Biochemistry 14, 3953, 1975).

The Mcg Bence-Jones dimer was compared with a Fab fragment from IgA murine immunoglobulin McPC 603 (Davies, D. R., et al., Ann. Rev. Biochem. 44, 639, 1975) in collaboration with Drs. D. R. Davies and E. A. Padlan of NIH. This Fab fragment has binding specificity toward phosphoryl choline. Partially refined atomic coordinates of the Bence-Jones protein were used for the comparison. Corresponding domains have very similar conformations, if allowances are made for insertions and deletions in the heavy and light chains of the Fab fragment. Furthermore, it was possible to define a core region in all eight domains of the Fab fragment and the Bence-Jones protein for which the relative positions of alpha carbons can be considered to be the same. The presence of these common cores allowed the direct comparison of the geometries of the two structures. The positions of the domains in the variable regions are very similar, with the distances between the residues in the interface essentially maintained. The same conclusion applies to the constant domains and the interface between them. The principal differences between the light chain dimer and the Fab fragment lie first in the positions of the variable and constant domains of the substituent chains, and second in the switch regions, which affect the orientation of the antigen binding site relative to the constant region of the molecule. The methods worked out for the comparisons will be applicable to other immunoglobulins as well.

The fact that the relative orientation of the domains within both the variable and constant regions is maintained in these unrelated immunoglobulins suggests that it might be a general rule of immunoglobulin structure. This property can then be used to solve related structures by molecular replacement methods (The Molecular Replacement Method: A Collection of Papers,

Ed. M. G. Rossman. Gordon and Breach, New York, 1972). The orientation of the domains in the Mcg IgG1 immunoglobulin was found using previously determined structures of the variable and constant domains of the Mcg Bence-Jones protein as search structures. Because one domain represents only a small part of the IgG molecule, a method to discriminate between true and false peaks was required. To find the correct peaks of the rotation function, sets were selected that had the relationships of the V-V or C-C domains in the Bence-Jones dimer. The application of molecular replacement methods should be easier for Fab and Bence-Jones proteins.

CRYSTALLIZATION OF κ-BENCE-JONES PROTEINS AND OTHER IMMUNOGLOBULINS

To understand how the amino acid sequence determines the three-dimensional structure, and hence the specificity, of the antigen binding site, more Bence-Jones proteins and Fab fragments have to be studied. The conformations of switch regions in different proteins will help in the understanding of how information is relayed from the binding site to the effector part of the molecule. Immunoglobulins supplied by Dr. A. Solomon of the University of Tennessee, Dr. G. N. Abraham of the University of Rochester, and Dr. J. E. Hopper of the University of Chicago are being screened to find suitable candidates for high resolution structural studies.

In collaboration with Dr. Solomon, 13 Bence-Jones proteins were characterized. Six of these have been purified on a Sephadex G-200 column in a Tris NaCl buffer system at pH 8; crystallization attempts were successful for three of the proteins. One of these is the noncovalently bound dimer of κ-type protein Fin, which forms orthorhombic crystals in a very narrow concentration range of 1.70-1.75 M ammonium sulfate; these crystals diffract to spacings smaller than 2.9 Å. This is the first crystallization of a κ-type protein. Another form of the Fin protein excreted by this patient is covalently bound and forms tetramers in solution.

IMPLICATIONS OF CONFORMATIONAL ISOMERISM AND ROTATIONAL ALLOMERISM TO THE BINDING OF SMALL MOLECULES BY THE Mcg BENCE-JONES DIMER*

A. B. Edmundson, E. E. Abola, K. R. Ely, J. R. Firca, N. C. Panagiotopoulos, M. Schiffer, and F. A. Westholm

In this article we have presented evidence that conformational isomerism and rotational allomerism are fundamental features of immunoglobulin structures. The differences between the V and C domains provide a striking example of divergent evolution. A common immunoglobulin fold is preserved by the maintenance of the hydrophobic character of key internal sites in cylindrical domains differing substantially in amino acid sequences. Modifications of the functions of the domains have occurred as a result of the rotational allomerism. Substitutions among important residues in both the three- and four-chain layers are required for a stable transition of one allomer to the other. The collective findings are incorporated into a possible evolutionary pathway.

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*Conclusion of a paper published in Antibodies in Human Diagnosis and Therapy, Eds. E. Haber and R. M. Krause. Raven Press, New York, pp. 135-152, 1977.

CONFORMATIONAL ISOMERISM, ROTATIONAL ALLOMERISM, AND DIVERGENT EVOLUTION IN IMMUNOGLOBULIN LIGHT CHAINS*

A. B. Edmundson, K. R. Ely, E. E. Abola, M. Schiffer, N. Panagiotopoulos, and H. F. Deutsch†

Immunoglobulin light chains are examples of single polypeptide chains synthesized under the control of two genes. The three-dimensional structure of a human (Mcg) λ-type light chain (Bence-Jones) dimer supports the hypothesis of a common primordial gene for the amino ("variable" or V) and carboxyl ("constant" or C) halves of each monomer. However, sequence homologies have been obscured by divergent evolution of the V and C regions ("domains"). The types of evolutionary changes that have occurred in the domains can be surmised by a comparison of the sequences, using the three-dimensional structures as a basis for alignment. Despite substantial differences in sequences, the hydrophobic character of key internal sites has been maintained in each domain. Regions present in only one domain are situated in positions appropriate for their functions, but not deleterious to the general structural integrity of a common fold. The divergence of the V and C domains can be

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*Abstract of a paper published in Fed. Proc., Fed. Am. Soc. Exp. Biol. 35, 2119 (1976).

†University of Wisconsin, Madison.

interpreted in terms of rotational allomerism. The cylinders of β-pleated sheets have rotated in such a way that homologous regions in the two domains perform different functions in their interactions with a second molecule of light or heavy chain. These regions include complementarity-determining sites for antigen binding in the V domains and crossover sites stabilizing dimer formation in the C domains. Differences in surface properties between the V_1-V_2 and C_1-C_2 dimeric modules may partially explain why the V regions have been implicated in the formation of amyloid fibrils and in the characteristic thermal behavior of Bence-Jones proteins.

14. MAMMALIAN CELL BIOLOGY

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GROUP LEADER'S INTRODUCTION

Mortimer M. Elkind, Group Leader

The research of the Mammalian Cell Biology Group continues to be based upon the cultivation of mammalian cells *in vitro*. Although a series of diverse topics is being pursued, these have in common efforts to understand the cell and molecular biology of functional changes produced by environmental agents including ionizing radiation, nonionizing radiation, and various chemicals.

In respect to ionizing radiation, studies of damage and repair relative to cell division have been extended to neoplastic transformation; further, the neoplastic changes induced by X-rays and by fission-spectrum neutrons from the JANUS reactor have been compared.

Relative to nonionizing radiation, further data were obtained on the comparative properties of ultraviolet light (UV, 254 nm) and a near-ultraviolet light emission simulating sunlight (NUV). These studies concern mechanisms of damage-repair interactions between X-rays and nonionizing radiations, and between UV and NUV, relative to cell killing and DNA damage.

And in regard to chemical actions, a new research area has been introduced involving the separate and combined effects of chemical carcinogens (polycyclic aromatic hydrocarbons) and radiation. These studies were initiated in part because the mechanism of such combined actions is of interest, but also in order to develop methodologies for studying damage interaction that may result from exposure to effluents from various fossil-fuel technologies and radiation (ionizing and nonionizing).

*Through the Fermi National Accelerator Laboratory.

Thus, while the group maintains a considerable interest in the radiobiology of ionizing radiation, its perspectives have broadened and shifted: first, to include the biological properties of various spectra of fast neutrons as well as X-rays; second, to inquire about sunlight effects and the interaction of sunlight with ionizing radiation damage; third, to include end effects in addition to cell killing and DNA damage such as neoplastic transformation; and fourth, to initiate studies of the action of chemical carcinogens by themselves and in conjunction with radiation.

MAMMALIAN CELL BIOLOGY STAFF

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*Now in Carcinogenesis Group.

†Terminated during 1976.

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MECHANISMS OF MAMMALIAN CELL KILLING

Mortimer M. Elkind, Mary E. Geroch, Antun Han, Michael Hagan,† *Eva E. Kautzky, Chin-Mei Liu, Melvin D. Long, Frank Q. H. Ngo, Warren K. Sinclair,*‡ *and Hiroshi Utsumi*

THE RADIOBIOLOGY OF FAST NEUTRONS

Further progress has been made in characterizing the radiobiological properties of neutrons produced at the Fermi National Accelerator Laboratory, and in comparing Fermi neutrons (average energy 25 MeV) to those produced by the JANUS reactor at Argonne (average energy 0.85 MeV) and at the Franklin McLean Institute of The University of Chicago (average energy 3 MeV). The Fermi beam was developed for cancer therapy, and the first treatments were started in September 1976.

Two lines of cultured cells have been used: V79 Chinese hamster cells, because they have been well characterized in respect to other radiations; and mouse C3H 10T1/2 cells, because they are being used in connection with studies of transformation to neoplastic properties.

The relative biological effectiveness (RBE) of neutrons compared to X-rays at a given level of survival decreases with increasing average neutron energy. In contrast, the oxygen enhancement ratio is essentially energy independent and drops from about 3.0 for X-rays to about 1.8 for fast neutrons. Also, the repair of sublethal damage is essentially energy independent and appreciably less than it is for X-rays.

Lastly, the induction of division delay was measured for JANUS fission-spectrum neutrons. As for 50 kVp X-rays, surviving and nonsurviving cells suffer equal delays which increase linearly with dose. From earlier results, and the RBE of 50 kVp relative to 250 kVp X-rays, the RBE of division delay of JANUS neutrons to 250 kVp X-rays is probably greater than 4 and independent of dose for neutron doses up to 350 rad.

REPAIR ABILITY AND THE AGE-RESPONSE PATTERN

Studies of the modification of irradiation survival throughout the cell cycle by N-ethylmaleimide (NEM) and hydroxyurea (HU), using V79 Chinese hamster cells, have shown that when repair is inhibited by these chemicals, the

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*Through the Fermi National Accelerator Laboratory.

†Laboratory Graduate Program Participant, University of Illinois, Urbana.

‡Associate Laboratory Director for Biomedical and Environmental Research.

age-response pattern becomes relatively flat. Mitotic cells, harvested by the dish shaking technique, are of special interest because they appear to lack repair capability and are insensitive to NEM or HU. Also, such populations generally consist of mother and daughter cells, differing by a factor of 2 in DNA content. Data thus far indicate that daughter cells are the more resistant of the two.

THE RADIOBIOLOGY OF "SUNLIGHT"

In the past, cellular studies of the effects of nonionizing radiation generally have been limited to ultraviolet light (UV, 254 nm) whether bacteria, yeasts, or mammalian cells were used. While this wavelength has certain advantages for studies of mechanism, it is effectively eliminated on the earth's surface by the ozone layer. We have compared the lethal effects produced by Westinghouse Sun Lamps--which emit a continuous spectrum of near ultraviolet light (NUV) light, starting at about 290 nm and having a maximum intensity at 315 nm--with those of low pressure Hg germicidal lamps (UV, 254 nm) for the purpose of examining the predictive value of UV for cell killing due to sunlight. Qualitative as well as quantitative differences were observed in the killing of V79 Chinese hamster, HeLa, and mouse C3H 10T1/2 cells; i.e., the survival curves after UV and NUV differ in shape since they are not related by a constant modification dose factor. These results suggest that if pyrimidine dimers are the principal lesion after UV responsible for cell killing, they are not after NUV. Thus, results obtained with UV may not, in general, be adequate to predict the effects produced by sunlight.

DNA SINGLE-STRAND LESIONS PRODUCED BY ULTRAVIOLET LIGHT AND "SUNLIGHT"

Further data have been obtained on the different ways in which mammalian cells handle single-strand lesions due to nonionizing radiation. Following UV exposure, the number of alkali labile lesions in single strands of DNA of V79 cells increases rapidly for the first 25 minutes and then remains relatively constant for the next 5 hours. The initial increase presumably represents the first step in an incision process related to repair. In contrast, using an equivalent thymidine dimer producing dose of sunlight-simulating NUV, the number of lesions does not change. Since DNA excision repair in bacteria can be inhibited by NUV (Tyrrell, R. M., and R. B. Webb, Mutat. Res. 19, 361, 1973), we examined the effect of NUV on the increase in lesions following UV. NUV did not inhibit the increase in breaks following UV.

Finally, a comparison of UV and NUV on a D_0 dose basis shows that they are equally effective in producing single-strand lesions. Thus, even though there are about 100 such lesions per D_0 (X-rays produce 1000 single-strand breaks per D_0), the fact that the number of breaks either increases or at least persists (after X-rays they are rapidly repaired), supports the possibility that single-strand lesions may have a connection with cell killing, particularly in rodent cells, which are deficient in the excision repair of dimers.

INTERACTION STUDIES WITH X-RAYS AND "SUNLIGHT"

Studies previously reported of the interaction of damage produced by X-rays and nonionizing radiation--UV (254 nm) or a near UV source simulating sunlight (NUV)--showed that exposure of V79 Chinese hamster cells to a conditioning dose of UV or NUV reduces the shoulder of the X-ray survival curve and *vice versa*. These observations have now been extended in order to permit a more detailed comparison between UV and NUV in regard to damage interaction with X-ray damage.

Experiments with synchronized cells treated in the middle of the DNA synthetic phase show that survival equivalent doses of UV and "sunlight" are not equally effective in reducing the shoulder of the X-ray survival curve (i.e., the capacity for sublethal X-ray damage). The sublethal damage produced by UV is essentially completely additive to that due to X-rays, since UV reduces the shoulder on the X-ray curve as effectively as an equal survival dose of X-rays. However, a dose of "sunlight" does not eliminate the shoulder on the X-ray survival curve as effectively. Thus, at equal levels of survival, damage due to "sunlight" is only partially additive to X-ray damage; furthermore, survival equivalent conditioning exposures of "sunlight" or X-rays reduce to the same extent the capacity of cells for sublethal damage from the other radiation.

Sublethal damage repair is more rapid after a dose of X-rays than after a dose of "sunlight" or UV, as may be inferred from the time of reappearance of shoulders on survival curves. When mid-S cells are exposed to "sunlight" (or UV) and 6 hours later to graded doses of X-rays, only a small portion of the X-ray survival curve shoulder is recovered. In contrast, cells exposed to X-rays and 6 hours later to graded doses of "sunlight" (or UV), completely recover their capacity for sublethal "sunlight" (or UV) damage. Further tests involving longer fractionation intervals have shown that the complete repair of sublethal damage due to nonionizing radiation takes at least one complete cell cycle. In spite of quantitative differences in the degree of additivity of damage due to UV or "sunlight" with X-ray damage, both repair rates are slow and they are about equal.

Pyrimidine dimers are the principal lesions associated with UV lethality, and bond breakage is the main result of X-ray absorption. The principal lesion(s) produced by our sunlight-like source is not known. Nevertheless, there are qualitative similarities between the NUV and X-ray interaction and the UV and X-ray interaction. Similar observations were obtained earlier with the DNA intercalator actinomycin D and X-rays. Hence, we propose that DNA distortion is the property in common between this antibiotic and UV or NUV in respect to enhanced X-ray killing.

MISREPAIR: A HYPOTHESIS FOR MAMMALIAN CELL KILLING

The induction of DNA single-strand lesions has been measured in V79 Chinese hamster cells; about 1000 lesions are registered per D_0. An estimate from preliminary data and the work of others indicate that about 40 DNA double-strand breaks are produced per D_0. Also, from earlier studies and those of others, it is known that single-strand lesions are rapidly repaired,

after moderate doses apparently completely. However, largely for technical reasons, studies of DNA integrity are invariably performed using supralethal doses; i.e., doses that would reduce the number of surviving cells to 1 in 1000 or fewer. Thus, almost invariably DNA breakage and repair studies are in effect performed with cells destined to die.

It follows from the considerable excess of DNA lesions per D_o--a D_o being a dose increment that reduces survival by the factor 1/e by producing one hit in a functional sense--that surviving cells must repair a very large amount of damage in their DNA. For example, a dose of 800 rad of 50 kVp X-rays, which reduces survival to a few percent, registers about 10,000 single-strand lesions, and about 400 double-strand breaks, per cell. Clearly, the cell that survives this dose either propagates a large amount of damage in its genome, or repairs that damage. However, because of the supralethal doses ordinarily used in DNA studies, it is not possible to be confident of the fate of DNA damage in surviving cells.

To address this question, V79 cells were repeatedly exposed to about 800 rad. Between each dose increment, the population was incubated long enough for the killed cells to lyse and the surviving cells to grow out. This procedure was used until about 22,000 rad had been accumulated. If DNA lesions were not repaired, the survivors should have contained about 2.8×10^5 single-strand lesions and about 1×10^4 double-strand breaks per cell. In terms of the single-strand unit referred to in earlier work as "main peak" DNA, whose size is 2×10^8 daltons, 22,000 rad would have resulted in 9.5 single-strand lesions per 2×10^8 daltons. The sedimentation techniques used permit the detection of less than 0.5 breaks per 2×10^8 daltons. It was found that survivors of 22,000 rad contained no detectable breaks per 2×10^8 daltons nor was their susceptibility to additional breakage different from parental, unirradiated populations.

These observations support a hypothesis that the radiation killing of mammalian cells results from an occasional misrepair of lesions whose repair, in the main, is error free. Conceptually, this notion departs from the classical biophysical ideas of "hit-target" theory in cell killing. Future studies will be aimed at testing the hypothesis and evaluating whether misrepair, in a similar sense, is involved in other functional changes.

THE MOLECULAR BIOLOGY OF LETHALITY IN CELLS LABELED WITH 5-BROMODEOXYURIDINE (BUdR) AND EXPOSED TO NEAR ULTRAVIOLET LIGHT

Earlier studies employing V79 Chinese hamster cells whose DNA was bifilarly labeled with BUdR (Ben-Hur, E., and M. M. Elkind, Mutat. Res. 14, 237, 1972; and Biophys. J. 12, 636, 1972) showed that: (1) cells are not killed by fluorescent light unless they have incorporated BUdR; (2) the cell sensitivity reflected the amount of BUdR incorporated; (3) the number of alkali labile lesions in DNA per D_o dose was independent of the level of BUdR incorporation; and (4) a cell suffers about 50,000 alkali labile lesions in its DNA per D_o. Since it is highly likely that lethality in cells containing BUdR involves damage in DNA, and in view of the fact that X-ray cell killing of unsubstituted cells also involves a large surfeit of DNA single-strand lesions (i.e., about 1,000 per D_o), we have pursued BUdR-NUV cell killing as a means of cross-illuminating the mechanism of X-ray cell killing.

For the NUV exposures the sunlight simulating source referred to above (Westinghouse Sun Lamp) was used, except that the shorter wavelengths were filtered out by polystyrene in order to eliminate lethality due to this light when used alone. Synchronized V79 cells were unifilarly labeled with BUdR for a portion of the S phase (i.e., 45-60 minutes) and were subsequently irradiated. The degree of sensitization of cells containing BUdR is dependent upon the time in S phase when they were labeled with BUdR and upon the subsequent cell cycle position during irradiation. Cells are minimally sensitized to NUV when given BUdR early in S phase, because of a large shoulder on the survival curve. Caffeine, 1 mM, administered after exposure reduced the shoulder to zero. The labeled cells are maximally sensitized to NUV at the approximate time of replication of the BUdR segment in the next S phase; survival at this time is exponential and is not modified by 1 mM caffeine. These observations indicate a major difference between expression of lethal damage in unifilarly substituted cells *vis a vis* cells containing bifilar BUdR substitution.

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INDUCTION OF FUNCTIONAL CHANGES IN MAMMALIAN CELLS

Mortimer M. Elkind, Mary E. Geroch, Antun Han, Eva E. Kautzky, and Hiroshi Utsumi

CELL TRANSFORMATION BY IONIZING RADIATION (X-RAYS AND FISSION SPECTRUM NEUTRONS)

A cell line derived from a mouse embryo, designated C3H 10T1/2, and a method of assessing neoplastic transformation *in vitro*, based on changes in colonial morphology (Reznikoff, C. A., et al., Cancer Res. 33, 3239, 1973), were used to study the frequency of transformation induced by 50 kVp X-rays and by fast neutrons from the JANUS reactor. The induction curve after X-rays rises to a plateau at about 400 rad of 3×10^{-3} transformants per survivor. The induction curve after fission neutrons is qualitatively similar to that after X-rays; however, its initial part shows a steeper increase, and it levels off at a higher level, at about 6.5×10^{-3} transformants per survivor.

Transformation frequency was measured after dose fractionation. After X-rays, it was reduced by about 7-fold, with an interfraction interval of 16 hours. After neutron fractionation there was much less reduction; that is, after a 24-hour interval transformation frequency decreased by a factor of 1.8.

The RBE for transformation varies from about 10 for low doses to about 2.5 for moderately high doses of neutrons compared to X-rays; in the plateau

regions an RBE cannot be specified according to the ICRU definition, because the neutron plateau exceeds that due to X-rays. The RBE for survival varies from 2.7 to 2.3, for 0.8 to 0.001 surviving fractions, respectively.

Preliminary data indicate that the X-ray induced transformation frequency was appreciably enhanced when X-irradiated cells were treated with the promoting agent TPA (12-O-tetradecanoylphorbol-13-acetate). The frequency was increased by a factor of 10 when cells were incubated in medium containing 0.1 μg/ml of TPA after exposure to 215 rad.

CARCINOGEN-INDUCED PHOTODYNAMIC CELL KILLING

In part to explore possible interactive effects between two or more carcinogens, and in part to develop a methodology for assessing the biological activity of effluents produced by different fossil fuel technologies, a search was made for possible enhanced responses due to the polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA) and radiation. Cell killing (i.e., proliferation suppression) using conventional cells was adopted for the initial end point in view of its ease of measurement and the body of knowledge that we have about the radiobiology relative to lethality. Treatment of V79 Chinese hamster cells for 30 minutes with concentrations up to 10 μg/ml of DMBA alone is without effect; X-ray or UV exposure (254 nm) immediately after DMBA did not significantly change survival compared to that after the radiation treatment alone. However, appreciably enhanced cell killing occurs in cells exposed to a near ultraviolet (NUV) sunlight-simulating source (Westinghouse Sun Lamp, described in the preceding report) following DMBA treatment. Introduction of a polystyrene filter to attenuate the shorter NUV wavelengths (e.g., by 100 times at 290 nm) largely eliminates cell killing by the NUV alone, but significant killing persists due to DMBA + NUV. Experiments with synchronized cells have shown that while all phases are affected, cells in S are preferentially made NUV sensitive by DMBA.

Studies with hypoxic V79 cells have shown an absolute requirement for O_2 for cell killing. However, administration of DMBA-7,12-endoperoxide does not result in synergism with "sunlight." Thus, a mechanism of action involving a photoinduced triplet-singlet conversion of O_2 is suggested.

In addition to V79 Chinese hamster cells, DMBA photosensitizes mouse C3H 10T1/2 cells and HeLa cells. It would appear, therefore, that this type of photodynamic action is general. Other polycyclic aromatic hydrocarbons--i.e., benzo(a)pyrene and 3-methylcholanthrene--are not particularly effective in enhancing cell killing with our "sunlight" source. However, a "black light" near-ultraviolet source (maximum emission at 365 nm) plus benzo(a)-pyrene or DMBA is able to enhance cell killing.

DNA DAMAGE AND ITS REPAIR IN HYPERTHERMIC MAMMALIAN CELLS: RELATION TO ENHANCED CELL KILLING*

E. Ben-Hur† *and M. M. Elkind*

In view of the size of the DNA molecule and its central role in information storage and transfer, it is not surprising that it has been implicated as a principal target of many radiation effects. Since the first reports that cells possess mechanisms by which they repair damage to their DNA, this field has been investigated intensively. Most of our information derives from studies with bacteria and much less is known relative to mammalian cells. A detailed understanding of the mechanisms by which cells preserve the integrity of their DNA has practical as well as theoretical importance. This is because in the absence of repair of DNA damage the probability of carcinogenesis is greatly enhanced. Also, treatments that kill cells by damaging their DNA--like radiation and alkylating agents--are used in cancer therapy and therefore repair of DNA, its absence, or misrepair is expected to play a major role in determining the efficacy of such treatments.

Recently it was reported that cultured mammalian cells are killed more readily under hyperthermia compared to normal conditions. In this presentation we summarize observations relative to the sensitizing effects of hyperthermia and several agents that are known to induce DNA damage. Correlations between cell killing and DNA damage and repair are noted, and the possible use of this approach in cancer therapy is discussed.

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*Introduction of a paper published in Radiation Research. Biomedical, Chemical, and Physical Perspectives, Eds. O. F. Nygaard, H. I. Adler, and W. K. Sinclair. Academic Press, New York, pp. 703-717, 1975.

†The Hebrew University, Jerusalem, Israel.

MECHANISMS FOR ENHANCED RADIATION-INDUCED CELL KILLING IN HYPERTHERMIC MAMMALIAN CELLS*

E. Ben-Hur† *and M. M. Elkind*

The effects of hyperthermia upon cell killing by radiation and methyl methansulfonate (MMS) are described and correlated with effects on DNA damage and repair in Chinese hamster cells. Hyperthermia enhances cell killing by

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both agents due to interference with repair of sublethal damage and an enhanced expression of lethal damage. Following x-irradiation, hyperthermia up to 42°C increases the rate of repair of single-strand breaks but slows the repair of the DNA complex. After MSS treatment, hyperthermia slows also the repair of MSS-induced single-strand breaks probably because breaks are inserted concomitantly with repair. Prolonged hyperthermia after either agent causes an endonucleolytic degradation of the DNA.

It is proposed that hyperthermia induces changes in chromatin and/or DNA super structure which makes it less susceptible to repair of sublethal damage. Enhancement of lethal damage expression may reflect accelerated hyperthermia-induced DNA degradation and related structural changes.

CELLULAR AND SUBCELLULAR BIOLOGY*

M. M. Elkind

The field of *radiation research*, from outward appearances, must seem to be a conglomeration of unrelated topics. Still, if there is one area I would identify as being a focus or crossroads of most of the others, it would be *cellular* and *subcellular biology*. Here the physics and chemistry of elementary absorption processes converge on studies of radiation effects in molecules, viruses, microbes, and even mammalian cells. And, it is from here, with cellular effects in mind, that the physiologist and physician seek to understand tissue and organ responses. Thus, our knowledge of the biochemistry and biology of cell effects *on the one hand* draws upon the physics of absorption processes and the chemistry following therefrom, and *on the other hand* is essential for the understanding of ecological and environmental effects of radiation, and for the improved use of radiation in medicine. The attraction of the gamut of science to the cell, in my opinion, has been a mainstream in the 5th Congress of Radiation Research.

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*Conclusion of a paper published in Radiation Research. Biomedical, Chemical, and Physical Perspectives, Eds. O. F. Nygaard, H. I. Adler, and W. K. Sinclair, Academic Press, New York, pp. 1349-1361, 1975.

FRACTIONATED DOSE RADIOTHERAPY AND ITS RELATIONSHIP TO SURVIVAL CURVE SHAPE*

M. M. Elkind

In the treatment of cancer by radiation, it is now generally accepted that of primary importance are the radiobiological properties of the relevant proliferating cells involved. Principal among these are: (1) the survival curve(s) (i.e., the dependence of surviving fraction of clonogenic or stem cells on absorbed dose); (2) repair of sublethal damage; (3) the oxygen effect; and (4) repopulation of partially depleted tissue(s). These cardinal properties reflect the quality of the radiation used (for example, fast neutrons or pimesons *versus* high energy X- or γ-rays) and there are those who believe that important roles are also played by potential lethal damage repair and cell cycle reassortment. While there are examples from rodent systems where the latter two phenomena are found to be observable, the generality of their influence in a significant way on the responses to fractionation of tumor or normal tissues has not been established.

The current era of rational radiotherapy began with the publication of the first mammalian cell survival curve in 1956 by Puck and Marcus followed 3 years later by a similar kind of demonstration by Hewitt and Wilson for mammalian cells assayed *in vivo*. In that same period, the general importance of oxygen as a radiation sensitizer became established (e.g. I and II), and repair of sublethal damage in mammalian cells was first demonstrated. Subsequently, repair of sublethal damage was observed in mammalian cells hypoxic during as well as between irradiations.

The observation that mammalian cells could withstand repeated cycles of damage and repair stimulated the application of such results to radiotherapy. An essential feature of these early analyses was the bridge formed conceptually between the radiobiologist's concept "isosurvival"--and its dependence on physical, chemical and biological parameters--and the radiotherapist's clinical equivalent "isoeffect." While the former term has a precise meaning in that it specifies the same quantitative effect for different regimes of dose administration, it was recognized that an isoeffect in clinical terms probably results from equal degrees of tissue damage for different protocols.

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*Introduction of a paper published in Cancer Treatment Rev. 3, 1 (1976).

THE INITIAL PART OF THE SURVIVAL CURVE: IMPLICATIONS FOR LOW DOSE, LOW DOSE RATE RADIATION RESPONSES*

M. M. Elkind

Aspects of radiation exposure that relate to epidemiological questions generally focus upon late effects due to low doses delivered at low dose rates. Among the questions of major concern, oncogenesis, mutagenesis, and teratogenesis are of primary interest but in each case cell killing must also be considered. At a cellular level, few data exist relative to the foregoing except for cell killing but even in the latter instance, technical difficulties impose limitations on the generalizations that may be inferred.

Using cell survival as a model, several formal dose-effect relationships are discussed, particularly in reference to the low dose region of the survival curve, and a summary of available survival data is presented. Of particular interest is the biophysics underlying so-called "single-hit" inactivation. The theory of exponential (or linear) dose-effect dependencies is presented from which the conclusion is reached that in "single-hit" killing, the level of damage expressed (i.e., the inactivation constant) may be modified by postirradiation cellular processes reflecting damage repair or enhanced damage expression.

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*Abstract of a paper to be published in Radiat. Res.

SPURIOUS PHOTOLABILITY OF DNA LABELED WITH [^{14}C]-THYMIDINE*

M. M. Elkind and R. D. Lay

Thymidine labeled in the methyl position with ^{14}C (Lot No. 824-137, Compound NEC-568, New England Nuclear Corporation) was used to label the DNA in V79 Chinese hamster cells and bacteriophage T4. In both cases, abnormally high ultraviolet and near ultraviolet light photolability resulted. A 1-2% contamination with 5-bromodeoxyuridine of the total thymidine in a [^{3}H]-thymidine stock produced an equivalent photolability. Neutron activation analyses showed that Lot No. 824-137 contained 1-2% Br relative to thymidine.

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*Summary of a paper published in Biochem. Biophys. Res. Commun. 68, 691 (1976).

ADDITIVE ACTION OF IONIZING AND NON-IONIZING RADIATIONS THROUGHOUT THE CHINESE HAMSTER CELL-CYCLE*

A. Han and M. M. Elkind

The age-dependent variations in survival of V79 Chinese hamster cells after combined administration of a fixed dose of X-rays and ultraviolet light show enhanced cell killing at all cell ages. The greatest interaction is observed in the middle of S-phase where the dose of UV reduces the ability of surviving cells to accumulate X-ray induced sublethal damage and *vice versa*. This reduction of the shoulder is, however, always greater when UV precedes X-rays. The repair of X-ray induced DNA single strand breaks is not significantly inhibited by preexposure of cells to a dose of UV, and it is concluded that enhanced cell killing is not due to UV-damage interference with the repair of X-ray induced DNA lesions.

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*Abstract of a paper published in Int. J. Radiat. Biol. 31, 275 (1977).

THE EFFECT OF *N*-ETHYLMALEIMIDE ON THE RESPONSE TO X RAYS OF SYNCHRONIZED HeLa CELLS*

Antun Han, Warren K. Sinclair, and Bruce F. Kimler

The presence of 0.75 μ*M* *N*-ethylmaleimide (NEM) during irradiation of synchronized HeLa cells at different stages of their cycle enhances cell killing (i.e., sensitizes) in early G_1 and late S, but does not affect mitotic cells or cells at the G_1/S border. The effect is primarily on the shoulder of the survival curve, rather than its slope. NEM given immediately before or after irradiation is as effective as during irradiation. When given as a function of time after exposure the effect decreases in magnitude, cells becoming insensitive to NEM more rapidly in S than in G_1. The drug is also effective if administered prior to irradiation, the effect generally decreasing the longer the interval between treatment and irradiation.

The addition of 1.0 m*M* hydroxyurea (HU) to synchronous HeLa cells in G_1 showed that survival changes occur as the cells proceed through the "cell cycle" even in the absence of DNA synthesis. The changes observed are qualitatively the same to those observed previously in Chinese hamster cells. The irradiation of HU-inhibited cells in the presence of 0.75 μ*M* NEM produced almost no variation in survival through "the cycle," the inhibited cells showing a sensitivity greater than normally observed in mitotic cells.

The results presented are consistent with the idea that NEM inhibits repair processes in the cell most likely due to its binding to a critical SH-containing enzyme(s) concerned with the repair of radiation damage.

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*Abstract of a paper published in Radiat. Res. 65, 337 (1976).

N-ETHYLMALEIMIDE SENSITIZATION OF X-IRRADIATED HYPOXIC CHINESE HAMSTER CELLS*

B. F. Kimler, W. K. Sinclair, and M. M. Elkind

Chinese hamster cells were X-irradiated either aerobically or hypoxically, after flushing with nitrogen plus carbon dioxide. In agreement with earlier data, for asynchronous cells, the oxygen enhancement ratio (OER) was approximately three. If the sulfhydryl-binding agent N-ethylmaleimide (NEM) was present during or immediately after irradiation, the principal effect was a pronounced decrease in the extrapolation number of the survival curve of NEM-treated cells compared to the non-treated cells. This was observed with hypoxic as well as aerobic cells and the OER for NEM treated cells was also about three. For NEM treatments which are essentially nontoxic, NEM acts synergistically with X-rays, suggestive of an inhibition by NEM of a cell's ability to repair sublethal damage.

For synchronous cells obtained by mitotic selection, a result consistent with the above was obtained; a dose three times as large was necessary to reduce survival to the same level for hypoxic and aerobic cells whether or not the cells were treated with NEM. Thus the OER was independent of NEM treatment throughout the entire cell cycle, with the possible exception of mitosis which could not be studied with the methods used.

It is concluded that the action of NEM at low concentrations (0.75 μM) is largely independent of oxygen tension. Oxygen acts to produce more damage per unit dose in the cell while NEM sensitizes apparently by preventing the repair of sublethal damage.

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*Abstract of a paper to be published in Radiat. Res.

COMPARATIVE RADIOBIOLOGY OF FAST NEUTRONS: RELEVANCE TO RADIOTHERAPY AND BASIC STUDIES*

F. Q. H. Ngo, A. Han, H. Utsumi, and M. M. Elkind

Comparative neutron radiobiological properties relevant to radiation therapy are being investigated using V79 Chinese hamster cells in culture. Three neutron beams are being used: linear accelerator-produced neutrons, $p^+ \rightarrow Be$, at the Cancer Therapy Facility of the Fermi National Accelerator Laboratory (Fermi); cyclotron-produced neutrons, $d^+ \rightarrow Be$, at the Franklin McLean Research Institute, University of Chicago (FMI); and fission-produced neutrons at the JANUS reactor of the Argonne National Laboratory (JANUS). The mean neutron energies of the Fermi, FMI, and JANUS beams are 25 MeV, 3.6 MeV and 0.85 MeV, respectively. The RBE values of cell survival relative to 250 kVp X-rays, at a given surviving fraction, decreased in the order

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*Abstract of a paper to be published in Int. J. Radiat. Oncology, Biol., and Physics.

JANUS, FMI, Fermi, a trend opposite to the mean neutron energy. The OER's measured with Fermi and JANUS neutrons were essentially the same, however.

Other radiobiological studies have been initiated with JANUS neutrons. Results from two-dose fractionation experiments showed that very little repair of neutron-induced sublethal damage occurred for incubation at 37°C for intervals up to 5 hours between exposures. Data from postirradiation growth kinetics indicated that neutron radiation produces cell division delays that increase linearly with dose. An RBE for cell division delay, relative to 55 kV X-rays, was calculated to be approximately 3.5, which is close to the upper limit of the RBE values for survivals. Hyperthermic treatment, immediately following neutron irradiation, enhanced cell killing. Postirradiation treatments of cells with hypo- and hypertonicity also resulted in enhanced cell killing.

MAMMALIAN CELL SENSITIZATION REPAIR AND THE CELL CYCLE*

W. K. Sinclair

Experiments with hydroxyurea and with NEM, first in fully oxygenated V79 Chinese hamster cells and more recently in HeLa cells, support the proposed two-component model for control of cell survival after x-irradiation during the cell cycle. One component is DNA synthesis; the other is determined by the action of NEM throughout the cell cycle and is probably a small fraction of the protein sulfhydryl located in critical molecules.

These results are generally consistent with the notion that during mitosis the cell is most sensitive because it lacks the ability to repair radiation damage. It may be noted that neither hydroxyurea nor NEM affects mitotic cells. At other stages of the cell cycle, hydroxyurea inhibits a repair mechanism that operates during DNA synthesis and thus appears to sensitize the cell, and NEM inhibits another repair mechanism that is usually most effective both early in G_1 and late in S. When both agents are used together, the response of cells is broadly (this is an oversimplification) flat through the cell cycle and roughly the same as that of mitotic cells.

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*Conclusion of a paper published in Radiation Research. Biomedical, Chemical, and Physical Perspectives, Eds. O. F. Nygaard, H. I. Adler, and W. K. Sinclair, Academic Press, New York, pp. 742-751, 1975.

UV-INDUCED DNA TO PROTEIN CROSS-LINKING IN MAMMALIAN CELLS*

P. Todd† *and A. Han*

Searches for photoproducts attributable to UV-induced DNA to protein cross-links have been partially successful but positive identifications of such photoproducts remain to be made. There is no proof that histones are linked to DNA.

There is sufficient evidence that proteins are involved in the UV response of mammalian cells. Todd and co-workers with Chinese hamster cells, and Rauth with L cells showed that exposures required to reduce survival to a given level are a firm function of wavelength, and the general patterns of the two action spectra are the same, both having a broad peak in the region of 270 nm, suggesting that DNA and protein are involved.

The incorporation of BrdUrd in the DNA of mammalian cells significantly enhances the amount of DNA cross-linked to proteins. There is about a 3 to 5-fold increase in cross-linking when cells are grown for two generations in the presence of 5×10^{-7} M BrdUrd. This drug is a very effective sensitizer of mammalian cells in the cases of both ionizing and UV irradiation.

The cell cycle dependent fluctuations in the yield of DNA cross-linked to proteins are almost identical to the changes in cell survival throughout the cycle. There are some other observations and results that indicate the importance of this damage in survival of UV-irradiated mammalian cells.

That formation of cross-links between DNA and proteins could contribute significantly to the killing of cells by UV radiation has been suggested by Alexander and Moroson as well as the possibility that in mammalian cells particular proteins may be involved in the expression of damage to DNA.

The formation of cross-links between DNA and enzymes or other proteins (histones) in its vicinity could prevent normal DNA repair processes. Consequently, damage would become irreparable and result in cell death.

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*Conclusion of a paper published in Aging, Carcinogenesis, and Radiation Biology: The Role of Nucleic Acid Addition Reactions, Ed. K. C. Smith, Plenum Press, New York, pp. 83-104, 1975.

†Pennsylvania State University.

15. GENETICS

ERDA RT-02-02
ANL 62200
ANL 62201

GROUP LEADER'S INTRODUCTION

Herbert E. Kubitschek, Group Leader

The major deleterious effects of chronic exposure of living cells to pollutants and other noxious agents occur from the production of genetic (DNA) lesions. This is obviously the case when the genetic system itself is the most sensitive in the cell, as with ultraviolet light (UV); but even when other cell components are more sensitive, physicochemical damage to these usually is negligible or has no detectable biological effects, whereas the rarer genetic lesions frequently are lethal or mutagenic. Furthermore, this disparity between genetic effects and those produced by interaction with other parts of the cell--wall, membrane, or cytoplasm--is accentuated at lower concentrations of pollutants. While genetic effects are decreased relatively proportionally, effects in other cellular components generally diminish far more rapidly because of the redundancy in those structures.

Because DNA has a relatively simple structure, the kinds of lesions that can be produced are limited. For this reason, we are concerned with mechanisms of lethality and mutagenesis produced not only by environmental pollutants, but by other agents as well, because these lesions can provide vital information on common mechanisms. Certain lesions specifically inhibit DNA replication, or alter initiation or termination of replication, so we also carry out associated research in these areas. Bacteria are chosen for studies of molecular mechanisms of mutagenesis and lethality since good genetic maps are presently available only for these organisms. Because DNA has the same molecular structure in all plants and animals, we can assume that the same kinds of molecular mechanisms are involved in man as in microorganisms. Other advantages of using bacteria are the availability of a tremendous number of different genetically marked strains and the fact that experiments can be done more economically, far more rapidly, and in greater numbers than possible with higher organisms. Mammalian cells also are used, because they are expected to have mutational and lethal sensitivities similar to those for human cells.

Research on mammalian genetics, formerly carried out in this group, is now being conducted in the Pathology and Risk Assessment Group, as it relates to certain specific radiation hazards in the nuclear fuel cycle.

Major findings of the Genetics Group during the year include:

1) A mutational process producing clustered, multiple mutations was observed to operate with other strong mutagens in addition to UV, namely, nitrosoguanidine, nitrous acid, sodium bisulfite, and acridine orange plus visible light (H. E. Kubitschek). These results support earlier findings of UV-induced clustered multiple mutation in an entirely different bacterial system, and indicate the operation of error-prone repair mutagenesis with a variety of strong mutagens.

2) High level mutagenesis is produced by acridine orange and 500-nm light in *recA* and *lexA* strains of *Escherichia coli* that are not mutated by most strong mutagens (R. B. Webb and B. S. Hass). The results provide strong evidence for photodynamic mutagenesis at this wavelength by intercalatory binding of the dye through a mechanism not dependent upon error-prone repair.

3) Experimental results for temporary associations of the chromosome origin and terminus with the cell membrane in *Bacillus subtilis* are inconsistent with the Jacob-Brenner-Cuzin mechanism for control of DNA segregation and imply the operation of a hitherto unknown regulatory mechanism in DNA replication (T. Matsushita).

GENETICS STAFF

REGULAR STAFF

Brown, Mickey S. (Scientific Associate)
Darby, Donna M. (Scientific Assistant)
*Frystak, Barbara H. (Scientific Assistant)
*Grahn, Douglas (Senior Biologist)
Krisch, Robert E. (Biophysicist)
Kubitschek, Herbert E. (Senior Biophysicist)
Matsushita, Tatsuo (Geneticist)
†Prioleau, John C. (Scientific Assistant)
Shotola, M. Anita (Scientific Assistant)
Venters, Dace (Scientific Assistant)
Webb, Robert B. (Bacteriologist)

TEMPORARY STAFF DURING 1976

Boye, Erik (Postdoctoral Appointee)
Hass, Bruce S. (Research Associate)
*Lee, Chung H. (Research Associate)
Newman, Chester N. (Postdoctoral Appointee)

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*Now in Pathology and Risk Assessment Group.

†Transferred to Affirmative Action Program in 1977.

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MOLECULAR GENETICS AND ENVIRONMENTAL MUTAGENS

Herbert E. Kubitschek, Mickey S. Brown, Donna M. Darby, Bruce S. Hass, Bernard N. Jaroslow, Ronald D. Ley,† Tatsuo Matsushita, Chester N. Newman, John Prioleau, Robert E. Krisch, Anita Shotola, Aaron Simms,‡ Robert B. Webb, Dace Venters, and Lisa Vukalcic***

CLUSTERED MULTIPLE MUTATIONS

This research, by H. E. Kubitschek and D. Venters, examines the extent of production of clustered, multiple mutations by ultraviolet light (UV) and other mutagens, and the degree to which recombination and other genetic repair mechanisms are involved. The possibility that more than one gene might be mutated during error-prone repair was recognized several years ago and first tested in *Bacillus subtilis* (Kubitschek, H. E., and G. Venema, Mutat. Res. 35, 325, 1976). Almost all streptomycin-resistant mutants induced at low doses of UV were also resistant to other antibiotics with genetic loci in the neighboring region of the genetic map, and, on average, three to four loci were observed to have been mutated in each mutant cell. That is, mutations were clustered and multiple. Because this phenomenon might have been peculiar to that genetic region in *B. subtilis*, we tested for the phenomenon in *Escherichia coli* by examining for frequencies of double mutants that were resistant both to azide and to bacteriophage T5. Although the corresponding genes are separated by about 1% of the genome and double mutants should be very infrequent, observed conjoint mutation frequencies were very high, approximately a thousandfold greater than can be accounted for by chance mutations of the individual genes.

Are large frequencies of multiple mutations also produced with other strong mutagens? To answer this question we again measured conjoint mutation frequencies to azide and T5 resistance in chemostat cultures of *E. coli*. Similar high frequencies were again induced by exposure to nitrosoguanidine, nitrous acid, sodium bisulfite, or photodynamically with acridine orange. These results support our working hypothesis that multiple mutations are produced by the error-prone "SOS-inducible repair" pathway of mutagenesis. In further support, neither of two other mutagens known not to involve this pathway, the base analog 2-aminopurine and the frameshift mutagen ICR-170, gave detectable levels of conjoint mutations, although both azide-resistant and T5-resistant mutants were induced.

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*Pathology and Risk Assessment Group.

†Carcinogenesis Group.

‡Summer 1976 participant in the Undergraduate Honors Research Participation Program, New York University, and Resident Associate under the Argonne Affirmative Action Program.

**Fall 1976 participant in the Undergraduate Honors Research Participation Program, Mount St. Mary's College, California.

Our results suggest that multiple mutations may be characteristic of the SOS-inducible repair pathway of mutagenesis, and that this pathway may account for mutagenesis with most strong mutagens. Because this pathway also produces multiple mutations, it also is a strong candidate for carcinogenesis.

LETHAL AND GENETIC EFFECTS OF RADIOISOTOPE DECAY

Decay of radioactive isotopes incorporated into the genetic material of living cells usually causes death or other genetic damage. The goal of our experimental research is to relate observed biological effects of such decays to specific physicochemical damage to the genetic material. Iodine-125 is utilized as a model source for the production of highly localized and severe damage to DNA molecules in living systems. Radioactive iodine is incorporated into bacteria or bacteriophage DNA from 5-iododeoxyuridine, and the microorganisms are then stored in liquid nitrogen for varying periods to accumulate different amounts of radioactive decay, after which damage to DNA and biological damage are assayed.

Earlier we found that ^{125}I decay in the DNA of *E. coli* induces double strand breaks (DSB's) with 100% efficiency, and that wild-type cells can repair about two thirds of these breaks, but *recA* mutants can repair none (Krisch, R. E., et al., Int. J. Radiat. Biol. 29, 37, 1976). Furthermore, each unrepaired DSB appears to be lethal. Studies of repair of DSB's in bacteriophage λ are now underway. In addition, studies were initiated on the effect of growth medium and cellular growth rate upon the capability of cells to repair DSB's, and preliminary results with X-irradiated cells suggest that this repair capability is strongly affected by growth rate. We have also recently found that K12 strains of *E. coli* are predisposed to filament formation at rapid growth rates. Although these bacteria have been used extensively by other investigators, this important property has apparently not been noted. R. E. Krisch and D. M. Darby are now carrying out experiments to define this property better so as to evaluate its implications for our studies, as well as for those of others.

Experiments were also initiated in collaboration with Dr. Y. E. Rahman (Liposomes as Biological Carriers Group) to explore the possibility of selectively incorporating ^{125}I into tumor cell DNA by means of the liposome system. Because ^{125}I decay severely damages DNA, this radioisotope may be an antineoplastic agent. Results to date indicate that liposome-encapsulated ^{125}I is readily incorporated into the DNA of mouse tumor cells, but selective incorporation has not yet been demonstrated.

CHROMOSOME REPLICATION AND THE DIVISION CYCLE OF *Escherichia coli*

There is a difference of opinion concerning DNA synthesis in slowly growing bacteria. One school believes that DNA synthesis occurs essentially over the first two thirds of the cell cycle (Helmstetter, C. E., et al., Cold Spring Harbor Symp. Quant. Biol. 33, 809, 1968), while the other finds that DNA replication occurs near the end of the division cycle, as occurs in eucaryotic organisms (Kubitschek, H. E., and M. L. Freedman, J. Bacteriol. 107, 95, 1971).

Current studies of C. N. Newman and H. E. Kubitschek use a technique free of perturbations from synchronizing procedures to examine cells in DNA synthesis. Exponential phase cultures of *Escherichia coli* are exposed briefly to ^{125}I-labeled iododeoxyuridine, which is incorporated only into cells synthesizing DNA. This labeled fraction of the population is killed by decay of the radioisotope. Results are shown in Figure 15.1.

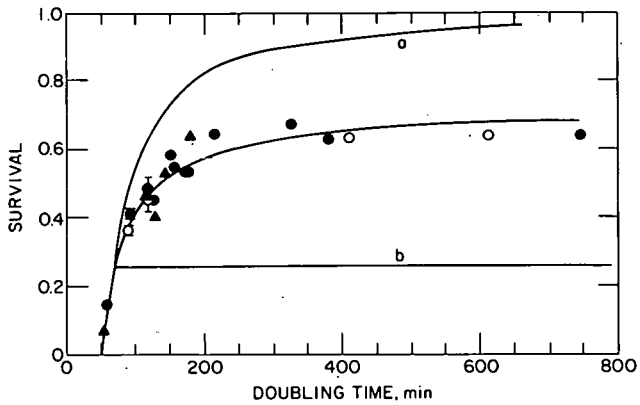

Fig. 15.1. Percentage of cells not replicating DNA as a function of generation time in three strains of *E. coli* B/r. Cultures were grown at various doubling times (generation times) and labeled for 4 minutes with ^{125}I-iododeoxyuridine. The cells were then stored at liquid nitrogen temperatures to allow accumulation of lethal radioactive decays. Cells survived only if they were not synthesizing DNA during labeling. The data are for three strains: o, B/r A; •, B/r K; ▲, B/r TT. The horizontal heavy line (b) is the survival predicted from the model of Helmstetter and co-workers. The heavy curved line (a) is the survival predicted from the observations of Kubitschek and Freedman (J. Bacteriol. 107, 95, 1971).

From the figure, it may be seen that three different strains give essentially the same results, contrary to earlier observations with cultures synchronized by the membrane elution technique. Thus the results in Figure 15.1 confirm earlier conclusions that slowly growing cells have their DNA synthesis periods perturbed by the synchronization technique (Kubitschek, H. E., and M. L. Freedman, J. Bacteriol. 107, 95, 1971).

The timing of DNA synthesis can be estimated from the fraction of cells killed. Preliminary estimates of the duration of C, the synthesis period, from the data in Figure 15.1 indicate that C increases with generation time, but is never more than approximately one third of a generation.

GENETIC EFFECTS OF DNA LESIONS SENSITIZED BY ENDOGENOUS AND EXOGENOUS AGENTS

In this program R. B. Webb, B. S. Hass, M. S. Brown, and R. D. Ley (Carcinogenesis Group) are investigating the genetic effects of chemical agents, especially polycyclic hydrocarbons, and nonionizing radiation. Current work places special emphasis on the mutagenicity, lethality, interactions, and repair of specific DNA lesions produced by processes sensitized by both endogenous and exogenous agents.

Genetic effects in bacteria can be induced by UV-B (290-320 nm), UV-A (320-400 nm), and visible light (400-750 nm) through sensitized processes involving both endogenous and exogenous chromophores. Pyrimidine dimers (Tyrrell, R. M., Photochem. Photobiol. 17, 69, 1973) and DNA single-strand breaks (Tyrrell, R. M., et al., Photochem. Photobiol. 20, 395, 1974) are readily produced by both UV-B and UV-A. The lethal consequences of these DNA lesions have been shown to be enhanced by damage to repair enzymes by UV-A radiation (Tyrrell, R. M., and R. B. Webb, Mutat. Res. 19, 361, 1973).

Action Spectra for Pyrimidine Dimers, DNA Breaks, and Mutagenesis

A biological action spectrum for the production of pyrimidine dimers has been obtained with *E. coli* K12 AB2480 (*recA uvrA*), a strain that shows no dark repair of pyrimidine dimers. Dimer yield calculations are based on the specificity of enzymatic photoreactivation (PR) for pyrimidine dimers. At 254 nm and 365 nm the number of dimers measured chemically (Tyrrell, R. M., Photochem. Photobiol. 17, 69, 1973) corresponds closely to the number of "PR events" in this strain. The action spectrum reveals an unexpectedly sharp minimum at approximately 350 nm and a maximum at approximately 365 nm (Figure 15.2). Based on both chemical (Tyrrell, R. M., personal communication) and biological assay, the dimer yield declines to a value below detection at 405 nm. These data suggest that dimer production is a sensitized process mediated by a non-DNA chromophore that absorbs between 350 and 390 nm.

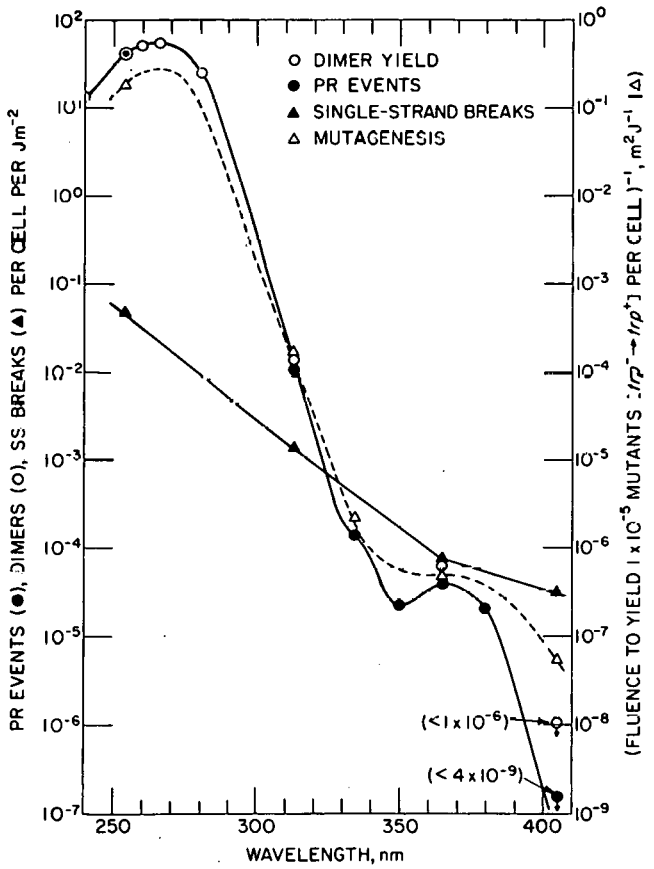

Fig. 15.2. Action spectra for pyrimidine dimers assayed chemically and biologically based on the slopes of the survival curves, k_o, in the absence of photoreactivation (PR) and k_{PR}, the slope in the presence of maximal PR, both in J m^{-2}. Thus, $k_o - k_{PR}$ = PR events per J m^{-2}. Single-strand breaks were based on alkaline sucrose gradient analysis. Mutagenesis was obtained with *E. coli* $WP2_s$ (*trp uvrA*) assayed on minimal plates supplemented with 2% nutrient broth.

A preliminary action spectrum for single-strand breaks, based on alkaline sucrose gradient analysis, revealed that DNA breaks were induced at 1/800 the rate of dimers at 254 nm (Figure 15.2). However, at 365 nm, twice as many single-strand breaks were induced as dimers. At 405 nm, the DNA break yield declined only twofold, whereas dimers were below detection. Since both DNA single-strand breaks and lethality are strongly oxygen dependent, and *uvrA* and wild-type strains show no photoreactivation in the UV-A range, DNA breaks are the most likely candidates for the lethal lesions for these strains (Webb, R. B., et al., Mutat. Res. 37, 164, 1976).

An initial action spectrum for mutation to tryptophan independence is also shown in Figure 15.2. Although mutagenesis was clearly associated with pyrimidine dimers at 254, 313, 334, and 365 nm, as demonstrated by almost complete photoreactivation of the premutational lesions at these wavelengths, the mutant yield per dimer was fivefold greater at 365 nm than at 254 nm. In contrast to data at 254 nm in which both lethality and mutagenesis are caused by dimers, data at 365 nm indicate that mutagenesis is largely caused by dimers, but lethality is clearly associated with an oxygen-dependent lesion.

Mutagenesis at 365 nm

The induction of mutation to tryptophan independence was linear at 365 nm under stringent anoxic conditions in which the major lesion is the pyrimidine dimer. However, in the presence of oxygen, single-strand breaks drastically changed the shape of the mutation curve to an upward bending, dose-square type. The dose-square mutation curve obtained at 365 nm under aerobic conditions is typical of the mutation response to 254-nm irradiation and to other strong mutagens. It is proposed that the dose-square mutation curve is caused by the interaction of two kinds of DNA lesions.

Mutagenesis at 365 nm at high fluence rates was similar to the 254-nm response in that the highest mutation rates were observed in *uvrA* and *polA* strains, and mutagenesis was below detection in *recA* strains. However, in contrast to the 254-nm response, the wild-type strain *E. coli* WP2 showed a complex mutation response with monochromatic 365-nm radiation and no mutagenesis with a broad spectrum UV-A source (fluorescent BLB 310-405 nm).

Results with high fluence rates are consistent with mutations occurring as a consequence of errors during postreplication repair (Witkin, E. M., Bacteriol. Rev. 40, 869, 1976) of UV-A-induced DNA lesions. In addition, DNA lesions clearly interact with damaged repair systems in both lethality and mutagenesis (Tyrrell, R. M., and R. B. Webb, Mutat. Res. 19, 361, 1973). Mutagenesis by UV-A radiation, especially in wild-type strains, is suggested to depend on the relative sensitivity of error-prone and error-free repair systems.

Mutagenic Evidence for Two Kinds of DNA Lesions Associated with Type of Binding of the Sensitizing Agent

Acridine orange (AO) binds to DNA in two modes: intercalation (absorption maximum about 500 nm) and external, dimeric (absorption maximum about 460 nm). We have examined the effect of low (0.1-1.0 W/m^2) fluence rates of monochromatic 460- or 500-nm light on chemostat cultures of different strains

of *E. coli* growing in the presence of 2 x 10^{-6} M AO. Bacteria that are deficient in recombination (*recA* and *lexA*) are known to be highly resistant to mutation by far-UV radiation and most strong mutagens. However, in the case of AO and 500-nm light, *recA* and *lexA* strains showed much greater mutation rates than wild-type and *uvrA* strains.

Observed mutation rates (phage T5 resistant mutants per 10^8 cells per day per W/m^2) 460- and 500-nm, respectively, were: 223 and 220 for strain WP2 (B/r wild type); 455 and 180 for strain $WP2_s$ (B/r *uvrA*); 398 and 1180 for strain B_{s-1} (*uvrB lexA fil*); and 516 and 2770 for strain WP10 (B/r *recA1*). It is evident that the mutagenesis observed at 500 nm is not due to error-prone repair, which requires the *rec*$^+$*lexA*$^+$ repair systems. In contrast, the functional *recA*$^+$*lexA*$^+$ repair systems strongly reduced the rate of mutation induction at 500 nm. We propose that lesions induced by AO plus 500 nm produce mutations by causing errors during replication. We further propose that AO plus 500-nm light produces DNA lesions that are capable of being repaired by the *recA*$^+$*lexA*$^+$ systems through an error-free process.

The 460-nm results show a different pattern from those at 500 nm. The *uvrA* strain had twice the mutation rate as the wild-type strain. Although the *recA* and *lexA* strains had higher mutation rates than wild type, the rates at 460 nm were only one third (*lexA*) and one fifth (*recA*) of the rates at 500 nm, indicating that there is a significant difference in the lesions produced at the two wavelengths.

CELLULAR CONTROL OF DNA REPLICATION

The basic objective of this study by T. Matsushita, A. Shotola, and L. Vukalcic has been to develop systems for studying control mechanisms for DNA replication. In particular, the role of cell membrane interaction with the chromosome is being examined as a specific control mechanism. The bacterium *Bacillus subtilis* is used because of its unique advantages for genetic studies, namely the availability of well-mapped mutants and a genetic transformation system. Sucrose-CsCl double density gradients are used to isolate the DNA associated with cell membranes, and transformation analyses are used to identify which parts of the chromosome are membrane associated.

Earlier, we found that protein synthesis is required for the release of the chromosome terminus from the cell membrane at the end of DNA replication. During the last year we have extended this finding to show reattachment of both the origin and terminus of the chromosome to the cell membrane in the next replication cycle.

The origin attachment was predicted by the well-known replicon model (Jacob, F., et al., Cold Spring Harbor Symp. Quant. Biol. 28, 329, 1963), which also suggested that newly replicated chromosomes were segregated to daughter cells by DNA-membrane attachments. Our results appear to require reconsideration of the segregation function, and the assignment of other, more complex roles, such as replication control, to the chromosomal origin and terminus attachments to the cell membrane. Since neoplastic cells usually differ from normal cells by inadequate control of chromosome replication, this work on mechanisms of DNA-membrane relationships in bacteria may be important for understanding the more complex relationships existing in eucaryotic cells.

MUTAGENESIS IN MOUSE MYELOMA CELLS

The major objective of these studies by T. Matsushita, A. Simms, and B. N. Jaroslow (Pathology and Risk Assessment Group) has been to examine the role of DNA synthesis and mutagenesis in complex developmental processes such as transformation, antibody formation, and DNA repair. To facilitate this objective, we are attempting to isolate a series of mutants that are affected in these processes, and which can also serve as mutational end points for screening environmental toxicants.

Isolation of eucaryotic mutants has necessitated the development of mouse myeloma cells into a usable mutagenic system. We have determined UV survival curves as a prelude to using UV light as an easily controllable mutagen. Conditions for mutagenesis have subsequently been established, and stable ouabain-resistant variants have been obtained. UV-induced mutants have been produced at frequences 4000 times greater than the spontaneous mutant background of 2×10^{-7}. In contrast to immunoglobulin gene mutations, ouabain resistance shows a high level of sensitivity to mutagen against a low background. Mouse myeloma cells are of special interest for studying contrasting high and low frequency classes of gene mutations, as well as providing an additional environmental mutagen screening system.

OXYGEN-DEPENDENT INACTIVATION OF *HAEMOPHILUS INFLUENZAE* TRANSFORMING DNA BY MONOCHROMATIC RADIATION: ACTION SPECTRUM, EFFECT OF HISTIDINE AND REPAIR*

E. Cabrera-Juarez,† *J. K. Setlow,*‡ *P. A. Swenson,*‡ *and M. J. Peak*

The action spectrum for the oxygen-independent inactivation of native transforming DNA from *Haemophilus influenzae* with near-UV radiation revealed a shoulder beginning at 334 and extending to 460 nm. The presence of 0.2 *M* histidine during irradiation produced a small increase in inactivation at 254, 290 and 313 nm, a large increase at 334 nm and a decrease in inactivation at 365, 405 and 460 nm. Photoreactivation did not reverse the DNA damage produced at pH 7.0 at 334, 365, 405 and 460 nm, but did reactivate the DNA after irradiation at 254, 290 and 313 nm. The inactivation of DNA irradiated at 254, 290 and 313 nm was considerably greater when the transforming ability was assayed in an excision-defective mutant compared with the wild type, although DNA irradiated at 334, 365, 405 and 460 nm showed smaller differences. These results suggest that the oxygen-independent inactivation of *H. influenzae* DNA at pH 7 by irradiation at 334, 365, 405 and 460 nm is caused by lesions other than pyrimidine dimers.

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*Abstract of a paper published in Photochem. Photobiol. 23, 309 (1976).

†Escuela Nacional de Ciencias Biologica, I.P.N., Mexico.

‡Oak Ridge National Laboratory.

LETHALITY AND DOUBLE-STRAND SCISSIONS FROM ^{14}C DECAY IN THE DNA OF MICRO-ORGANISMS*

Robert E. Krisch

^{14}C-2-Thymidine was incorporated into the DNA of *E. coli* B/r and of coliphage T4. The labelled organisms were stored for several years at -196°C. Both were periodically assayed for loss of viability, and the coliphage also for the appearance of double-strand breaks (DSBs) in DNA. *E. coli* B/r exhibited a survival curve with a substantial initial shoulder, extrapolation number 5.2 ± 2.3, and a final exponential portion corresponding to a lethal efficiency per ^{14}C decay per 2.5×10^9 daltons of DNA, of 0.009 ± 0.002. For coliphage T4, our best estimate for the lethal efficiency per ^{14}C decay is 0.03 ± 0.04, and that for the DNA breakage efficiency is -0.002 ± 0.004. The large standard errors result from the very small number of ^{14}C decays occurring in each phage. These results suggest that ^{14}C decay in the DNA of micro-organisms does not cause DSBs but does cause potentially lethal damage to the thymine bases in which decay occurs, and that wild-type *E. coli* can repair a large number of such DNA lesions.

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*Abstract of a paper published in Int. J. Radiat. Biol. 29, 249 (1976).

DNA BREAKAGE, REPAIR AND LETHALITY AFTER ^{125}I DECAY IN *rec*$^+$ AND *rec*A STRAINS OF *Escherichia coli*[*]

Robert E. Krisch, Frank Krasin, and Catherine J. Sauri

Iodine-125 decays by electron capture and is known to cause extensive molecular fragmentation via the Auger effect. ^{125}I was incorporated into the DNA of exponentially growing *E. coli* K12 AB2487, a *rec*A mutant, and *E. coli* K12 AB2497, the corresponding *rec*$^+$ strain, as 5-iododeoxyuridine (IUdR), an analogue of thymidine. Radioactive bacteria were stored at -196°C, and samples were periodically assayed for loss of viability and for the induction of double-strand breaks (DSBs) in DNA. Each ^{125}I decay in the DNA of either strain induces one DSB, i.e. α(DSB)=1.0. For the *rec*A strain, α(lethal)=0.9 and for the *rec*$^+$ strain, 0.4. Assays for biological repair of DSBs, involving incubation of thawed samples in growth-medium at 37°C before the extraction of DNA, demonstrate significant repair of ^{125}I-induced DSBs by *rec*$^+$ cells but none by *rec*A cells. For small numbers of decays, there is approximately a 1:1 correlation, for either strain, between lethal decays and post-incubation residual DSBs. Comparison with data for larger numbers of decays indicates that a typical *rec*$^+$ cell can repair no more than three to four DSBs per completed genome (2.5×10^9 daltons).

[*]Abstract of a paper published in Int. J. Radiat. Biol. 29, 37 (1976).

ACTION SPECTRA FOR LETHALITY IN RECOMBINATIONLESS STRAINS OF *Salmonella typhimurium* AND *Escherichia coli*[*]

Donna Mackay, A. Eisenstark,[†] *R. B. Webb, and M. S. Brown*

Action spectra for lethality of both stationary and exponentially growing cells of recombinationless (*recA*) mutants of *Salmonella typhimurium* and *Escherichia coli* were obtained. Maximum sensitivity was observed at 260 nm which corresponds to the maximum absorbance of DNA. However, a shoulder occurred in the 280-300 nm range that departed significantly from the absorption spectrum of DNA. At wavelengths longer than 320 nm, the shapes of inactivation curves departed significantly from those at wavelengths shorter than 320 nm and survival curves at wavelengths longer than 320 nm had a large shoulder. A small peak or shoulder occurred in the 330-340 nm region of the action spectra. The special sensitivity of *recA* mutants to broad spectrum near-UV radiation may be due to synergistic effects of different wavelengths. Parallels between the inactivation of *recA* mutants and the induction of a photoproduct of L-tryptophan toxic for *recA* mutants (now known

[*]Abstract of a paper published in Photochem. Photobiol. 24, 337 (1976).

[†]Kansas State University.

to be H_2O_2) suggest that H_2O_2 photoproduct from endogenous tryptophan may be involved in the high sensitivity of these strains to broad spectrum near-UV radiation.

PROTECTION BY AET AGAINST INACTIVATION OF TRANSFORMING DNA BY NEAR-ULTRAVIOLET LIGHT: ACTION SPECTRUM*

M. J. Peak and J. G. Peak

We have previously shown that histidine protects *Bacillus subtilis* DNA from inactivation by near-ultraviolet (near UV) radiation, but not by far-ultraviolet (far UV) radiation. This observation indicated that the processes of inactivation of transforming DNA by near UV are not the same as for far UV. In this note, we describe the protective action of another compound, 2-aminoethylisothiouronium bromide hydrobromide (AET). The fact that histidine protects against the actions of both near UV and X rays prompted us to compare the histidine protection against near UV with AET protection against near UV, since this latter compound also protects biological material against X-ray action.

*Abstract of a paper published in Photochem. Photobiol. 22, 147 (1975).

SENSITIVITY OF STRAINS OF *Escherichia coli* DIFFERING IN REPAIR CAPABILITY TO FAR UV, NEAR UV AND VISIBLE RADIATIONS*

Robert B. Webb and Mickey S. Brown

In stationary phase, strains of *Escherichia coli* deficient in excision (B/r Hcr) or recombination repair (K12 AB2463) were more sensitive than a repair proficient strain (B/r) to monochromatic near-ultraviolet (365 nm) and visible (460 nm) radiations. The relative increase in sensitivity of mutants deficient in excision or recombination repair, in comparison to the wildtype, was less at 365 nm than at 254 nm. However, a strain deficient in both excision and recombination repair (K12 AB2480) showed a large, almost equal, increase in sensitivity over mutants deficient in either excision or recombination repair at 365 nm and 254 nm. All strains tested were highly resistant to 650 nm radiation. Action spectra for lethality of strains B/r and B/r Hcr in stationary phase reveal small peaks or shoulders in the 330-340, 400-410 and 490-510 nm wavelength ranges. The presence of 5 μg/ml acriflavine (an inhibitor of repair) in the plating medium greatly increased the sensitivity of strain B/r to radiation at 254, 365 and 460 nm,

*Abstract of a paper published in Photochem. Photobiol. 24, 425 (1976).

while strains *E. coli* B/r Hcr and K12 AB2463 were sensitized by small amounts. At each of the wavelengths tested, acriflavine in the plating medium had at most a small effect on *E. coli* K12 AB2480. Acriflavine failed to sensitize any strain tested at 650 nm. Evidence supports the interpretation that lesions induced in DNA by 365 nm and 460 nm radiations play the major role in the inactivation of *E. coli* by these wavelengths. Single-strand breaks (or alkali-labile bonds), but not pyrimidine dimers are candidates for the lethal DNA lesions in *uvrA* and repair proficient strains. At high fluences lethality may be enhanced by damage to the excision and recombination repair systems.

PROTEIN SYNTHESIS AND THE RELEASE OF THE REPLICATION TERMINUS FROM THE CELL MEMBRANE IN *Bacillus subtilis*[*]

Scott Winston and Tatsuo Matsushita

Thus far we have studied three systems in which protein synthesis and initiation are decreased--*B. subtilis* cells treated with toluene or rifampin, and *B. subtilis* cells amino acid starved. All three systems showed an increase enrichment of the terminus in the membrane fraction, indicating that termini were not released from the cell membrane when protein synthesis was inhibited. When protein synthesis resumed after rifampin treatment and amino acid starvation, the terminus enrichment index decreased markedly, indicating the release of the termini from the membrane. These results suggest that protein synthesis is necessary for the release of the terminus portion of the chromosome from the cell membrane, presumably sometime after the end of DNA replication.

The reason for the decrease in the origin attachment to the membrane in all three systems after the loss of protein synthesis is still not clear. One explanation is that initiator proteins are required to stabilize the origin-membrane complex. Under our experimental conditions of decreased protein synthesis and initiation, these required proteins are not synthesized and the origin-membrane binding site may be less stable, making it difficult to isolate.

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[*]Summary of a paper published in Microbiology--1976, Ed. D. Schlessinger. American Society for Microbiology, Washington, DC, pp. 123-127, 1976.

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16. MOLECULAR ANATOMY PROGRAM

ERDA RT-02-01
ANL 61203

GROUP LEADER'S INTRODUCTION

Norman G. Anderson, Group Leader

The complete resolution of all the metabolites and gene products that compose human cells is now theoretically possible. While a very great amount of developmental and experimental work remains to be done, some of which is described here, it is of interest to review briefly the concepts and work that led us to this point, and the directions of future research.

The science of anatomy has progressed slowly from the level of gross morphology through microscopic anatomy, first at the light microscope level and then at the level of the electron microscope, and on to the level of large molecules which self-assemble to make the structures of which cells and tissues are composed. While biochemists have explored a large number of cellular constituents, the gulf between the number of gene products found in cells (an estimated 25,000 structural genes exist in the human genome) and the number of proteins known and associated with a specific function (estimated at less than 1,000) is quite large. More to the point, the viewpoints of the molecular anatomists and the biochemists are quite different. Anatomy seeks complete structural descriptions, and it is incomplete if any entitites are omitted. To the extent that one does not "see" all of the gene products of a cell, descriptions of differences between normal and cancer cells (or any other diseased cell for that matter) are incomplete.

The history of the Molecular Anatomy Program (first at Oak Ridge National Laboratory, and now at Argonne National Laboratory) has been one of the systematic development and application of the basic tools that molecular anatomy requires. The underlying concept has been that of a hierarchical series of fractionations, beginning with tissues and ending with molecules. It led to the invention and development of the zonal centrifuge for separating different cell types and subcellular particles, to the development of the K centrifuge for large scale vaccine purification, to high pressure liquid chromatography systems for separating and analyzing compounds of intermediate molecular weight in human body fluids, to the invention and development of the centrifugal fast analyzer to analyze the large number of fractions separated, and to the development of the Cyclum system for rapid recycling chromatography for the separation of either groups of proteins or of individual proteins. This is not a complete list of the systems and techniques developed; rather it includes the ones that have come into wide use and have contributed

measurably to improvements in human health. The list is, however, incomplete in another respect. Except for the results of the high-pressure chromatographic systems, mapping cannot be done down to the molecular level.

The initial effort at Argonne National Laboratory has been, therefore, to develop methods for mapping quantitatively the thousands of gene products found in cells and subcellular structures, and to identify those whose functions are known. This effort is based on a long history of development of two-dimensional electrophoresis beginning in 1956 with the first two-dimensional separations of Smithies and Poulik, and extending on to the high resolution separations independently developed in four different laboratories in 1975-76 by O'Farrell, Klose, Scheele, and Iborra and Buhler. These authors described the use of isoelectric focusing in urea, in the first dimension, followed by electrophoresis in the presence of sodium dodecyl sulfate, in the second. This technique yields a map based on two independent characteristics: charge and molecular weight.

We do not merely seek lists of substances found in cells; rather we ask very basic questions concerning the organization of such lists. Are genes turned on and off in sets or groups so that we can organize our lists into such sets? Is there a discoverable sequence or program for sequencing these sets during human embryonic development? Do cancer and other diseases involve the reexpression of gene sets normally active only during early embryonic development? These questions are very basic to all studies on the toxicology of energy-related pollutants, including radiation. It is in terms of alterations in subcellular gene product sets that pathology must ultimately be rewritten. Hence the emphasis is on the development of the tools required for this work, and specifically on the development of the ISO-DALT two-dimensional electrophoresis system at ANL.

BASIC STRATEGY

The basic strategy of this work is initially to separate as many subcellular organelles as possible (including the cell sap), and then to map each of these. The objective is to see how many constituents are common to the same organelle in different tissues, and to different organelles in the same tissue. To do this, high repeatability of mapping is required and tests for identity must be made. As a final check, however, each sample must be run mixed with every other sample to see which spots co-electrophorese. This work, pursued to completion, should allow the base, or constitutive set of genes to be determined, and may allow organ or germ line sets also to be distinguished. Eventually, using radiolabels and autoradiography, the method can be applied to embryonic tissue and, hopefully, to the problem of deciphering the program of differentiation.

Identification of map spots is a quite different matter, and may be based on one of several techniques. If pure, known proteins are present, and if the map spots can be quantitated densitometrically, simple addition of the pure protein to the mixture from which it is derived will allow localization. If monospecific antibodies are available, an immunoprecipitate may be prepared, and mapped quite easily since dissociation of antigen and antibody occurs under the run conditions employed. A more general approach is to fractionate each subcellular mixture by a series of different methods, then determine the activity of a large number of different enzymes in each fraction, and in

addition map each fraction quantitatively by two-dimensional electrophoresis. A computerized analysis of the resulting data will locate map spots that are correlated with activity in the fractions obtained by each separations technique. The GeMSAEC centrifugal fast analyzer is ideally suited to this work. It is obvious that a very large number of electrophoretic analyses must be done. Hence, the first priority has been the development of systems for doing large numbers of analyses in a reproducible manner, for automatically densitometering the resulting gels, and for computerized data reduction and management. (This does not exhaust the methods planned for use.)

MOLECULAR ANATOMY PROGRAM STAFF

REGULAR STAFF

Anderson, Norman G. (Senior Physiologist)

TEMPORARY STAFF DURING 1976

Anderson, N. Leigh (Research Associate)

ERDA RT-02-01
ANL 61203

EXPERIMENTAL STUDIES

N. Leigh Anderson and Norman G. Anderson

THE ISO-DALT SYSTEM

The central effort during 1977 has been on the development of a two-dimensional electrophoretic system capable of resolving the complex mixtures of proteins and protein subunits found in living cells. The technique used, called the ISO-DALT system, uses isoelectric focusing in urea in one dimension, and electrophoresis in sodium dodecyl sulfate in the other. The effort is a truly interdisciplinary one and is a classic example of what can be done in a national laboratory. The first semiautomatic systems we have designed ourselves and have had constructed at Argonne. These prototypes allow a small staff to run up to 40 complete analyses per day. Fixing, staining, destaining, and photography are still done manually and are the present bottlenecks. We now enter the second phase of the project, a complete review and redesign of the entire system with engineers and specialists to optimize each aspect of it. There are more than a few parallels between building these basic biological tools and the development of the large tools of modern physics, and many of the same people can contribute effectively to both. The innovation, research, development, and engineering required to realize the full potential of this system will require several years of intense effort.

HUMAN SERUM PROTEINS

Approximately 60 human serum proteins have been seen by previous analytical techniques, and a little over 40 have been given names. For nearly all that have been studied quantitatively, it has been found that the amount varies in disease and may indeed be an indicator of disease. Hence, it is of interest to develop economical ways of "seeing" as many human proteins as possible. Several hundred spots appear in ISO-DALT patterns of human serum or plasma, and the initial problem is to find out which of these spots correspond to known proteins. Antisera to approximately 30 human proteins are available, and we have used them to obtain immunoprecipitates containing a specific protein and antibody. These precipitates are dissociated and analyzed using the ISO-DALT system. In this way, 29 polypeptides have been identified. In many instances multiple spots appear, caused by a variable number of sialic acid groups added during synthesis, or removed during circulation. In addition, molecular weight heterogeneities are often seen due apparently to the addition of variable amounts of neutral carbohydrate. This heterogeneity gives us a true picture of the state of the proteins in the circulation.

A map of proteins identified in ISO-DALT systems is shown in Figure 16.1.

It is evident that the ISO-DALT system is an extraordinarily useful one for detecting mutant proteins in man, both to detect genetic disease, or to determine the number of polymorphisms that may be present. Such work forms

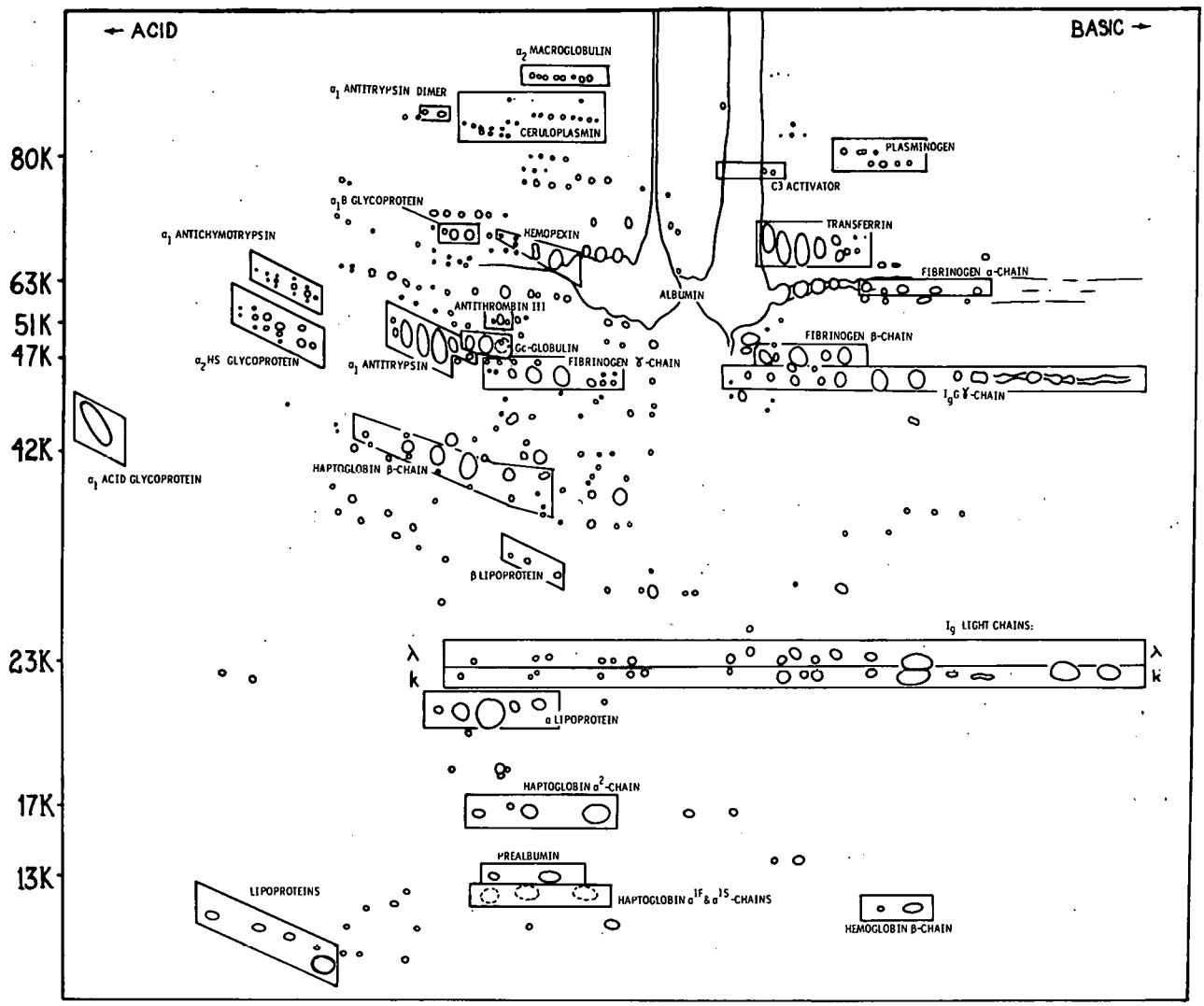

Fig. 16.1. Diagram of a two-dimensional electrophoretic (ISO-DALT) separation of human plasma proteins. The horizontal dimension is isoelectric focusing in 9 M urea, while the vertical dimension is electrophoresis in sodium dodecyl sulfate which separates the proteins according to molecular weight.

the basis for the determination of the background mutation rate in man. Alterations in this rate, produced by irradiation or other energy-related or environmental pollutants, would have profound consequences for mankind.

TISSUE PROTEINS

The problem of mapping tissue proteins is being approached by starting with relatively simple protein mixtures such as red cell lysates, then progressing on to human platelets and subcellular tissue fractions. Up to 677 discrete spots have been obtained with some of the latter. The problem, of

course, is to find out what activities or functions are associated with each spot. Actin turns out to be an almost universal cell constituent which can serve as a landmark in these patterns. For the remainder, special strategies are required, of which several are under development. The most generally applicable of these strategies involves the fractionation of a mixture such as cytosol by a variety of procedures including chromatography, gel filtration, and salting out, followed by analysis of all fractions for a battery of enzymes using the centrifugal fast analyzer. In addition, all fractions are analyzed by the ISO-DALT technique. One asks at the end what spots always correlate with a particular activity, regardless of the separation method used. Alternate methods based on two-dimensional electrophoresis under nondenaturing conditions, and on immunochemical methods for fractionating complex mixtures, are also being explored.

THE BIRTH AND EARLY CHILDHOOD OF CENTRIFUGAL ANALYZERS*

Norman G. Anderson

The future of a technology-based society such as ours depends on continued innovation. Given a high rate of technology export, and increasing uncertainty as to how, where, and at what level research and innovation should and will be supported, it becomes increasingly important to ask how innovations actually occur. Fact (that individuals invent) and accepted doctrine (that laboratories, corporations, programs originate) are so much at variance that one would hesitate to discuss either the problem or specific instances were it not that so much is at stake nationally. We must understand as honestly as we can how inventions occur, who actually makes them, and how continued invention can be fostered. It is for this reason only that I think it worthwhile to describe the early history of the development of centrifugal analyzers.

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*From introduction of a paper to be published in Clin. Chem.

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17. SUPPORT FACILITIES

COMPUTER SUPPORT FACILITIES

Frank S. Williamson, Jeanne A. Blomquist, and Carol A. Fox

We continue to support approximately 50 computer users who employ the remote access data station (RADS). This support particularly involves consultation at all levels of programming and job management.

Highlights of the year's programming activities include:

1) A set of scheduling programs for the Radiation Toxicity in Dogs project. These programs permit the management of a file containing coded descriptions of the clinical procedures, experimental protocols, and physical relocations which are projected for the animals. They also generate schedule reports which are used to manage the dog colony.

2) An Animal Inventory System, interfaced to the JANUS animal data files, has been created and is in use. This involves a time-sharing processor for the entry and verification of transactions, with a diversity of report generating programs. A transaction file will facilitate the addition of full accounting at a later date.

3) A set of programs to manage paper tape data on rat body temperature, for C. F. Ehret, Molecular Studies Group.

4) Programs to display processed paper-tape nephelometry data on two- or three-dimensional plots, also for C. F. Ehret.

5) A system to process paper-tape data recorded by another data-acquisition system, for G. A. Sacher, Pathology and Risk Assessment Group. In this case, interpretation of single missing data values is provided, using the Aitken-Lagrange algorithm, and bursts of missing values are flagged. Multiparameter plots are generated, and color slides have been produced for formal presentations.

COMPUTER SUPPORT ACTIVITIES STAFF

Blomquist, Jeanne A. (Programmer)
Fox, Carol A. (Programmer)
*Kraimer, Martin R. (Computer Scientist)
Williamson, Frank S. (Physicist)

*Transferred to Applied Mathematics Division.

ELECTRON MICROSCOPE CENTER

Thomas M. Seed

The Electron Microscopy Center provides specialized electron microscopic service to both the Division and to the Laboratory as a whole. During 1976, there were some twenty-two users, including six staff biologists, two post-doctoral appointees, three students, and five faculty researchers from neighboring educational institutions. The remainder were from other Argonne divisions, including Radiological and Environmental Research, Environmental Impact Studies, Chemistry, Physics, and Center for Educational Affairs.

To assist in our support efforts, a new tissue sectioning room was established during the year which allows multiple users to work under much improved conditions. In addition, one of the older electron microscopes (RCA 3F) was replaced with a newer model (RCA 4A) with improved operational features.

The major effort of the staff of the Center was in collaborative work with four Divisional research groups (listed below) which utilized one or more staff microscopists on a full time basis. Mr. G. T. Chubb continues to provide support to all users of the Center.

1) Radiation Toxicity in Dogs Group. The more prominent late ultrastructural effects of low dose irradiation are being described to supplement and extend histological findings. At present, the very prominent canine erythro- and myelogenous leukemias are being examined in depth, both in terms of mechanisms of induction and descriptions of the terminal disease state. (See report by T. M. Seed et al. in Section 3 of this report.) Other pathological entities, such as the late developing aplastic anemias, degenerative liver disease, and reproductive failure within dogs previously irradiated *in utero* with low doses of gamma irradiation, are being evaluated by electron microscopy.

2) Neutron and Gamma-Ray Toxicity Group. Comparative ultrastructural studies are being carried out to evaluate late pathological changes of the microvasculature of $B6CF_1$ mice treated with either single or fractionated doses of gamma or neutron irradiation. (See report by S. P. Stearner et al. in Section 2 of this report.) These studies, along with future ones, should

provide insight into assessing the potential added risk of radiation therapy in humans.

3) Carcinogenesis Group. The ultrastructure of the Harderian gland of gamma- and neutron-irradiated $B6CF_1$ mice is being studied in conjunction with the incidence, metastases, and possible life-shortening effects of the tumor in irradiated mice. (See report by R. N. Feinstein et al. in Section 5 of this report.) In addition, other types of tumors in $B6CF_1$ mice are being examined ultrastructurally for the purpose of classification.

4) Liposomes as Biological Carriers Group. Interactions of liposome-encapsulated cancer drugs with target cells are being evaluated at the ultrastructural level. (See report by Y. E. Rahman et al. in Section 9 of this report.) The intent is to elucidate mechanisms of uptake, therapeutic mode of action, and intracellular release.

ELECTRON MICROSCOPE CENTER STAFF

Chubb, G. Theodore (Engineering Specialist)
Devine, Rosemarie L. (Scientific Associate)
Sanderson, Margaret M. (Scientific Associate)
Seed, Thomas M. (Assistant Biologist)
Wright, Betty J. (Scientific Associate)

LABORATORY ANIMAL FACILITIES

Thomas E. Fritz

The use of laboratory animals for studies on the health effects of energy generation and utilization continues to be a highly significant part of the research program of the Division. During the past year, there were significant changes in the personnel, management, and physical plant of the animal facilities. These changes have been directed toward the goal of Dr. R. J. Flynn, former director of the Facilities--support of Divisional research by provision of the highest quality experimental animals, in the most economical and efficient manner.

Since the operational costs of the Animal Facilities approach 20% of the Division's annual budget, it is important to distribute these costs fairly and to assure the efficient utilization of the space, equipment, animals, and manpower of the Facilities. During the past year, a first approach to the fair distribution of costs and to efficient scheduling of facilities utilization was the development of a computer-based animal inventory system (AIS). This record system, its operational philosophy, and essential programming have been largely developed through the efforts of F. S. Williamson, J. A. Blomquist, and C. A. Fox, of the Computer Support Facilities. The AIS now contains the majority of the experimental and all of the stock animals in the Facilities.

Because of the importance of maintaining the physical plant, equipment, and adequate supplies, R. J. Robertson, Staff Assistant, was assigned on a part-time basis to assist the Director and the Animal Care Supervisors in activities relating to maintenance, repairs, and purchases. Similarly, new responsibilities for purchases of animal food, bedding, and supplies and the scheduling of work assignments were delegated to the Chief Animal Care Specialists, D. D. Banister and W. H. Hart.

Renovations to E-wing, which were designed to provide a barrier facility for maintenance of pathogen-free rodents, were largely completed in 1976.

Two new ^{60}Co gamma irradiation rooms designed for protracted studies in dogs were completed and occupied during the year. Tornado damage to these facilities required extensive repairs, but the commendable cooperation of all Divisional and Animal Facilities personnel prevented major problems in continuing the studies in the damaged areas.

Plans for a modest building addition to provide a separate wing for breeding of rodents were completed, and the facility should be operational during 1977. The breeding program, operated by L. O. Bibbs with the assistance of J. M. Angerman, regularly provides rodents to the Neutron and Gamma-Ray Toxicity Group, as well as other staff research programs.

To increase efficiency and reduce manpower costs in the processing of rodent water bottles, an engineering study was initiated on the mechanization and automation of the handling, cleaning, and filling of the bottles. The results of this study support the economic feasibility of purchasing, designing, and engineering such equipment. During the next year, the first of three stages for automating this work will be completed. The costs of this equipment will be recovered over a short period of time.

Plans to renovate the five dog kennels presently in operation, and to reactivate a kennel that has been unoccupied for many years, have had preliminary approval. These renovations are aimed at greater efficiency as well as providing a better environment for the dogs and better working conditions for the animal care personnel. Related to the kennel renovations are the plans to build a comprehensive clinical facility adjacent to the kennels. A new wing would consolidate, update, and expand existing facilities for diagnostic radiology, surgery, physiologic monitoring, necropsy, photography, postsurgical and intensive care, and physical examinations. Planning for this construction should be completed during the coming year and construction begun the following year.

LABORATORY ANIMAL FACILITIES STAFF

*Brennan, Patricia C. (Biologist)
Fritz, Thomas E. (Veterinary Pathologist)
†Flynn, Robert J. (Senior Veterinarian)
Keenan, William G. (Scientific Associate)
Poole, Calvin M. (Veterinarian)
Robertson, Robert J. (Staff Assistant)
*Simkins, Richard C. (Scientific Assistant)
*Tolle, David V. (Scientific Associate)

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*Now in Pathology and Risk Assessment Group.

†Now in Viral, Radiation, and Environmental Oncology Group.

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18. EDUCATIONAL ACTIVITIES

POSTGRADUATE TRAINING

During 1976, a total of 36 postdoctoral appointees, visiting scientists, and research associates contributed to the research programs of the Division. Five of these were new appointments in 1976, 2 less than the number who finished their assignments during the year.

The temporary appointees, their schools, and the staff members with whom they were affiliated were as follows:

Enrique E. Abola	University of Pittsburgh	A. B. Edmundson
N. Leigh Anderson*	Cambridge University, Cambridge, England	S. S. Danyluk
Erik Boye	Norwegian Radium Hospital, Oslo, Norway	R. E. Krisch
Charles D. Brown*	University of Illinois Medical Center	D. Grahn
David A. Crouse	University of Iowa	E. J. Ainsworth
Diana K. Dixon-Davis*	University of California, Berkeley	D. Grahn/ L. J. Hoover (EES)
Kenneth W. Dobra	Indiana University, Bloomington	C. F. Ehret
Fouad S. Ezra	University of Rochester	S. S. Danyluk
Joseph R. Firca	University of Cincinnati	A. B. Edmundson
Raymond A. Guilmette	New York University	A. Lindenbaum
Diana L. Gutzeit	University of Illinois, Urbana	M. P. Finkel/ W. P. Norris
Antun Han†	Laboratory for Experimental Cancerology (Yugoslavia)	M. M. Elkind
Wayne R. Hanson	University of Iowa	R. J. M. Fry
Bruce S. Hass*	Texas A & M University	R. B. Webb
Gunnard K. Jacobson*	University of Chicago	R. D. Ley
Margaret M. Jonah*	Columbia University	Y. E. Rahman
Eva E. Kautzky*	Academy of Sciences, Czechoslovakia	M. M. Elkind
Chung Hee Ryu Lee*	University of Illinois, Urbana	D. Grahn

*Research Associate.

†Visiting Scientist, now Biophysicist.

Chung K. Lee*	University of Illinois, Urbana	M. P. Finkel
Karin A. Mede	University of Illinois Medical Center	P. D. Klein
Chester N. Newman	Indiana University Medical Center, Indianapolis	H. E. Kubitschek
Frank Q. Ngo	Wayne State University	M. M. Elkind
Nicolas C. Panagiotopoulos	University of Pittsburgh	A. B. Edmundson
Dale A. Schoeller	Indiana University, Bloomington	P. D. Klein
Bobby R. Scott	University of Illinois, Urbana	E. J. Ainsworth
Elsie M. Sorenson	University of Texas Austin	A. Lindenbaum
Surendra T. Shenoy	University of California, Davis	C. Peraino
Frans Stellaard†	University of Technology, The Netherlands	P. D. Klein
Ravindra Tewari	Tata Institute of Fundamental Research, Bombay, India	S. S. Danyluk
Kou-Yi Tserng	University of Michigan	P. D. Klein
Hiroshi Utsumi	Kyoto University, Japan	M. M. Elkind
Alice M. Wyrwicz	University of Chicago	S. S. Danyluk
Vivian V. Yang	University of Chicago	S. P. Stearner

In addition, there were nine Faculty Research Participation appointments, supported by the Argonne Center for Educational Affairs (CEA); these appointments enable college and university faculty members to participate in the research activities of the Laboratory in order to broaden their perspectives for teaching and research on their home campuses. The names of the Faculty Research Participants during 1976, their schools, and their staff sponsors were as follows:

Alonzo J. Fairbanks	Trinity College, Illinois	S. S. Danyluk
William G. Gensler	University of Arizona	J. Shen-Miller
Gordon L. Jendrasiak	University of Illinois, Urbana	Y. E. Rahman
Eugene W. McArdle	Northeastern Illinois University	C. F. Ehret
William F. Millington	Marquette University	J. Shen-Miller
Daniel G. Oldfield	DePaul University	R. J. M. Fry
John F. Schneider	University of Chicago	P. D. Klein
Thomas A. Victor	Northwestern University	S. S. Danyluk
Ruth L. Willey	University of Illinois, Circle Campus	R. J. M. Fry

SUMMER GRADUATE STUDENT PROGRAM IN BIOLOGY

Ten students from nine different universities were enrolled in the 1976 program. Dr. Frederic Giere, Chairman of the Department of Biology at Lake Forest College, served as coordinator of the course, in cooperation with Dr. Walter Kisieleski. The program, which ran for 12 weeks, consisted of

*Now Assistant Biologist.

†Research Associate.

"core" lectures in radiation biology and techniques by Dr. Giere and Dr. Kisieleski, and a series of 11 "specialized" lectures given by Drs. Thomas Borak, Arthur Lindenbaum, David Crouse, Ronald Ley, Thomas Seed, David Haugen, Christopher Reilly, Douglas Grahn, Steven Spigarelli (Division of Radiological and Environmental Research), and Norman Frigerio (Environmental Impact Studies).

An additional feature of the course this year was a series of workshop and lecture demonstrations designed to give the students practical experience with a spectrum of radiobiological techniques. At Argonne these were given by Messrs. C. J. Smyros (Reactor Research Operations Division), Gordon Holmblad, Emil Johnson, Norbert Kretz, and George Chubb. Mr. Donald Moore gave a demonstration of liquid scintillation counting methods at Packard Instrument Company, and Mr. Michael Sloan showed radioimmunoassay methods and gamma counting techniques at Beckman Instruments Company.

Each student spent the remainder of his time working in a laboratory of a staff member. Most of the students received academic credit for the course from their home institutions.

The students, their schools, and their staff supervisors were as follows:

John P. Albers	San Diego State University	W. P. Norris
Angelica Bauer	St. Louis University	G. A. Sacher
Michael J. Blend	Chicago College of Osteopathic Medicine	R. J. M. Fry
Margarette Douyon	Tuskegee Institute	D. A. Haugen
Russell C. Holpuch	University of Illinois Medical Center	S. A. Spigarelli (RER)
Arlene M. Magon	University of Illinois, Circle Campus	H. E. Kubitschek
Edward C. Piller	Creighton University	T. M. Seed
Mohamed A. Sharaf	St. Louis University	S. P. Stearner
Phillip W. Urnezis	University of Michigan	R. P. Larsen (RER)
Eric C. Zickgraf	Illinois Institute of Technology	R. E. Krisch

OTHER GRADUATE PROGRAMS

Five graduate students were Laboratory Graduate Participants working in the Division on research for their PhD degrees in a program administered by the Center for Educational Affairs. The Laboratory Graduate Participants, their schools, and their staff sponsors were as follows:

Avrom M. Brendzel	University of Illinois, Circle Campus	Y. E. Rahman
Charles D. Brown	University of Illinois Medical Center	S. S. Danyluk
John P. Christopher	Oregon State University	C. Peraino
Michael P. Hagen	University of Illinois, Urbana	M. M. Elkind
Roy M. Vigneulle	University of Illinois, Urbana	E. J. Ainsworth

A related program, called Thesis Parts, allows graduate students to perform pertinent parts of their research at Argonne. In 1976, two students held such appointments in the Division:

Jay H. Jones	Southern Illinois University	P. D. Klein
Bradford B. Smith	San Diego State University	J. F. Thomson

In addition, three students held Guest Graduate Student appointments. They were Avrom M. Brendzel (until October, 1976), University of Illinois, Circle Campus, under the supervision of Y. E. Rahman, Roy A. Goodson, Northeastern Illinois University, under the supervision of C. F. Ehret, and Michael P. Hagen (until April, 1976), University of Illinois, Urbana, under the supervision of M. M. Elkind.

Aaron D. Simms worked under the supervision of Dr. T. Matsushita, under the sponsorship of the Affirmative Action Program.

UNDERGRADUATE TRAINING

During 1976, a total of 26 college undergraduates received training in the Division of Biological and Medical Research through the CEA-sponsored Spring, Summer, and Fall Honors Research Participation Programs. The students, their schools, and their staff supervisors are listed below:

SPRING PROGRAM

David C. Caldwell	High Point College	W. E. Kisieleski
Jannette L. Carey	Central Connecticut State College	J. Shen-Miller
Robert S. Decker	University of Rhode Island	T. Matsushita
Sue E. DeWalt	Millikin University	R. E. Krisch
Carol F. Farver	Pacific Lutheran University	E. J. Ainsworth
James M. Fenton	Kent State University	A. B. Edmundson
Steven H. Gray	Carthage College	S. S. Danyluk
Jay L. Schlabach	Goshen College	C. F. Ehret
Grace H. Van Epps	Pennsylvania State University	T. E. Fritz

SUMMER PROGRAM

Janice L. Arnold	Illinois Institute of Technology	R. B. Webb
Frank C. Chao	University of Illinois, Urbana	A. Lindenbaum
Debora E. Dyett	Harvard University	C. Peraino
Thomas P. Fagedes	Xavier University, Ohio	T. B. Borak
James M. Fenton	Kent State University	A. B. Edmundson
Barbra Gabriel	Elmhurst College	A. B. Edmundson
Candis D. Grace	South Carolina State College	R. N. Feinstein
Steve H. Gray	Carthage College	S. S. Danyluk
Mary B. Martin	Jackson State University	P. C. Brennan
Leo D. Savare, Jr.	LeMoyne-Owen College, Memphis	J. Shen-Miller
Aaron D. Simms	New York University	T. Matsushita
Edward M. Williams	Tuskegee Institute	T. E. Fritz
Lucille A. Wilson	Allen University	C. F. Ehret

FALL PROGRAM

Lois J. Ayash	Southeastern Massachusetts University	H. E. Kubitschek
Daniel Cameron	University of Minnesota	M. MacCoss
Barbara G. D'Arcy	The Colorado College	C. Peraino
Grant J. Price	Cornell College, Iowa	D. A. Haugen
Valeria L. Riotte	Rosary College	E. M. Sorenson
Lisa Vukalcic	Mount St. Mary's College, California	T. Matsushita
Dale R. Worley	Grinell College	F. S. Williamson

JOINT ARGONNE-UNIVERSITY APPOINTMENTS

During 1976, 20 staff members held a total of 28 faculty appointments at universities in the Chicago area. These appointments usually comprise limited teaching activities, generally of a specialized nature, at the graduate level, which involve regular contact with students. They have led to cosponsorship of graduate students and to collaborative research efforts with faculty members, some of which are described in this report.

The affiliations with Chicago area universities were as follows:

University of Chicago

Mortimer M. Elkind	Peter D. Klein
Robert N. Feinstein	Timothy E. O'Connor
R. J. Michael Fry	George A. Sacher
David L. Hachey	Patricia Szczepanik

University of Illinois, Circle Campus

Douglas Grahn	Carl Peraino
Bernard N. Jaroslow	Jane Shen-Miller
Herbert E. Kubitschek	John F. Thomson

Loyola University

Thomas E. Fritz	Walter E. Kisieleski
Bernard N. Jaroslow	Arthur Lindenbaum

Northern Illinois University

R. J. Michael Fry	Y. E. Rahman
Douglas Grahn	Christopher A. Reilly, Jr.
Bernard N. Jaroslow	John F. Thomson
Herbert E. Kubitschek	Robert B. Webb
Carl Peraino	

Northwestern University

Peter D. Klein

AUA-ANL BICENTENNIAL CONFERENCE

In 1976, the annual biology symposium recognized the bicentennial theme with a conference entitled Accomplishments and Challenges for American Life Sciences. The conference was held at Argonne National Laboratory on October 11-13, under the joint sponsorship of the Division of Biological and Medical Research, the Division of Radiological and Environmental Research, the Division of Environmental Impact Studies, the Argonne Center for Educational Affairs, and the AUA-ANL Biology Committee of the Argonne Universities Association. Drs. George W. Beadle, retired president of The University of Chicago, and Mortimer M. Elkind, Division of Biological and Medical Research, were co-chairmen.

The conference had a broader scope than the preceding nine annual symposia. The program was designed to survey for five areas of biology and medicine the significant progress in the past 200 years, the current status, and the outlook for the future of scientific research in each area. The five conference sessions were: Ecological Determinants in Environmental Change; Food and Energy: A World Problem; "Yankee Ingenuity" in the Life Sciences; Molecular Biology and Genetic Engineering; and Frontiers in Health Research.

The conference was directed toward first- and second-year graduate students who were primarily, but not exclusively, from member institutions of the Argonne Universities Association. Over 225 students and faculty members attended and participated in discussions.

The theme of the conference was set in the introduction to the program:

> "Two hundred years comprise a short period in the recorded history of man's experiences with organized society. At its birth, our Nation had a Benjamin Franklin and a Thomas Jefferson, each of whom had an interest in science. To our good fortune, we also have had Franklins and Jeffersons in the intervening years. The achievements of science in America, especially in the last few decades, have been remarkable, particularly in the life sciences. We may look to this Conference, therefore, as an occasion for joy and celebration as we reflect upon the accomplishments of American scientists.
>
> "Advances in the world's understanding of nature and life processes, to which Americans have contributed in good measure, justify optimism for the future. These advances occurred in a society that continues to involve a fruitful interaction between private and governmental structures--private and public universities, philanthropic and nonprofit institutions, and federally supported national laboratories. This dialog has been vital in the development of productive research programs and in the responsible expenditure of public funds.

"America faces many problems, as does the world. Resolutions will not be found through science and the wisdom of scientists alone. The continuing achievements of our Franklins and Jeffersons, however, provide not only pride but also confidence that America's life sciences will meet the critical challenges of tomorrow."

G. W. Beadle
M. M. Elkind

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19. PUBLICATIONS APPEARING IN CALENDAR YEAR 1976

JOURNAL ARTICLES

Ainsworth, E. J., D. L. Jordan, M. Miller, E. M. Cooke, and J. S. Hulesch. Dose rate studies with fission spectrum neutrons. Radiat. Res. 67, 30-45 (1976).

Bailey, J. M., W. D. Hill, A. G. Fiscus, C. A. Reilly, Jr., and M. P. Finkel. Plasma alkaline phosphatase in mice with experimentally-induced osteosarcomas. Lab. Anim. Sci. 26, 66-69 (1976).

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Duffy, P. H., and G. A. Sacher. Age-dependence of body weight and linear dimensions in adult *Mus* and *Peromyscus*. Growth 40, 19-31 (1976).

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Elkind, M. M. Fractionated dose radiotherapy and its relationship to survival curve shape. Cancer Treatment Review 3, 1-15 (1976).

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Feinstein, R. N. Increasing the flexibility of polyacrylamide gel-slab isoelectric focusing. Anal. Biochem. 72, 533-538 (1976).

Feinstein, R. N., R. J. M. Fry, E. C. Cameron, C. Peraino, and H. P. Morris. New aldehyde dehydrogenase isozymes in chemically induced liver tumors. Proc. Soc. Exp. Biol. Med. 152, 463-468 (1976).

Friedman, A. M., J. C. Sullivan, S. L. Ruby, A. Lindenbaum, J. J. Russell, B. J. Zabransky, and G. V. S. Rayudu. Studies of tumor metabolism--I: By use of Mössbauer spectroscopy and autoradiography of ^{153}Sm. Int. J. Nucl. Med. Biol. 3, 37-40 (1976).

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Granot, J., and D. Fiat. Proton magnetic resonance study of the dysprosium(III) aqueous complex. J. Magn. Reson. 19, 372-381 (1975).

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